Laboratory Procedures in Clinical Microbiology

Second Edition

Laboratory Procedures in Clinical Microbiology

Second Edition

Edited by
John A. Washington

With Contributions by Members of the
Section of Clinical Microbiology
Department of Laboratory Medicine
Mayo Clinic, Rochester, Minnesota

With 117 Figures (24 in Color)

Springer-Verlag New York Heidelberg Berlin Tokyo

John A. Washington II
Section of Clinical Microbiology
Mayo Clinic
200 Southwest First Street
Rochester, Minnesota 55905

Library of Congress Cataloging in Publication Data
Main entry under title:
Laboratory procedures in clinical microbiology.
Includes bibliographies and index.
1. Diagnostic microbiology—Laboratory manuals.
I. Washington, John A., 1936- . II. Mayo Clinic.
Section of Clinical Microbiology. [DNLM: 1. Diagnosis,
Laboratory. 2. Microbiology—laboratory manuals.
QW 25 L12348]
QR67.L33 1985 616'.01'028 84-23659

© 1981, 1985 by Mayo Foundation.
Softcover reprint of the hardcover 2nd edition 1985

All rights reserved. No part of this book may be translated or reproduced in any form without written permission from Springer-Verlag, 175 Fifth Avenue, New York, New York 10010, USA.

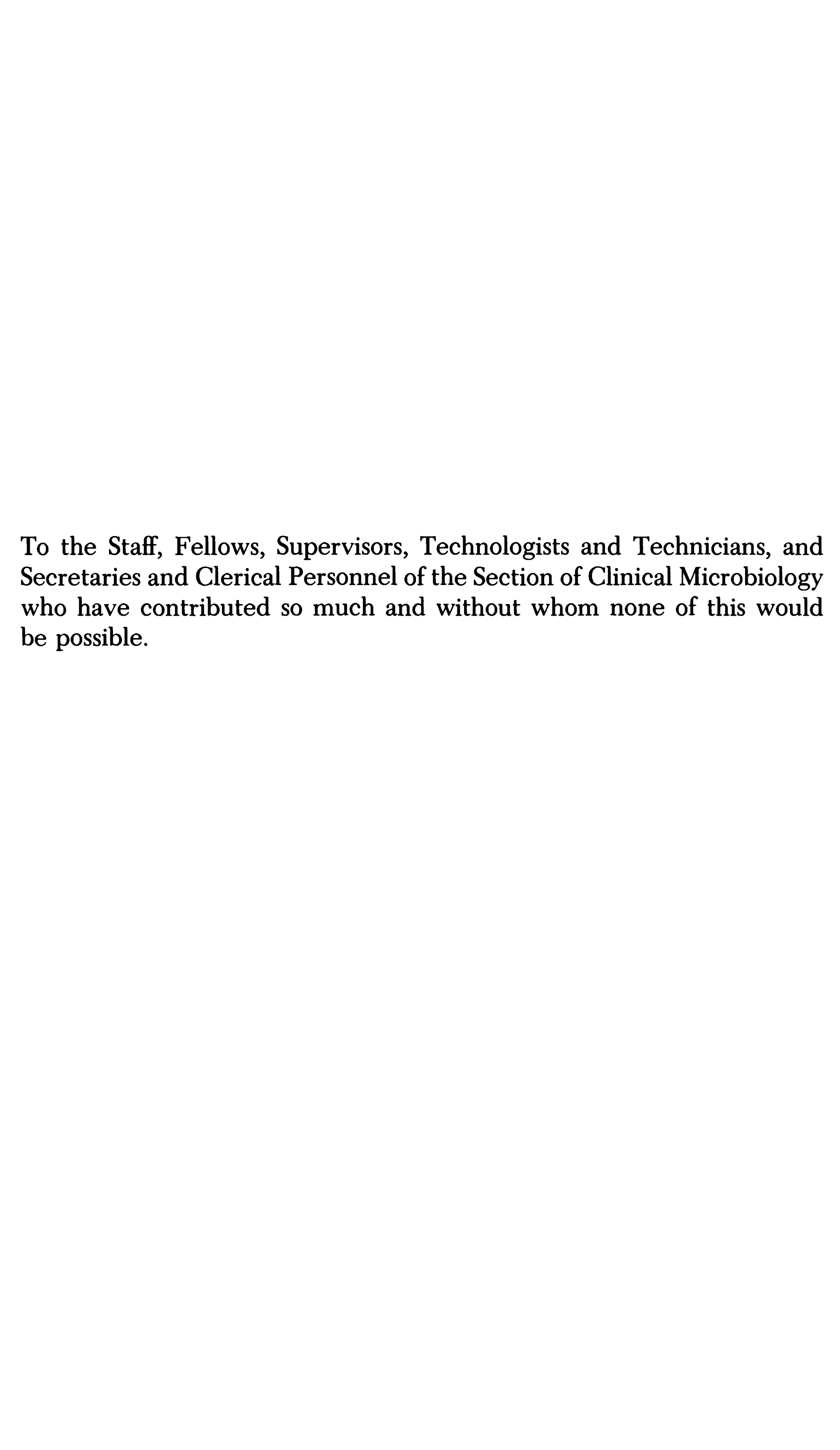
The use of general descriptive names, trade names, trademarks, etc. in this publication, even if the former are not especially identified, is not to be taken as a sign that such names, as understood by the Trade Marks and Merchandise Marks Act, may accordingly be used freely by anyone.

While the advice and information in this book are believed to be true and accurate at the date of going to press, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Typeset by Kingsport Press, Kingsport, Tennessee.

9 8 7 6 5 4 3 2 1

ISBN-13: 978-1-4612-9550-1 e-ISBN-13: 978-1-4612-5070-8
DOI: 10.1007/978-1-4612-5070-8



To the Staff, Fellows, Supervisors, Technologists and Technicians, and Secretaries and Clerical Personnel of the Section of Clinical Microbiology who have contributed so much and without whom none of this would be possible.

Preface

Although there are a number of comprehensive books in clinical microbiology, there remains a need for a manual that can be used in the clinical laboratory to guide the daily performance of its work. Most of the existing publications provide detailed and precise information, for example, by which a microorganism can be characterized and identified beyond any doubt; however, the number of tests involved in this process exceeds the capabilities and resources of most clinical laboratories and are irrelevant for patient care. It is, therefore, necessary in any clinical laboratory to extract from reference manuals, textbooks, and journals those tests and procedures that are to be used to complete the daily workload as efficiently and accurately as possible. It is also essential in the clinical laboratory to determine, on the basis of the kind of specimen being examined, which microorganisms are clinically relevant and require isolation and identification and which should either be excluded selectively or simply regarded as indigenous flora and, therefore, not specifically identified. Cost and time limit a laboratory's resources, and priorities must be established for handling the workload.

The procedures described in the second edition of this manual are those selected by our staff for use in the clinical laboratory on the basis of clinical relevance, accuracy, reproducibility, and efficiency. Alternative procedures, when considered equivalent on the basis of personal or published experience, have been included where appropriate. Since the output or reports from the laboratory are only as good or valid as the quality of its input, since microbiologists are often consulted about the types of specimens recommended for particular examinations, and since most laboratory procedures are based on the kind of specimen submitted to the

laboratory, emphasis has been placed on proper techniques of specimen selection, collection, transport, and processing.

Clearly, not all the procedures we have described will be universally applicable. As has been recognized in all inspection and accreditation programs, laboratories vary considerably in the levels or extents of their work. Since, however, the rational basis for selecting procedures should be based on evaluations performed in laboratories with a sufficiently large workload to generate statistically valid data, it is hoped that the procedures described in this manual will provide a basis for other laboratories' selection of procedures.

Little emphasis has been placed in this manual on the use of kits or automated equipment since the reasons for replacing conventional techniques with such devices vary so widely among laboratories. Nonetheless, it must be emphasized that the validation of any device is based on comparisons of its results with those of conventional techniques. It, therefore, remains essential for users of devices to understand the principles and applications of conventional techniques.

Since the last edition of this manual, substantial changes have occurred in the taxonomy and nomenclature of the Enterobacteriaceae. Novel approaches have provided increased sensitivity and speed in detecting bacteremia and fungemia, as well as for detecting infections due to *Yersinia enterocolitica, Legionella, Mycobacterium, Chlamydia trachomatis,* herpes viruses, and cytomegaloviruses. Simple, rapid, and specific immunoassays are rapidly replacing biological and chromatographic assays for determining aminoglycoside and vancomycin levels in serum and the body fluids. Immunological procedures for rapid detection of all sorts of microbial antigens are in various stages of development and evaluation. The extent to which the sensitivity and specificity of such procedures approaches those of cultures will determine their role in the clinical laboratory of the future.

Specific products and, in certain instances, their manufacturers are cited when their performance has been proved satisfactory or when their use has been shown to be essential for a test's accuracy. Acceptable substitutes may often be available; however, generic equivalence in product performance should never be assumed because of differences in production among manufacturers.

A special note of gratitude is due to Lola Jaeger and Roberta Kondert for their care in setting and their patience and perseverance in resetting our ideas into manuscript form.

John A. Washington II, M.D.

Contents

Preface vii

Contributors xiii

Chapter 1 Collection and Handling of Specimens 1

I. General Considerations 1
II. General Instructions for Specimen Collecting 5
III. Specific Guidelines for Specimen Collection 24
References 67

Chapter 2 Direct Examination of Specimens 71

I. General Considerations 71
II. Specific Indications 73
III. Stains and Wet Mount Preparations 85
References 92

Chapter 3 Initial Processing for Cultures of Specimens 95

I. Blood Cultures 95
II. Cultures of Normally Sterile Body Fluids, Tissue, Wounds, and Abscesses 97
III. Cultures of Specimens from the Upper Respiratory Tract 103
IV. Cultures of Specimens from the Lower Respiratory Tract 106
V. Cultures of Urine 110
VI. Cultures of Feces 113
VII. Cultures of Genital Tract Infections 115
VIII. Cultures of Integumentary Infections 119

IX. Cultures of Material for Epidemiological Purposes 119
References 121

Chapter 4 Aerobic and Facultatively Anaerobic Bacteria 125

Section 4.1 General Classification of Bacteria 127

Section 4.2 Identification of Aerobic and Facultatively Anaerobic Bacteria 131
I. Gram-Positive Cocci 131
II. Gram-Negative Cocci 157
III. Gram-Positive Bacilli 164
IV. Gram-Negative Bacilli 179
References 241

Section 4.3 Fluorescent Antibody Procedures and Counterimmunoelectrophoresis 251
I. Overview 251
II. Fluorescent Antibody Techniques 253
III. Counterimmunoelectrophoresis 270
References 278

Section 4.4 Antimicrobial Susceptibility Tests of Aerobic and Facultatively Anaerobic Bacteria 281
I. Overview 281
II. Preparation of Antimicrobial Solutions 288
III. Testing Procedures 291
References 310

Chapter 5 Anaerobic Bacteria 315
I. General Considerations 315
II. Isolation of Anaerobic Bacteria 321
III. Identification of Anaerobic Bacteria 330
IV. Antimicrobial Susceptibility Testing of Anaerobic Bacteria 373
References 377

Chapter 6 Mycobacteria and Nocardia 379
I. General Considerations 379
II. Identification 381
III. Antimicrobial Susceptibility Testing 408
IV. Common Aerobic Actinomycetes 411
References 417

Chapter 7 Fungi 419
I. General Considerations 419
II. Identification of Yeasts 426
III. Definitive Tests 440

IV. Identification of Filamentous Fungi 446
V. Serodiagnosis of Fungal Infections 487
VI. Antimicrobial Susceptibility Tests of Fungi 487
References 497

Chapter 8 Chlamydiae **501**
I. General Considerations 501
II. Cultures for Chlamydia 503
References 514

Chapter 9 Mycoplasmas **519**
I. General Considerations 519
II. Identification 521
References 532

Chapter 10 Viruses **537**
I. General Considerations 537
II. Processing of Specimens and Cultures 539
III. Cell Cultures 539
IV. Identification of RNA-Containing Viruses 543
V. Identification of DNA-Containing Viruses 585
VI. Reporting of Viral Isolates 611
VII. Viral Serology 611
References 616

Chapter 11 Parasites **625**
I. General Considerations 625
II. Procedures Suggested for Use in Examination of Clinical Specimens for Parasitic Infection 634
III. Processing of Stool Specimens 637
IV. Examination for Blood and Tissue Parasites 659
V. Examination of Specimens Other Than Feces and Blood 669
VI. Serology for Parasitic Diseases 674
VII. Miscellaneous Procedures 686
References 685

Chapter 12 Special Tests of Antimicrobial Activity **689**

Section 12.1 Antimicrobial Assays **691**
I. Overview 691
II. Bioassays 695
III. Liquid Chromatographic Assays 709
References 726

Section 12.2 Bactericidal Tests 731
I. Minimal Bactericidal Concentration (MBC) 732
II. Serum Antibacterial Titer (SIT) and Serum Bactericidal Titer (SBT) 736
III. Combination Studies 739
References 743

Appendix A Laboratory Safety 747
I. Introduction 747
II. Routes of Laboratory-Acquired Infections 748
III. Dangerous Chemicals 749
IV. Procedures following Exposure or Accident 750
V. Guidelines for Prevention or Containment of Contamination, Biohazards, and Infectious Agents 752
VI. Environmental Control 763
VII. Occupational Health 765
References 766

Appendix B Media and Reagents 769
I. Media for Aerobic and Facultatively Anaerobic Bacteria 770
II. Media and Reagents for Anaerobic Bacteria 800
III. Media and Reagents for Mycobacteria and Nocardia 808
IV. Media for Fungi 811
V. Media, Reagents, and Buffers for Chlamydiae, Mycoplasmas, and Viruses 816
References 829

Appendix C Quality Control 831
I. Overview 831
II. Media and Reagents 833
III. Equipment 861
References 864

Index 865

Contributors

John P. Anhalt, Ph.D., M.D.
Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Associate Professor of Microbiology and of Laboratory Medicine, Mayo Medical School.

Glenn D. Roberts, Ph.D.
Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Associate Professor of Microbiology and of Laboratory Medicine, Mayo Medical School.

Jon E. Rosenblatt, M.D.
Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine, and Division of Infectious Diseases, Department of Internal Medicine, Mayo Clinic and Mayo Foundation; Professor of Microbiology and of Internal Medicine, Mayo Medical School.

Thomas F. Smith, Ph.D.
Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Associate Professor of Microbiology and of Laboratory Medicine, Mayo Medical School.

John H. Thompson, Ph.D.
Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Professor of Laboratory Medicine, Mayo Medical School.

Edward Warren
Administrative Coordinator, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation.

John A. Washington II, M.D.
Head, Section of Clinical Microbiology and Consultant, Section of Clinical Microbiology, Department of Laboratory Medicine; Professor of Microbiology and of Laboratory Medicine, Mayo Medical School; Professor of Microbiology, University of Minnesota.

Walter R. Wilson, M.D.
Consultant, Division of Infectious Diseases, Department of Internal Medicine, and Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Professor of Internal Medicine and Assistant Professor of Microbiology, Mayo Medical School.

Pauline K. W. Yu, M.S.
Associate, Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic and Mayo Foundation; Assistant Professor of Microbiology and of Laboratory Medicine, Mayo Medical School.

1

Collection and Handling of Specimens

John A. Washington II, M.D. *Walter R. Wilson, M.D.*

I. General Considerations

Knowledge about the microbial etiology of infectious diseases has therapeutic, prognostic, and epidemiologic value. Complicating factors in achieving this knowledge include the type, location, and duration of disease; prior antimicrobial therapy; and contamination of the specimen with indigenous flora.

A. Indigenous Flora

1. Distribution

The prevalence of indigenous microbial flora is listed by anatomic site in Table 1-1. An understanding of the composition and distribution of microbial flora is essential to specimen collection and the interpretation of results of cultures. Alterations in the composition and distribution of indigenous flora may result from disease itself or from therapy with antimicrobials, irradiation, or antimetabolites. Alterations may occur in hospital personnel and in seriously ill, debilitated, hospitalized patients.

Contamination of specimens with indigenous flora is a frequent and serious problem both for the laboratory and the clinician in that identification of such flora is time consuming and costly and reporting its presence provides potentially misleading information regarding the etiology of an infection.

Table 1–1. Microorganisms Encountered on Healthy Human Body Surfaces[a]

Organism	Skin	Conjunc-tiva	Upper respiratory tract	Mouth	Terminal ileum, cecum, large intestine	Genitourinary tract		
						External genitalia	Anterior urethra	Vagina
Bacteria								
Actinomyces			+	+	±			
Bacteroides			+	++	++	+	+	+
Bifidobacteria				+	++			
Clostridia	±			±	++		±	±
Corynebacteria	++	+	+	+	+	+	+	+
Enterobacteriaceae	±		±	±	++	+	+	±
Eubacteria	±		±	+	++			±
Fusobacteria			+	++	+	+	+	±
Haemophili		±	++	+				+[b]
Lactobacilli				+	+		±	++
Neisseriae		±	++	+			±	±
Propionibacteria	++	+	+	±	±			+
Staphylococci	++	+	+	+	±	++	+	+
Streptococci								
Enterococcal			±	+	+	+	+	+
Pyogenic			±	±				± to ++[c]
Viridans	±	±	±	++	+	+	+	+

Cocci, anaerobic								
Gram-positive	+	±	+	++	++	+	±	+
Gram-negative			+	++	+		±	+
Chlamydiae							±	±
Mycoplasmas			+	+			+	+
Spirochetes		+		±	+			
Fungi								
Aspergillus	±	±		+				
Candida	±	±	+	++	+			+
Cephalosporium	±	±	±					±
Cryptococcus	±			±				
Fusarium	±	±		±				
Penicillium	+	±		+				
Rhodotorula	±	±		+				

[a] ±, irregular or infrequent; +, common; ++, prominent.
[b] *Gardnerella vaginalis.*
[c] Group B.

2. Methods for Circumventing Problems with Indigenous Flora

a. Antisepsis

Antiseptics, such as tincture of iodine, iodophors, or chlorhexidine, should be applied to the skin prior to aspiration of abscesses and various normally sterile body fluids. *N.B.* No antiseptic is instantly effective, and all require application for a minimum of 1 to 2 min to remove vegetative bacteria from the skin surface.

b. Decontamination

Some specimens can be subjected to decontamination procedures that will selectively inhibit or destroy microorganisms other than those of specific interest. A typical example is sputum that is treated with NaOH or benzalkonium chloride (Zephiran) prior to culture for mycobacteria.

c. Selective Media

Media containing inhibitory compounds are in common use for the selective isolation of a particular microorganism or related groups of microorganisms. Examples include enteric selective media for salmonellae and shigellae or fungal media containing antibacterial compounds.

d. Quantitation

Quantitative cultures are used to distinguish between bacteria likely to represent contaminants and those likely to be clinically significant. The most common example of a specimen cultured quantitatively is urine.

e. Microscopy

Cytological examination of urine, sputum, and wounds is useful for distinguishing between improperly or contaminated and properly collected specimens on the basis of the prevalence or rarity of squamous epithelial cells.

f. Invasive Procedures

The percutaneous transtracheal aspirate (TTA) and suprapubic aspirate (SPA) are invasive procedures performed to bypass the indigenous flora of the oropharynx and the urethra, respectively. Examples of other invasive procedures include bronchoscopy and the transbronchoscopic,

transthoracic and other percutaneous needle biopsies, as well as open lung biopsy.

II. General Instructions for Specimen Collecting

Physicians' offices and nursing stations should have instructions on hand regarding types of specimens, suggested volumes, and containers to be used. An example of such instructions, taken from the Mayo Clinic's and affiliated hospitals' procedure guides, is shown in Table 1–2. These instructions are reviewed and revised annually.

A. Identification

Specimen containers and request forms must be clearly identified with the

1. Patient's name.
2. Patient's registration number.
3. Patient's location.
4. Patient's physician.
5. Site or source of specimen.
6. Type of examination requested.
7. Tentative diagnosis.
8. Date and hour of specimen collection.
9. Date and hour of receipt in the laboratory.

Request forms accompanying blood or other body fluid to be tested for antibiotic activity (assays, serum bactericidal titer) should contain, in addition to the information listed above, the following:

1. Dosage of antibiotic(s) being administered.
2. Name(s) of all antibiotics being administered.
3. Times of the last dose of antibiotic and of blood collection.

B. Transport Containers, Devices, and Media

1. Swabs

a. Types

Combined swab-transport medium devices are desirable to prevent dessication of the specimen with loss of viability of bacteria.[3] Throat swabs represent one possible exception in the group A streptococci survive

Table 1–2. Instructions for Specimen Collection and Transport[a]

Specimen	Synonyms	Container or transport device	Volume (ml)	Other considerations
BLOOD (for culture bacteria)		blood culture bottles containing liquid media; lysis-centrifugation tube (Isolator™)	10 ml/100 ml bottle or 10 ml lysis-centrifugation tube	In the majority of cases, 3 **separate** cultures over a 24-hour interval suffice. Intervals between cultures are determined by urgency of clinical situation. Fewer than 3 cultures/24 hours will lessen chance of recovery of etiologic agent; therefore, when only a single culture is ordered, a second will automatically be collected. Moreover, if more than 4 cultures/24 hours are ordered, the laboratory will require confirmation of the request.
Brucella		Castaneda bottle (TS) or lysis-centrifugation tube	10	As for bacterial blood culture.
fungi		Castaneda bottle (BHI) or lysis-centrifugation tube	10	As for bacterial blood culture.
Leptospira		sterile, heparinized tube	1	Useful only during 1st week of illness.
malaria filaria trypanosomes		thick and thin films for malaria; anticoagulated blood (5 ml) for filaria and trypanosomes		These special procedures involve finger prick or venipuncture to obtain blood films or anticoagulated blood for concentration technique in Parasitology Laboratory.
CATHETER venous, arterial	cannula	Culturette™	N.A.	Disinfect surrounding entry site, remove catheter, clip off tip aseptically into tube.

suction, drainage		sterile, screw-capped tube	N.A.	
urinary		sterile, screw-capped tube or Weed bottle	N.A.	
EXUDATES Transudates, Drainages, Ulcers		swab (Culturette™) or anaerobe transporter vial	1–5 3–5 for TB 3–5 for fungi	Specimens from such sources are generally not suitable for anaerobic bacteriology, unless received in vial.
EYES conjunctiva		swab (Culturette™)	N.A.	
corneal lesion	ulcer	See "Other considerations"	N.A.	Call Microbiology. Technologists will bring necessary slides and media for direct smears and cultures of swab material and corneal scrapings.
FECAL MATERIAL stool bacteria	feces	sterile, screw-capped jar	N.A.	Freshly collected specimen mandatory, especially for **shigellae.** Less desirable alternative is to use Hajna transport medium.
fungal		sterile, screw-capped jar	N.A.	As for bacterial stool culture. Send to laboratory **immediately.** If storage time exceeds 1 hour, specimen should be refrigerated
O & P		stool carton sealed in plastic bag for transport to the laboratory	N.A.	Stool is collected from bed pan to water-proof carton, sent to hospital laboratory where it is sealed in a plastic bag for delivery to Parasitology Laboratory. The specimen should not be incubated, but rather, kept cool.

Table 1–2 (*continued*)

Specimen	Synonyms	Container or transport device	Volume (ml)	Other considerations
rectal swab		swab (Culturette™)	N.A.	As for bacterial stool culture.
anal swab for pinworms		special sterile plastic swab in stoppered tube (SWUBE)	N.A.	This special swab is sent from Parasitology Laboratory to bedside or home where the physician, nurse, or parent then swabs the perianal fold. Swabbing is best done on arising and prior to bathing or defecation.
FLUIDS				
cerebrospinal		sterile, screw-capped tube	1–5 additional { = 2 for TB; = 2 for fungi	Must be sent to Microbiology Laboratory **immediately** via pneumatic tube.
abdominal	peritoneal paracentesis dialysis bile	anaerobe transporter vial sterile, screw-capped tube	1–5 { 10–15 for TB; 10–15 for fungi	Collect with sterile needle and syringe* from which air bubbles are expelled before injection into vial.
chest	pleural thoracentesis empyema	anaerobe transporter vial sterile, screw-capped tube	1–5 { 10–15 for TB; 10–15 for fungi	Collect with sterile needle and syringe* from which air bubbles are expelled before injection into vial.
pericardial		anaerobe transporter vial sterile, screw-capped vial	1–5 { 10–15 for TB; 10–15 for fungi	Collect with sterile needle and syringe from which air bubbles are expelled before injection into vial.

synovial	joint	anaerobe transporter vial sterile, screw-capped tube	1–5 2–3 for TB 3–5 for fungi	Collect with sterile needle and syringe* from which air bubbles are expelled before injection into vial.
other		anaerobe transporter vial sterile, screw-capped tube	1–5 2–3 for TB 3–5 for fungi	Collect with sterile needle and syringe* from which air bubbles are expelled before injection into vial.
GENITOURINARY				
cervix	endocervix	swab (Culturette™) or anaerobe transporter vial	N.A. N.A.	If anaerobic infection of the uterus is suspected, disinfect cervix and attempt to aspirate material through the cervical os, expel air bubbles from syringe, and inject into vial.
culdocentesis fluid		anaerobe transporter vial	N.A.	
NEISSERIA GONORRHOEAE	GC	JEMBEC	N.A.	**Women:** **Cervix**—moisten speculum with water, **not** lubricant, insert swab into cervical canal. **Anal canal**—insert swab approximately 1″ into canal and move from side to side to sample crypts. **Urethral or vaginal**—indicated when cervical culture not obtainable. **Men:** **Urethral**—swab may be used if there is discharge; otherwise, a sterile bacteriological loop is used to obtain specimen from anterior urethra by gently scraping the mucosa. Smear should be prepared on clean glass slide for Gram stain. **Anal canal**—as for women.

Table 1–2 (*continued*)

Specimen	Synonyms	Container or transport device	Volume (ml)	Other considerations
				Swabs should be used for direct inoculation on agar in JEMBEC plate (pre-warmed to room temperature). Tablet is added to well; chamber is closed and is placed in Ziploc incubation bag.
TRICHOMONAS VAGINALIS		swab (Culturette™)	N.A.	This procedure is preferably done by a fresh swab of the vaginal discharge obtained with a speculum. A drop of the material, or the swab rolled in saline on a slide, is cover-slipped and examined as a fresh, wet preparation. The Culturette™ can be used as a transport device.
CHLAMYDIA		2SP transport medium	N.A.	Swab extracted into 2SP transport medium and sent to Microbiology Laboratory immediately via pneumatic tube. During nights, weekends and holidays, freeze specimen, then send to Laboratory on the next working day.
RESPIRATORY TRACT				
nasopharynx	NP	flexible wire calcium alginate–tipped swab (Calgiswab™) or #8 French 16″ catheter	N.A.	Used to detect carrier states of **Neisseria meningitidis, Corynebacterium diphtheriae,** and **Bordetella pertussis** and for diagnosis of whooping cough and of pneumonia in infants and children unable to expectorate. Call Microbiology for swab or catheter and techni-

				cian who will bring appropriate media for direct culture.
oral cavity		swab (Culturette™)	N.A.	
throat β-strep only		swab (Culturette™)	N.A.	For detection of **Streptococcus pyogenes** (group A). Swab tonsillar areas, posterior pharynx, and areas of inflammation, ulceration, exudation, capsule formation.
other		swab (Culturette™)	N.A.	Requests for isolation of **Neisseria meningitidis, N. gonorrhoeae, Corynebacterium diphtheriae, Bordetella pertussis,** fungi or yeasts, and mycoplasma must be specified.
virus		swab (Culturette™)	N.A.	See directions pages 17–19.
sputum bacteria		2 oz. sterile, screw-capped jar	N.A.	Collect fresh, clean specimen resulting from deep cough and send to laboratory **immediately.** If possible, have patient rinse mouth and gargle with water prior to collection. In a seriously ill, debilitated patient, transtracheal aspiration must be considered to bypass oropharyngeal contamination. Sputa with >25 epithelial cells/lpf are unsatisfactory for culture.
mycobacteria	TB	2 oz. sterile, screw-capped jar	5–10	Three early-morning, fresh, clean specimens resulting from deep cough or expectorated sputum induced by heated aqueous aerosol of 10% glycerin and 15% NaCl. If possible have patient brush teeth and gargle with water
fungi		2 oz. sterile, screw-capped jar	3–5	

Table 1–2 (*continued*)

Specimen	Synonyms	Container or transport device	Volume (ml)	Other considerations
				prior to collection. Send specimen to laboratory **immediately.** Storage in excess of 1 hour should be in refrigerator. **Do not send 24-hour sputum specimens.**
transtracheal aspirate	TTA	anaerobe transporter vial	3–5	Collect with sterile syringe by aspirating material from transtracheal catheter, expel air bubbles, and inject into vial.
bronchial washings		sterile, screw-capped vial	N.A.	Not generally suitable for bacterial cultural because of likelihood of oropharyngeal contamination.
protected catheter brush	Bartlett brush	sterile, screw-capped vial containing 1 ml Ringer's lactate	N.A.	Brush is clipped off into vial for quantitative bacterial culture.
tracheal aspirate		sterile, screw-capped vial	N.A.	
gastric washings		2 oz. sterile, screw-capped jar	≥1.5	Send specimen to laboratory immediately.
SKIN AND NAIL SCRAPINGS FOR DERMATOPHYTES fungi		sterile petri dish	N.A.	**Skin**—cleanse with 70% alcohol and remove skin at active border—including some of healthy area.

				Nails—cleanse with 70% alcohol. Using sterile scalpel blade, scrape surface and discard initial portion. Send deeper scrapings for culture.
TISSUE		sterile screw-capped bottle or anaerobe tube	Representative samples	Place aseptically into appropriate sterile container. Few viable organisms may be present in a lesion, especially if it is chronic. Specimen must be large enough to permit their recovery.
URINE midstream— clean-voided or clean catch		sterile, screw-capped tube 16 × 150 mm. 4 oz. screw-capped bottle	1–10 ≥10 for TB ≥10 for fungi	Urine is an excellent culture medium; therefore, specimens should be delivered **promptly** to the laboratory. Storage at room temperature in excess of 1 hour may invalidate results of culture. Storage in excess of 1 hr. should be in the refrigerator or on ice.
catheterized bacteria mycobacteria fungi	TB	sterile, screw-capped tube 16 × 150 mm. 2 sterile, 16 × 150 mm. screw-capped tubes filled to ¾ volume	1–10 15/tube for TB 10/tube for fungi	**Single urethral catheterization**—not recommended for collection of routine specimens. **Indwelling urethral catheter**—specimen is collected by direct aspiration through the catheter wall by sterile needle and syringe.
suprapubic aspiration	SPA	2 sterile, 16 × 150 mm. screw-capped tubes filled to ¾ volume or anaerobe vial	1–10 (5–10 for TB, 5–10 for fungi)	In anaerobic bacteriuria is suspected, SPA is the **only** valid means of establishing diagnosis. In such instances, expel air bubbles from syringe, and inject into anaerobe vial.
WOUNDS abscesses, pus		anaerobe transporter vial	1–5	Collect with sterile needle and syringe from which air bubbles are expelled before injection into vial. If additional fluid is required to obtain culture, bacteriostatic-free water should be used. Swabs should not be used since they provide limited material and are not amenable to anaerobic transport.

Table 1–2 (*continued*)

INSTRUCTIONS FOR VIRUS SPECIMEN COLLECTION AND TRANSPORT

General disease categories	Viruses frequently associated	Specimens to be submitted	Container or transport device	Volume	Serology available	Other considerations
Central nervous system (aseptic meningitis and encephalitis	California virus*	throat and rectal swabs, CSF and serum; biopsy material if available; urine also if possibility of mumps	Culturette™ for swabs; sterile, screw-capped tube for urine or CSF; blood collection tube	5–10 ml urine; 1–2 ml CSF; 5 ml sterile clotted blood	+	No serology done for enterovirus infections.
	enteroviruses				No	In general, for serologic evidence of acute infections 2 or more serum specimens are required taken 2–3 weeks apart. Single serum only required for determination of immune status after vaccination.
	herpes simplex virus				+	
	mumps					
	rubeola virus*				+	
	St. Louis encephalitis*				+	
	Western equine encephalitis*				+	
	rabies*	serum		5 ml sterile clotted blood		Neutralizing antibody determined by Centers for Disease Control only for specimens obtained from immu-

						nosuppressed individuals or those receiving the duck embryo vaccine.
Congenital	cytomegalovirus	throat swab, urine (fresh morning); vesicular fluid (preferred, if applicable) or swab of vesicle; serum	Culturette™ for swabs; sterile screw-capped tube for urine; 1 ml tuberculin syringe for vesicular fluid (send complete syringe); blood collection tube	5–10 ml urine; few drops vesicular fluid; 5 ml sterile clotted blood.	+	Two or more serum specimens are required from both mother and infant taken at 1-month intervals.
	herpes simplex virus				+	
	rubella virus*				+	
Exanthem (maculopapular)	adenovirus	throat swab serum	Culturette™ for swabs; blood collection tube	5 ml sterile clotted blood.	+	Single serum only required for determination of immune status. For serologic evidence of acute infections, 2 or more serum specimens are needed taken 2–3 weeks apart.
	enteroviruses				No	
	rubella virus*				+	
	rubeola virus*				+	
	less frequently: parainfluenza virus				+	
	respiratory syncytial virus				+	

Table 1–2 (*continued*)

General disease categories	Viruses frequently associated	Specimens to be submitted	Container or transport device	Volume	Serology available	Other considerations
Exanthem (vesicular)	enterovirus (Coxsackie virus A)		1 ml tuberculin syringe (send complete syringe).	few drops vesicular fluid	No	For serologic evidence of acute infections, 2 or more specimens are required taken 2–3 weeks apart. No serology done for enteroviruses.
	herpes simplex virus	vesicular fluid (preferred), or swab of vesicle; vesicle scrapings on slide for direct FA test; serum. Place small drop of saline in each of 3 separate areas of a glass slide 5 mm to 10 mm apart. Transfer skin scrapings from a scalpel blade to the saline and spread the cells over a small circular area (5 mm to 10 mm in diameter). Dry the slide at room temperature	Culturette™ for swabs; blood collection tube; petri dish for slide	5 ml sterile clotted blood.	+	
	varicella-zoster virus					

Infectious mononucleosis	Epstein-Barr*	serum	blood collection tube	5 ml sterile clotted blood	+	Immunofluorescent antibody test indicated in those patients with heterophile-negative determinations.
	CMV	serum, urine, throat swab	blood collection tube; sterile, screw-capped tube for urine; Culturette™ for swabs.	5 ml sterile clotted blood	+	
Respiratory: pharyngitis, croup, bronchitis, pneumonia	adenovirus	throat swab, sputum; serum	Culturette™ for swab; 2 oz. sterile, screw-capped jar for sputum; blood collection tube	5 ml sterile clotted blood	+	Transport swabs to virus laboratory rapidly. Do not freeze specimen. No serology done for enteroviruses.
	cytomegalovirus				+	
	enteroviruses				No	
	herpes simplex virus				+	
	influenza virus					
	mumps virus				+	
	parainfluenza virus				No	
	respiratory syncytial virus				+	
	rhinovirus					
Immunocompromised	cytomegalovirus	throat swab, blood, urine; serum	Culturette™ for swabs; sterile screw-capped tubes for urine; blood collection tube	5 ml sterile heparinized blood; 5–10 ml urine; 5 ml sterile clotted blood	+	
		serum				
	Epstein-Barr	throat swab, vesicular fluid; serum			+	
	herpes simplex				+	
	varicella-zoster				+	
Gastroenteritis	rotavirus	feces	screw-capped jar		No	

Table 1–2 (*continued*)

INSTRUCTIONS FOR LEGIONELLA, MYCOPLASMA, Q-FEVER AND CHLAMYDIA SPECIMEN COLLECTION

General disease categories	Organisms	Specimens to be submitted	Container or transport device	Volume	Serology available	Other considerations
Respiratory pneumonia	*Legionella*	sputum, pleural fluid, lung tissue	sterile screw-capped jar	not applicable		
		serum (for serologic tests)		5 ml sterile clotted blood for serologic tests	+	Because of high background antibody titers in some populations, two or more serum specimens taken 2–3 weeks apart are recommended.
	Mycoplasma pneumoniae	sputum, throat swab	sterile screw-capped jar, Culturette™	not applicable	+	Two or more serum specimens required taken 2–3 weeks apart.
	Q-fever*	serum (for serologic tests)		5 ml sterile clotted blood for serologic tests	+	Two or more serum specimens required taken 2–3 weeks apart.
Chlamydia	*C. trachomatis*	serum (for serologic tests) Culturette™	blood collection tube; 2SP transport	5 ml sterile clotted blood for serologic	+	Extract swab in 2SP or mix nasopharyngeal aspi-

	nasopharyngeal aspirate	medium for culture	tests		rate with equal volume of 2SP. Send to Microbiology Laboratory immediately. During nights, week ends and holidays freeze specimen then send to Laboratory on the next working day. Single serum specimen usually sufficient for diagnosis of infant pneumonia. Serology not useful for diagnosis of uncomplicated sexually transmitted disease.
*C. psittaci**	serum (for serologic tests)	blood collection tube	5 ml sterile clotted blood for serologic tests	+	For serology, two or more specimen required taken 2-weeks apart.

[a] From *Mayo Clinic Procedure Guide*.
* Serology only, isolation not attempted.

well for several days on dry cotton or Dacron swabs but may be overgrown in the same length of time by indigenous oropharyngeal flora when transport medium is used.

b. Advantages and Disadvantages

Swabs represent convenient and economical specimen collection devices; however, they often provide an inadequate amount of specimen for both microscopic examination and multiple types of culture. At least 10^6 organisms must be present on a swab for their detection in a Gram-stained smear.[27] Another disadvantage is that the recovery of bacteria from swabs is less than 10% of the original inoculum.[7,32] Marked qualitative differences in the recovery of bacteria, particularly anaerobes, have been demonstrated between cultures of swabs and syringe aspirates of abscess material.[15]

c. Indication for Use

(1) Collection of material from skin and mucous membranes for bacterial (aerobic and facultatively anaerobic only) fungal, and viral cultures.
(2) Collection of cervical, vaginal, and urethral secretions or exudates for microscopic examination and/or culture for *Trichomonas vaginalis, Gardnerella vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasma urealyticum,* fungi, and herpesviruses.
(3) Anal swabs of crypts for cultures of *N. gonorrhoeae.*
(4) Rectal swabs for cultures of *Salmonella, Shigella* and *Campylobacter.*

d. Contraindications to Use

(1) Pus or Exudates

Material should be aspirated with needle and syringe for transport directly to the laboratory or for injection into anaerobic transport vial. If volume of material is small, sterile bacteriostat-free fluid (e.g., Ringer's lactate) should be used as irrigating fluid.

(2) Surgical Specimens

Fluid should be collected as described under (d,1). Portions, rather than swabs, of tissue suspected of being infected should always be sent for microbiological, as well as histopathological, examination.

(3) Cultures for Mycobacteria

Tissue or fluid aspirated from closed body cavities or wounds should always be obtained for microscopic examination and culture for mycobacteria. Material collected on swabs is simply inadequate for this purpose.

(4) Cultures for Anaerobic Bacteria
Some specially designed anaerobic swab transport systems may be more efficient than conventional swab systems for the collection and transport of anaerobes, but needle and syringe or anaerobic vial techniques (see Section II,B,2 and 3 below) are preferred.

2. Syringe

A sterile syringe can serve as a transport device for fluids by plugging the tip of the needle with a sterile rubber stopper.

3. Anaerobic Transport Tube or Vial

A simple anaerobic transport device can be prepared with a butyl rubber stoppered (size 00, no. 8820-B, Arthur H. Thomas Co., Philadelphia), screw-capped tube (20 × 125 mm, Kimax or Pyrex) that has been flushed with 99.9% pure CO_2. Samples are injected through the stopper or are placed into the tube by removing the stopper briefly and keeping the tube upright to minimize loss of CO_2.

Commercially prepared vials (Pre-duced Transporter, cat. no. B3062, Gibco Laboratories) contain an atmosphere of oxygen-free gas and a small volume of transport medium with an indicator that remains colorless under anaerobic conditions. A few vials from each lot should be vented to ensure coloration of the indicator upon exposure to air.

4. Tubes, Jars, and Bottles

A variety of tubes, jars, and bottles must be available for purposes of transporting fluids and tissues. It is essential that they be sterile and leakproof. In certain instances it may be necessary to test randomly selected containers, including anaerobic transport containers and blood culture bottles, for sterility and for the presence of nonviable but stainable bacteria.

N.B. Body fluids that are shipped for purposes of diagnostic examination are considered to be *diagnostic specimens* and are, therefore, subject to minimum packaging requirements. Standard procedures for the storage and transport of such specimens are described in detail elsewhere (see Appendix A).

5. Fecal Transport Systems

Whenever possible, feces should be examined microscopically for parasites or cultured for bacteria within a few hours of passage. Should longer periods of transport and storage be anticipated, it is recommended that portions of the specimen be placed into a preservative or transport medium.

a. Buffered Glycerol–Saline (BGS) Solution

BGS solution (see Appendix B) is preferred to *Shigella,* and is one of several transport media that are suitable for *Salmonella.*[10] It is not suitable for *Campylobacter.*

b. Polyvinyl Alcohol (PVA) Fixative*

PVA fixative (see Ch. 11) should be used to preserve parasites in feces that require overnight storage or mailing to a reference laboratory.

6. SWUBE Disposable Paddle (Falcon 2012)

This disposable device consists of a tube containing a smooth plastic paddle coated on one side with tacky adhesive that is applied to the perianal folds, replaced in the tube for transport, and then removed for microscopic examination for pinworm eggs and adult female pinworms.

7. Sexually Transmitted Disease (STD)—Related Specimen Transport Systems

a. Neisseria gonorrhoeae

(1) Combined Swab-Transport Medium

Such devices are usually satisfactory, provided the period of transport does not exceed six hours.[22] Optimal results are, however, obtained when modified Thayer-Martin medium (MTM) is inoculated and incubated in an atmosphere with increased CO_2 immediately.

(2) Transgrow

Transgrow consists of a bottle containing an agar slant of MTM and an atmosphere of CO_2. The slant is inoculated with a swab of the infected site.

PRECAUTIONS

(a) To minimize loss of CO_2, the bottle must be held in an upright position while uncapped for inoculation.
(b) The bottle should be incubated overnight at 35°C prior to shipment, since failure to do so will decrease frequency of recovery of gonococci.

(3) JEMBEC

JEMBEC consists of a plastic rectangular plate that contains MTM, to which is added a CO_2 -generating tablet and which is placed into a Ziploc

* Available commercially from Delkote, Inc., Penns Grove, NJ, and Med Chem Corp., Santa Monica, Ca, or in a collection kit from Marion Laboratories, Inc., Kansas City, Mo.

bag prior to incubation. Moisture is formed from the medium within the bag and activates the CO_2-generating tablet. Advantages of JEMBEC over Transgrow include its assured CO_2 content and better visibility of MTM.

PRECAUTION
Plate should be incubated overnight at 35°C prior to shipment.

b. Chlamydia trachomatis and Ureaplasma urealyticum

Urethral and cervical swabs and other material from patients with suspected chlamydial or mycoplasmal infections should be placed immediately into 2SP transport medium (Ch. 8). If a single specimen is to be used for the recovery of both organisms, no antibiotics should be present in the 2SP medium.[33] This medium should be frozen (−70°C) when the specimen is shipped or if it cannot be processed within 24 hr.

C. Transport

Prompt transport of specimens to the laboratory is required to preserve the viability of fastidious microorganisms, prevent overgrowth of fastidious microorganisms by more rapidly growing ones, and prevent multiplication of clinically insignificant numbers of bacteria to numbers that are erroneously interpreted as being clinically significant.

Specimens should be transported in sterile, leakproof containers to prevent loss of sample, prevent extrinsic contamination, and avoid exposure of others to potentially hazardous materials.

D. Accession Requirements in the Laboratory

1. Patient Identification

Request forms should be checked to ensure that the patient's name and registration number match.

2. Specimen Identification

Specimen containers should be identified by patient name and registration number. The laboratory should not assume responsibility for processing unidentified specimens that have become separated from accompanying request forms. The request form should clearly identify the specimen type or source.

3. Test Specification

The request form should specify the tests requested. The tentative diagnosis should also be included.

4. Time and Date

The times and dates of collection and of receipt in the laboratory should be written or stamped on the request form.

III. Specific Guidelines for Specimen Collection

A. Septicemia

Septicemia is defined as a systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood (*Dorland's Medical Dictionary,* 1981), although use of the term septicemia is customarily restricted to systemic infections associated with the presence of aerobic, facultatively anaerobic, and anaerobic bacteria, as well as yeasts, in the blood. Strictly defined, however, septicemia may also be associated with spirochetes, sporozoa, hemoflagellates, and microfilariae. The requirements for specimen collection from patients suspected of having bacteremia or fungemia are similar; therefore, they will be considered together in the section that follows (III,A,1). Specimen requirements in instances of septicemia suspected of being due to spirochetes, sporozoa, hemoflagellates, and microfilariae will be considered separately (Section III,A,2 and 3).

1. Bacteremia and Fungemia[30,36]

a. Etiology

Predominant among gram-positive microorganisms causing bacteremia are *Staphylococcus aureus,* enterococcal group D streptococci, and *Streptococcus pneumoniae.* Predominant among gram-negative causes of bacteremia are *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Bacteroides fragilis.* The most common cause of fungemia is *Candida albicans.*

b. Collection

(1) Timing

Timing of blood collection is not critical in endocarditis or endarteritis, uncontrolled infections, and early in the course of typhoid or brucellosis, since bacteremia is continuous. Timing is, however, difficult in other situations because bacteremia is usually intermittent and has been shown experimentally in animals to precede the onset of fever or chills.

(2) Number of Cultures

Two or three separate cultures at hourly intervals are nearly always sufficient in patients with suspected endocarditis or endarteritis; however, the intervals between culture may be reduced in patients requiring urgent antimicrobial therapy. For other types of bacteremia, three separate cultures should be collected within a 24-hr period, the intervals being determined by clinical circumstances and the urgency of initiating therapy. Approximately 80% of bacteremias will be detected by the first of three cultures, 90% by the first two of three cultures, and 99% by three separate cultures (Figure 1–1). A single blood culture within a 24-hr interval, therefore, lacks sensitivity; however, more than three cultures per septic episode are seldom required. One exception may be the patient who has recently received antimicrobial therapy and from whom one or two additional cultures may have to be collected.

It is, therefore, suggested that within a 24-hr period, at least two sepa-

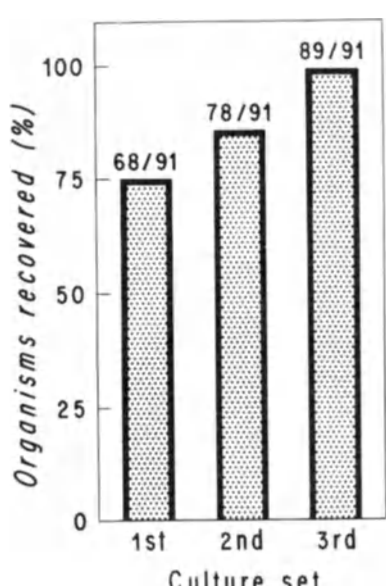


Figure 1–1. Cumulative rates of positivity in each of three blood culture sets from 80 bacteremic patients. (From Washington, J. A., II. Blood cultures: Principles and techniques. *Mayo Clin. Proc.* **50**:91, 1975.)

rate sets of cultures be performed and a limit of four separate cultures be established. Exceptions to either of these suggestions can be made after consultation with the laboratory director.

(3) Precautions to Minimize Risk of Contamination

(a) Skin Antisepsis

The phlebotomy site should be scrubbed with 70 to 95% alcohol and then prepared by concentric application of 2% iodine, an iodophor, or cycloheximide.

(b) Closed Bottle System

Blood should be injected into bottles sealed with a rubber diaphragm to minimize airborne contamination of the medium.

(c) Sterile Evacuated Blood Collection Tubes

Only sterile tubes should be used when this system is selected for transport of blood to the laboratory for culture; however, care should be taken to prevent backflow of the tube's contents during phlebotomy, and non-sterile tube holders may serve as a source of contamination if not cleaned regularly.

(4) Volume of Blood

(a) Adults

The order of magnitude of bacteremia in adults is usually small and often <1 colony-forming unit (CFU)/ml.[16] There is a direct relationship between the volume of blood cultured and the rate of isolation of bacteria from

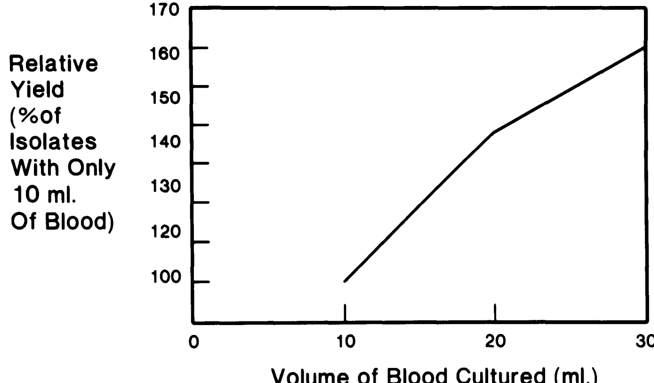


Figure 1–2. Yield of organisms relative to volume of blood cultured. (From Ilstrup, D. M., and Washington, J. A., II. The importance of volume of blood cultured in the detection of bacteremia and fungemia. *Diagn. Microbiol. Infect. Dis.* **1**:107, 1983.)

blood (Figure 1–2). It is important to understand that this is a variable separate from that related to the intermittency of bacteremia. It is recommended that 20 to 30 ml of blood be collected from adults for each set of cultures.

(b) Infants and Children

The order of magnitude of bacteremia in this age group is substantially higher than that in adults; therefore, 1 to 3 ml of blood per culture set is adequate.

(5) Specimen Handling

Three alternatives are available.

(a) Direct Inoculation into Media

Blood is inoculated at the bedside directly into bottles containing media, either with a syringe and needle or with a collection set. The bottles are then sent to the laboratory for processing.

(b) Transport

Blood is collected in a sterile evacuated blood collection tube containing sodium polyanetholsulfonate (SPS) and transported to the laboratory, where appropriate media are inoculated.

(c) Lysis-Concentration

Blood is collected and mixed in a lysis-centrifugation blood culture tube (Isolator™, Du Pont Co.) containing SPS, saponin, propylene glycol, disodium ethylenediamine-tetraacetic acid (EDTA), and inert fluorochemical. The tube is transported to the laboratory, where it is centrifuged, and the sediment is removed and inoculated onto bacterial and fungal isolation media.

c. Media

(1) Composition

Numerous broth formulations are available commercially including soybean–casein digest [Tryptic or Trypticase soy (TSB)], brain heart infusion (BHIB), Brucella (BB), Thiol, thioglycollate, Columbia, and supplemented peptone (SPB).

(a) Selection

Selection is complicated by differences in production among manufacturers, including the addition of proprietary growth factors to the media, varying amounts of vacuum and CO_2 in the atmosphere, and variations in procedures among laboratories evaluating the product. Comparative

clinical studies of two blood culture bottles should meet the following minimum criteria:

(i) The volumes of blood cultured should be equal.
(ii) The bottles should be examined and subcultured at the same time, with equal frequency, and in the same manner.
(iii) The number of cultures examined should be sufficiently large to permit a statistically valid comparison to be made (usually at least 300 positive cultures).[36]

(b) Evaluations

To date, clinical studies at the Mayo Clinic have demonstrated equivalency in recovery of bacteria from Difco Laboratories' TSB, BHIB, and BB, as well as BBL Microbiology System's TSB.[13] Compared with TSB, significantly fewer *Staphylococcus aureus* were recovered from Columbia broth and *Pseudomonas* and *Candida* from both Thiol and thioglycollate.[36] In no instance were anaerobic bacteria isolated significantly more frequently from Thiol, thioglycollate, or prereduced anaerobically sterilized BHIB when these were compared to TSB.[36]

Biphasic medium blood culture bottles have been shown to increase or accelerate the detection of bacteria and fungi[14,31]; however, they have posed considerable problems in production and transportation. These problems were resolved by the introduction of the Roche SeptiChek™ blood culture bottle, which has an attachable agar-containing slide chamber and allows repeated subcultures to be made rapidly.[17]

The Isolator™ has provided significantly more rapid and more frequent detection of septic episodes due to fungi and bacteria, including those occurring in patients receiving antibiotics, than conventional broth and biphasic medium systems.[5,16,18] Disadvantages include significantly more frequent contamination and significantly less frequent detection of pneumococcal and anaerobic bacteremias.[16] The Isolator™ should, therefore, be used in conjunction with a broth bottle that is suitable for the recovery of pneumococci and anaerobic bacteria. Current practice at the Mayo Clinic is to process 30 ml of blood from adults for each of two to three separate cultures, as shown in Table 1–3.

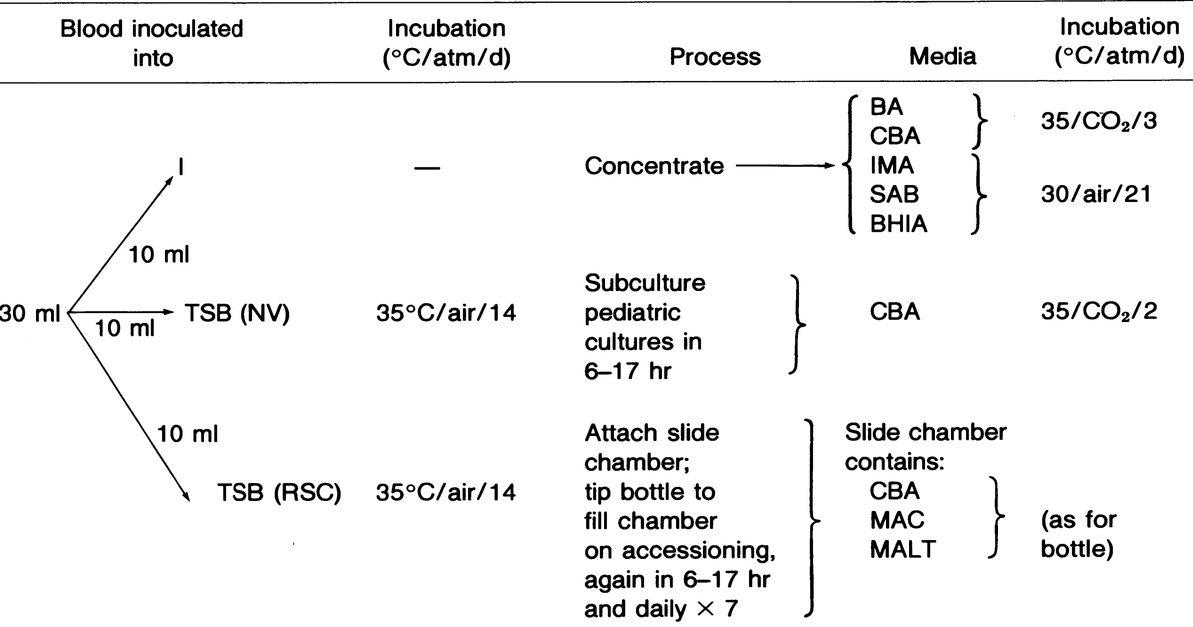
(2) Volume of Media

In general, blood should be diluted 1:10 (10% v/v) in broth with sodium polyanetholsulfonate (SPS) to neutralize the normal bactericidal properties of blood. A lesser (20% v/v) dilution in broth with SPS is adequate as long as two separate blood cultures are obtained.[2]

(3) Atmosphere of Incubation

Because of different optimal atmospheric growth conditions of bacteria and fungi,[19] blood should routinely be inoculated into media that are

Table 1–3. Mayo Clinic Blood Culture Procedure[a]



Blood inoculated into	Incubation (°C/atm/d)	Process	Media	Incubation (°C/atm/d)
30 ml → 10 ml → I	—	Concentrate →	BA CBA	$35/CO_2/3$
			IMA SAB BHIA	30/air/21
30 ml → 10 ml → TSB (NV)	35°C/air/14	Subculture pediatric cultures in 6–17 hr	CBA	$35/CO_2/2$
30 ml → 10 ml → TSB (RSC)	35°C/air/14	Attach slide chamber; tip bottle to fill chamber on accessioning, again in 6–17 hr and daily × 7	Slide chamber contains: CBA MAC MALT	(as for bottle)

[a] I, Isolator; TSB (NV), nonvented vacuum bottle containing 100 ml tryptic soy broth with 0.025% SPS and CO_2 (Difco Laboratories): TSB (RSC), vacuum bottle containing 70 ml tryptic soy broth with 0.05% SPS, CO_2, and SeptiChek slide chamber attachment (Roche); BA, blood agar; CBA, chocolate blood agar; IMA, inhibitory mold agar; SAB, Sabouraud dextrose agar; BHIA, brain heart infusion agar with blood; MAC, MacConkey agar; MALT, malt agar.

incubated aerobically (e.g., vented vacuum bottle) and anaerobically (e.g., nonvented vacuum bottle).

d. Additives

(1) Sodium Polyanetholsulfonate (SPS)

SPS serves as an anticoagulant but also has anticomplementary, antilysozymal, and antiphagocytic properties. Other properties of SPS include precipitation of β-lipoprotein, fibrinogen, and C_3, C_4, IgG and inhibition of aminoglycoside and polymyxin activity. SPS significantly increases the yield of bacteria from blood cultures.

SPS is inhibitory to some meningococci, gonococci, and *Peptostreptococcus anaerobius;* however, the inhibitory effect of SPS is neutralized by the inclusion of 1.2% gelatin in the medium.

(2) Sucrose or Sorbitol

Hypertonicity of blood culture media by the addition of sucrose has been reported by some investigators to increase the isolation rate of bacteria.[29] The value of sucrose may be medium or system dependent; however, studies at the Mayo Clinic comparing TSB with TSB containing sucrose or sorbitol and with BHIB containing sorbitol have not only failed to demonstrate any significant advantages of hypertonic media but have also demonstrated significant disadvantages of such media.[36]

(3) Penicillinase

There has been no recent study specifically addressing the value of adding penicillinase to blood culture media for more than 30 years. Nonetheless, the addition of penicillinase should inactivate high concentrations of penicillins in the medium following inoculation of blood from patients receiving high dosages of such antibiotics parenterally.

Contamination of penicillinase in preparation or in use may lead to spuriously positive blood cultures ("pseudosepticemia"). Therefore, when penicillinase is added, it should be tested concurrently for sterility.

Other means of antibiotic inhibition or neutralization include a 1:10 ratio of blood to broth, the presence of SPS in the medium, use of lysis-centrifugation (Isolator™), and, according to some studies, exposure of blood to antibiotic-adsorbent resins (ARD, Marion Scientific; BACTEC 16B or 17B, Johnston Laboratories).

2. Spirochetemia

a. Leptospirosis

(1) Collection

Leptospiremia is limited to the first 7 to 10 days of infection; therefore, cultures of blood should be restricted to this time interval. Darkfield

examination of blood is generally not recommended because of the formation of pseudospirochetes by red cell extrusions.

(2) Transport

Blood may be inoculated directly into the medium or be anticoagulated (by heparin, oxalate, or sodium polyanetholsulfonate but *not* citrate derivatives) for shipment to the laboratory with subsequent inoculation of the medium.

(3) Medium

Four tubes, each containing 5 ml of Fletcher's or EMJH semisolid medium, are inoculated with 0.05 ml of blood.

(4) Other

Urine should be cultured after the first 10 days of illness. Blood should be obtained for serological testing.

b. Borreliosis (Relapsing Fever)

Blood should be collected during febrile periods to prepare thin and thick films (see p. 32) for microscopic examination. As the number of relapses become shorter and milder, the number of circulating spirochetes also decrease and may be impossible to detect. *Borrelia* may be cultured in Kelly's medium.[23]

c. Lyme Disease

The *Ixodes dammini* spirochete, *Borrelia burgdorferi*, which has been found to cause this disease, can be recovered infrequently and only with great difficulty in a modified Kelly's medium[20] from blood, skin, or cerebrospinal fluid. A serological test is under investigation.

3. Parasitemia

a. Etiology

Sporozoa
- *Plasmodium vivax*
- *P. falciparum*
- *P. malariae*
- *P. ovale*
- *Babesia microti*

Microfilariae
Wuchereria bancrofti
Brugia malayi
Loa loa
Dipetalonema perstans
Mansonella ozzardi
Hemoflagellates
Trypanosoma brucei
T. cruzi
T. rangeli

b. Collection

The time of collection of blood for preparing smears depends on individual circumstances and the urgency of the situation. In patients suspected of having malaria, the optimal time is midway between febrile paroxysms; the least optimal time is during or immediately following febrile paroxysms when species differentiation is difficult to make because the red cells have ruptured and freed the parasites. *Wuchereria* and *Brugia* have nocturnal periodicity so that the microfilariae are most likely to be found in smears of blood collected between 10 P.M. and 2 A.M. *Loa loa,* on the other hand, has diurnal periodicity, so that the microfilariae are best found in smears of blood collected around noon.

Smears may be prepared from blood obtained by finger or ear lobe puncture or by venipuncture. Blood should not be anticoagulated when it is to be examined for *Plasmodium.* Smears must, therefore, be prepared at bedside. Blood may be anticoagulated when it is to be examined for filariae or trypanosomes. When alcohol is used as a skin antiseptic, it should be allowed to dry or wiped off to prevent its acting as a fixative for the blood films.

c. Preparation of Films

(1) Slide

Glass microscope slides must be free of all grease, dust, and residue of the manufacturing process. They should be cleaned with alcohol and stored in dust-free containers.

(2) Thick Film

The thick film may be prepared with the first drop from a syringe or, most often, from a drop of blood exuding from the puncture wound of the skin.

Holding the clean glass slide along the edges to prevent smearing its surface with oil from the fingers, the slide is touched to the drop of

blood. The flow of blood will determine whether an adequate thick film can be made from a single large droplet or whether several smaller pooled droplets are required. With the corner of another clean glass slide, the blood on the slide is then stirred and spread in a circular motion to form a film approximately the size of a dime. The blood must "wet" the glass surface to produce a film that will withstand drying and will not float away in the staining solution. Wetting is best accomplished by scratching the carrier slide lightly with the corner of the stirring slide as the droplet is being spread. The thick film should be made at least ½ in. from either end of the carrier slide to facilitate its examination microscopically with the aid of a mechanical stage. Properly prepared, the film will then be many cell layers thick in its central area and consist of only a few layers or a single-cell layer at its periphery. It is recommended that two thick films be prepared.

Drying of the thick film should be gentle, and may be accomplished by placing the slide for a brief period in a 35°C incubator or by exposing it to gentle heat from a light bulb or hair dryer. Exposure to excessive heat, dust, and insects should be avoided. If drying occurs too rapidly or if the slide is left exposed too long to heat, the film will become brittle and will crack and be lost during staining. Dust can be avoided by allowing the slide to dry in a covered petri dish. If insects have access to the smear, they will eat the blood film and may deposit their excrement in the wet blood. Such fecal material may contain insect protozoa that, when seen stained under oil immersion, have been known to arouse great furor in the laboratory.

(3) Thin Film

Thin films are prepared by a technique identical to that used for any hematological purpose, requiring clean slides and smearing to produce a film no more than 1 or 2 cell layers in thickness, and with a feather-end to ensure an area having only a single cell layer. While drying, the thin film also should be protected from dust. Alcohol is used for its fixation.

Some workers prefer to prepare both thick and thin films on a single slide. Caution must be taken to avoid excessive handling of a single slide and to protect the thick film against fixation by alcohol vapors while the thin film is being processed. The thick film is protected by covering it with a soft paper towel or tissue. Both films are then placed into Giemsa staining solution.

d. Purpose of Thick and Thin Films

The essential purposes of the two types of smears, whether prepared on a single slide or on multiple slides, are to allow scanning of the thick film for organisms and examination of the thin film for details of organisms'

structures. Red blood cells will be "laked" in the thick film during the staining process so that only microorganisms, leukocytes, platelets, and cellular debris remain.

Fever in malarial patients is generally believed to reflect the concentration of plasmodia and to indicate that they have ruptured one or more red cells per 100,000 red cells. Scanning approximately 100,000 red cells in a thin film requires 30 min of careful viewing; in a thick film, this number of cells can be screened within 3 to 5 min. Thus, if only thin films are used for diagnosis, they should be studied for at least 30 min before being discarded as free of microorganisms.

B. Infections of Normally Sterile Body Fluids

1. Central Nervous System (CNS)

a. Etiology

The microbial etiology of CNS infections is very diverse; therefore, only those of major importance or frequent occurrence in the United States are listed.

(1) Bacterial meningitis

(a) Neonates: *Escherichia coli, Streptococcus agalactiae* (Lancefield's group B), other Enterobacteriaceae.
(b) Infants and children: *Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae.*
(c) Adults: *Neisseria meningitidis; Streptococcus pneumoniae, Staphylococcus aureus,* Enterobacteriaceae, *Pseudomonas aeruginosa, Listeria monocytogenes.*

(2) Aseptic Meningitis and Encephalitis

(a) Viral: enteroviruses (polio, Coxsackie, ECHO), mumps, arboviruses (St. Louis, Western equine, California), herpesvirus.
(b) Fungal: *Cryptococcus neoformans, Coccidioides immitis.*
(c) Other: *Mycobacterium tuberculosis, Leptospira interrogans, Toxoplasma gondii, Naegleria fowleri, Strongyloides stercoralis* (autoinfection).

(3) Intracranial Abscess

Microbiology varies by primary focus of infection and is often mixed. Prominent among bacteria causing brain abscesses are *Staphylococcus*

aureus; facultatively anaerobic, microaerophilic, and anaerobic strepto-cocci; *Bacteroides;* and *Nocardia asteroides.* Less commonly isolated from brain abscesses are Enterobacteriaceae, *Entamoeba histolytica,* and *Cladosporium.*

b. Specimen Collection and Transport

(1) Collection

(a) Cerebrospinal Fluid (CSF)

CSF must be collected aseptically. Specimen tubes in lumbar puncture (LP) trays should be checked to ensure that they are sterile, that they are free from nonviable but stainable bacteria, and that they are leakproof. It is suggested that tubes from randomly selected LP trays be checked by filling them with filter-sterilized CSF remaining from other laboratory tests and handling them as blind, unknown specimens submitted to the laboratory for microscopic examination and culture.

(b) Brain Abscess

Pus from a brain abscess should be placed into an anaerobic transport device (see Section II,B,3 above).

(c) Serum or Urine

Acute and convalescent sera should be collected from patients with aseptic meningitis or encephalitis for appropriate serological studies (Table 1–2). Serum and/or urine may also be collected for testing for the presence of antigens of *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* (see Ch. 4.3).

(2) Volume

See Table 1–2.

(3) Transport

Specimens should be sent to the laboratory as rapidly as possible. Care should be taken to avoid refrigeration of specimens for culture.

2. Other Fluids

Pleural, pericardial, peritoneal, and synovial fluids should be collected in sufficient volume to ensure adequate examination.

C. Wounds

1. Etiology

Wound infections, whether endogenous or exogenous in origin, are usually due to bacteria and only occasionally to mycobacteria or fungi.

a. Traumatically Acquired Wound Infections

Posttraumatic wound infections commonly are associated with staphylococci and streptococci; however, those following contamination with soil, vegetation, or water are frequently polymicrobial and associated with gram-negative bacilli including *Escherichia coli, Klebsiella, Enterobacter, Proteus, Serratia,* and *Pseudomonas.*[35] Posttraumatic wound infections due to histotoxic clostridia occur infrequently today, but there has been increasing recognition of the importance of nonsporulating anaerobic bacteria, notably *Bacteroides* and anaerobic cocci, in such situations.

b. Postoperative Wound Infections

Predominant in this group are *Staphylococcus aureus, Escherichia coli, Pseudomonas aeriginosa, Serratia marcescens* enterococcal group D streptococci, and *Bacteroides fragilis.* Polymicrobial infections including *Bacteroides* and anaerobic cocci are most frequent following colorectal and gynecological surgery.

2. Specimen Collection

a. Avoid Contamination

Previously opened and chronically draining wounds and sinus tracts often become contaminated with flora indigenous to the skin, mucous membranes, and environment. There is often a poor correlation between cultures of sinus tracts and those of operative specimens in chronic osteomyelitis.[28] Biopsy of chronic lesions is often necessary to obtain material for culture and histopathological examination with special stains.

b. Obtain Sufficient Material for Examination

Swabs rarely contain sufficient material for both microscopic examinations and cultures; therefore, pus and exudate should be aspirated with a needle and syringe and sent to the laboratory promptly or injected into an anaerobic transport vial (see p. 21). Biopsies may be necessary when drainage is chronic or minimal. Quantitative smears and cultures of biopsies of

burn wounds and thoroughly debrided operative or traumatically acquired wounds may have prognostic value relative to the risk of developing sepsis or perhaps in some cases to predict advisability of wound closure or skin grafting (see Ch. 3).

D. Upper Respiratory Tract Infections

1. Etiology

The causes of infections in the upper respiratory tract are diverse and are listed according to the most likely clinical syndrome with which they are known to be associated (Table 1–4). It should be noted that there is no clear association between non-group A β-hemolytic streptococci, *Staphylococcus aureus, Haemophilus influenzae, Neisseria meningitidis,* or Enterobacteriaceae and pharyngitis or tonsillitis. It should also be noted that orolaryngeal lesions may occur in the acute and chronic forms of blastomycosis, paracoccidioidomycosis, histoplasmosis, leishmaniasis, and tuberculosis.

2. Specimen Collection

A throat swab suffices in the majority of instances for cultures for bacteria, mycoplasmas, and viruses (Table 1–2); however, *Bordetella pertussis* is preferentially detected by immunofluorescent staining and culture of nasopharyngeal secretions aspirated with a soft rubber catheter. Also, respiratory syncytial virus (RSV) is significantly more frequently isolated from children from a nasal wash than from a nasopharyngeal swab.[12]

The microbial etiology of sinusitis and of otitis media can only be established accurately by direct aspiration of the middle ear (tympanocentesis) or the sinus cavity.

Sputum may be examined for the eggs of *Paragonimus westermani* (lung fluke) and protoscolices and hooklets of *Echinococcus* from hydatid cysts that may have ruptured in the lungs, as well as for structures suggesting the presence of pulmonary amebiasis, ascariasis, strongyloidiasis, hookworm larvae, and occasionally *Pneumocystis carinii.*

Biopsies of oral lesions, particularly those of chronic duration, may be necessary to rule out malignancy, to provide information regarding the nature of the inflammatory process, and to prompt preparation of special stains for detecting mycobacteria, fungi, and parasites. Blood cultures are suggested in cases suspected of having *Haemophilus influenzae* epiglottitis and cellulitis because this species is normally present in the oropharynx of many children and attempting to swab the pharynx of a child with epiglottitis may precipitate acute airway obstruction. Blood cultures nearly always yield *H. influenzae* in such cases.

Table 1-4. Etiology of Acute Upper Respiratory Tract Infections

Clinical syndrome	Etiological agents
Common cold Coryza Rhinitis Rhinopharyngitis Catarrhal tonsillopharyngitis	Picornaviruses Rhinovirus Coxsackievirus Echovirus Myxovirus Parainfluenza Respiratory syncytial virus (RSV) Adenovirus *Bordetella pertussis*
Acute necrotizing ulcerative gingivitis (Vincent's)	Spirochetes and fusobacteria
Gingivostomatitis	Herpesvirus Coxsackievirus type A Adenovirus *Candida albicans*
Pharyngitis-tonsillitis	*Streptococcus pyogenes* *Corynebacterium diphtheriae* *Neisseria gonorrhoeae* Epstein-Barr (EB) virus Adenovirus Herpesvirus Myxovirus, paramyxovirus *Mycoplasma pneumoniae*
Acute epiglottitis Laryngitis Laryngotracheobronchitis	*Haemophilus influenzae* *Corynebacterium diphtheriae* *Bordetella pertussis* *Streptococcus pneumoniae* *Streptococcus pyogenes* Viruses Parainfluenza Influenza Respiratory syncytial virus (RSV)
Acute otitis media	*Streptococcus pneumoniae* *Streptococcus pyogenes* *Haemophilus influenzae* *Branhamella catarrhalis* *Staphylococcus aureus* *Staphylococcus epidermidis* *Escherichia coli* *Klebsiella pneumoniae* *Pseudomonas aeruginosa*
Serous and mucoid otitis media	*Haemophilus influenzae* *Streptococcus pneumoniae* *Staphylococcus epidermidis*

Table 1–4 (*continued*)

Clinical syndrome	Etiological agents
	Staphylococcus aureus *Mycoplasma pneumoniae*
Sinusitis	*Haemophilus influenzae* *Streptococcus pneumoniae* *Streptococcus,* nonpneumococcal *Branhamella catarrhalis* *Escherichia coli* *Pseudomonas aeruginosa* *Bacteroides* *Fusobacterium* Rhinoviruses Influenza virus Parainfluenza virus *Penicillium*

E. Lower Respiratory Tract Infections

1. Etiology

a. Pneumonia

Bacteria
- *Streptococcus pneumoniae*
- *Staphylococcus aureus*
- *Klebsiella pneumoniae*
- Enterobacteriaceae, other species
- *Pseudomonas aeruginosa*
- *Haemophilus influenzae*
- *Legionella* sp.
- *Neisseria meningitidis*
- *Branhamella catarrhalis*
- *Streptococcus pyogenes*
- *Actinomyces israelii*
- *Bacillus anthracis*
- *Francisella tularensis*
- *Pseudomonas pseudomallei*
- *Nocardia asteroides*

Mycobacteria
- *Mycobacterium tuberculosis*
- *Mycobacterium,* other species

Chlamydiae
- *Chlamydia trachomatis*
- *Chlamydia psittaci*

Mycoplasmas
- *Mycoplasma pneumoniae*

Rickettsiae
- Q fever

Fungi
- *Histoplasma capsulatum*
- *Coccidioides immitis*
- *Aspergillus fumigatus*
- *Candida albicans*
- *Cryptococcus neoformans*
- *Zygomycetes*

Protozoa (?)
- *Pneumocystis carinii*

Viruses
- influenza
- parainfluenza
- respiratory syncytial
- adenovirus
- cytomegalovirus
- herpes simplex
- variola
- varicella
- measles

b. Lung Abscess

Bacteroides
Fusobacterium
Anaerobic cocci
Nocardia asteroides
Pseudomonas pseudomallei
Entamoeba histolytica
Staphylococcus aureus
Streptococcus pyogenes
Klebsiella pneumoniae
Pseudomonas aeruginosa
Streptococcus pneumoniae (type 3)

2. Specimen Selection and Collection

The type of specimen required for establishing the diagnosis is usually predicated on the suspected diagnosis based on the clinical presentation,

physical findings, laboratory and radiographic findings, occupational and travel history, age, underlying conditions and diseases, and prior history of antimicrobial, antimetabolite, or radiation therapy.

Guidelines for specimen selection are listed in Table 1–5.

a. Specimen Type

(1) Expectorated Sputum

Patients should be instructed to rinse their mouths with water and then to expectorate a specimen resulting from a deep cough. Specimens grossly or microscopically consisting primarily of saliva (see Ch. 2) are *not* acceptable for bacterial culture. Single, early morning, expectorated specimens are also suggested for mycobacterial and fungal cultures; 24-hr sputum collections are unacceptable for this purpose.

(2) Induced Sputum

Sputum to be examined for mycobacteria and fungi may be induced from patients with unproductive coughs by ultrasonic nebulization with 10% NaCl solution.

(3) Bronchoscopy Specimens

Bronchial washings and brushings or transbronchial biopsies may be obtained by bronchoscopy. By using telescoping double catheters with distal occlusion, it is possible to obtain bronchial secretions that are not contaminated by oropharyngeal flora.[38]

(4) Tracheostomy Aspirate

Readily available from patients with tracheostomies, these specimens pose great problems in interpretation. Neither cytological studies nor quantitative microbiology are of any help in interpreting results of cultures of tracheostomy aspirates.[4]

(5) Transtracheal Aspirate (TTA)

TTA provides a specimen devoid of oropharyneal contamination and is especially suitable when studies of expectorated sputum are inconclusive, in patients with severe, usually nosocomially acquired, pneumonias in which invasive techniques are necessary to establish an etiology, and in patients suspected of having anaerobic pleuropulmonary infections.

(6) Lung Puncture

Percutaneous needle aspiration of the lung has been used to establish the etiology of lower respiratory infections in infants and children with minimal complications.[24]

Table 1–5. Specimens for Isolation or Identification of Lower Respiratory Tract Pathogens[a]

Organism	Specimen	Test[b]			
		Microscopy	Culture	Serology	Other
Bacteria	Expectorated sputum, infralaryngeal aspirate, empyema fluid, lung biopsy	Gram stain	X		
Legionella sp.	Lung biopsy, pleural fluid, expectorated sputum, serum	IFA or Dieterle stain	X	Micro-IFA	
Nocardia	Expectorated sputum, infralaryngeal aspirate, bronchial washings, tissue	Gram and/or carbol fuchsin stain	X		
Chlamydia	Nasopharyngeal swab, lung aspirate or biopsy, serum		X	Micro-IFA, CF	
Mycoplasma	Expectorated sputum, nasopharyngeal swab, serum		X	CF, IFA, or MI; cold agglutinins	
Mycobacteria	Expectorated or induced sputum, tissue, gastric washings	Carbol fuchsin or fluorochrome stain	X		PPD
Fungi					
Deep-seated					
Blastomyces	Expectorated or induced sputum, bronchial washing or biopsy, tissue, serum	KOH with phase-contrast; GMS stain	X	CF, ID	
Coccidioides				CF, ID, LA	
Histoplasma				CF, ID	
Opportunistic					
Aspergillus	Lung biopsy, serum	H & E, GMS stain	X	ID	
Cryptococcus	Expectorated sputum, serum	H & E, GMS stain	X	LA	
Zygomycetes	Expectorated sputum, tissue	H & E, GMS stain	X		
Viruses	Nasal washings, nasopharyngeal swab, serum		X	IFA	
Pneumocystis	Lung biopsy, infralaryngeal aspirate, bronchial brushings or washings; expectorated sputum from AIDS patients	Toluidine blue, Giemsa, or GMS stain			

[a] From Bartlett, J. G., Brewer, N. S., and Ryan, K. J. *Cumitech 7: Laboratory Diagnosis of Lower Respiratory Tract Infections* (Washington, J. A., II, Coordinating ed.). Washington, D.C., American Society for Microbiology, 1978.

[b] IFA, Immunofluorescent antibody; CF, complement fixation; ID, immunodiffusion; LA, latex agglutination; MI, metabolic inhibition; CIE, counterimmunoelectrophoresis; GMS, Gomori methenamine silver.

(7) Open Lung Biopsy

Generally, performed in the immunocompromised host with an otherwise undiagnosed pulmonary infection, open lung biopsy provides a specimen of adequate size, obtained aseptically under direct visualization. Such specimens, also obtained at some risk and considerable expense to the patient, require special attention and should undergo both histopathological and microbiological examination (Table 1–6). The results of examination of the frozen section and specially stained smears are available within 3 hr of the specimen's arrival in the laboratory and should be reported to the patient's physician by phone. A specific protocol is essential to ensure that all of the necessary tests are carried out.

(8) Pleural Fluid

Pleural fluid, when present, may yield the etiological agent in many types of pulmonary infection.

(9) Blood Culture

Blood cultures may be helpful in establishing the diagnosis of bacterial and fungal pneumonias.

(10) Serum

Serum may be submitted for serological testing (Table 1–2).

Table 1–6. Method for Processing Open Lung Biopsy Specimens

Lung specimen

Surgical pathology	Clinical microbiology	
Frozen section		
Fixed section	*Stain*	*Cultures*
hematoxylin-eosin silver chromate Gram, auramine-rhodamine, and other stains as indicated	Gram (bacteria) potassium-hydroxide (fungi) auramine-rhodamine (mycobacteria) touch preparation toluidine-blue 0 or silver chromate (*Pneumocystis carinii*) fluorescent antibody (*Legionella*) modified Kinyoun (*Nocardia*)	general bacteria anaerobic bacteria fungi mycobacteria *Actinomyces* *Nocardia* *Legionella* virus

F. Urinary Infection

1. Etiology

Escherichia coli
Klebsiella pneumoniae
Enterobacter
Serratia marcescens
Proteus mirabilis
Providencia
Pseudomonas aeruginosa
Streptococcus, enterococcal group D
Staphylococcus saprophyticus
Candida albicans
Mycobacterium tuberculosis

2. Specimen Collection

a. Clean Voided Midstream

This is a convenient, practical, and economical approach that is reliable when performed properly. In females, the procedure is preferably performed by a trained aide who, holding the labia apart, cleanses the urethral meatus and labia minora with dilute hexachlorophene solution, instructs the patient to void, and collects a midstream specimen for microscopic examination and culture. In males, the glans is cleansed with dilute hexachlorophene solution, and a midstream specimen is then collected.

Clean voided midstream urine specimens are suitable for bacterial, mycobacterial, fungal, and viral cultures. Twenty-four hr collections are not acceptable for examination for *Schistosoma haematobium* and microfilariae, especially *Onchocerca volvulus*. A single clean voided urine specimen following prostatic massage may be examined for *Trichomonas vaginalis*.

b. Urethral Catheterization

This approach is useful when the patient is unable to void or when a catheter is being inserted for diagnostic purposes or to relieve obstruction; however, it is associated with a small but definite risk of infection.

c. Suprapubic Aspiration (SPA) (Figure 1–3)

SPA is preferred for collecting urine from infants and small children; it is the only acceptable specimen for anaerobic culture of urine; and it is

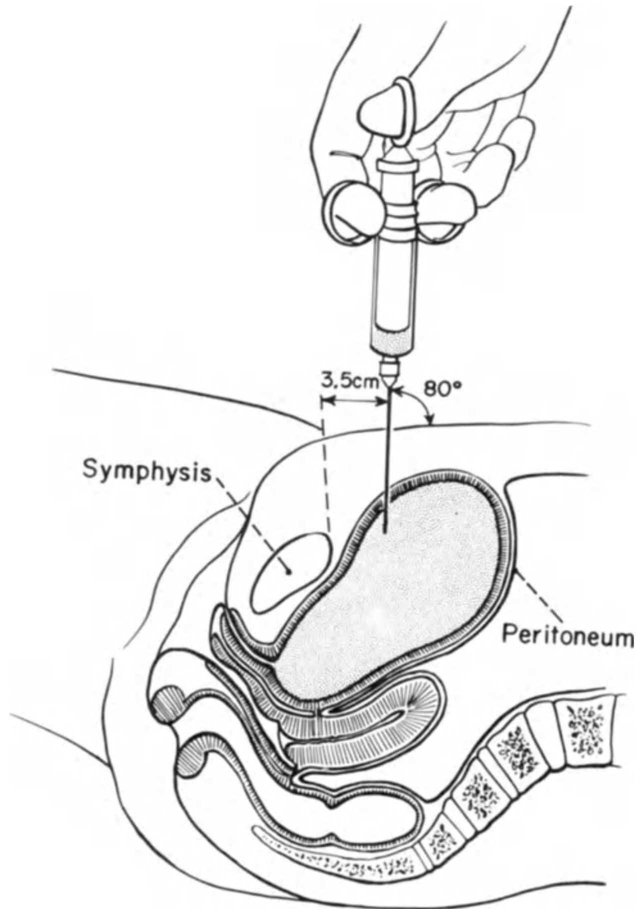


Figure 1–3. Sagittal view of anatomical structures and suprapubic bladder aspiration in the female. (Courtesy of J. W. Segura, M.D., Mayo Clinic.)

helpful in instances of inconclusive midstream cultures. There is minimal risk of complications.

d. Localization Studies

(1) Segmented Urine Cultures for Diagnosis of Prostatitis[34]

Quantitative cultures are made sequentially of an initially voided 10 ml of urine; the midstream urine; secretions following prostatic massage; and voided urine (10 ml) following massage.

Interpretation:
Prostatitis is indicated by higher colony counts in prostatic secretion and

finally voided urine cultures than in initially voided and midstream urine cultures.

(2) Bladder Washout for Differentiation between Renal and Bladder Bacteriuria (Fairley Test[11])

(a) Insert indwelling urethral catheter.
(b) Collect residual urine for quantitative culture.
(c) Instill 50 ml saline containing neomycin (40 mg), polymyxin (20 mg), and a mixture of fibrinolysin and deoxyribonuclease (Elase, Parke-Davis) into bladder.
(d) Clamp catheter for 30 min.
(e) Irrigate bladder with 2000 ml sterile water.
(f) Collect three consecutive urine specimens at 10-min intervals for quantitative cultures.

Interpretation:

Bladder bacteriuria
Prewashout residual urine: $>10^5$ CFU/ml
Postwashout urine: negative

Renal bacteriuria
Prewashout residual urine: $>10^5$ CFU/ml
Postwashout urine: $>3\times10^3$ CFU/ml

(3) Cystoscopic Differentiation between Renal and Bladder Bacteriuria (Stamey Test[34])

(a) Insert cystoscope into bladder.
(b) Collect bladder urine ("CB") for quantitative culture.
(c) Introduce ureteral catheters into bladder, irrigate with 50 to 100 ml irrigating fluid and collect through ureteral catheters ("WB" for "washed bladder") for quantitative culture.
(d) Pass ureteral catheters to renal pelves.
(e) Collect four consecutive paired specimens (5 to 10 ml in volume) from ureteral catheters (label LK_1, RK_1, LK_2, RK_2, etc.) for quantitative cultures.

Interpretation:
Bilateral renal bacteriuria: $>10^5$ CFU/ml in RK and LK.
Unilateral renal bacteriuria: $>10^2$ CFU/ml in RK or LK; 0 CFU/ml in contralateral kidney urine.
Bladder bacteriuria: $>10^5$ CFU/ml in CB; 0 to $<10^3$ CFU/ml in WB; 0 CFU/ml in RK and LK.

3. Specimen Transport

Urine is an excellent culture medium. Urine should, therefore, be cultured within 2 hr of collection or stored at 5°C if the interval between collection and culture exceeds 2 hr.

G. Gastroenteritis

1. Etiology

The known causes of gastroenteritis are diverse (Table 1–7) and are expanding rapidly as new techniques are applied to the detection of microbial antigens, antibodies, and toxic properties (i.e., enterotoxigenicity and invasiveness). Host, environmental factors, and travel history may be helpful in assessing the most likely cause of gastroenteritis; however, symptoms are often nonspecific.

2. Specimen Collection and Transport for Bacteria

Stool examination is generally reserved for patients with serious or unremitting illness. Moreover, practicality limits stool examination to certain bacteria, especially *Salmonella, Shigella, Campylobacter,* and *Yersinia.* One approach to the patient with gastroenteritis is depicted by DuPont in an algorithm (Figure 1–4).

A single negative examination of feces has limited value, and it is recommended that a minimum of three separate specimens be examined before ruling out diseases due to bacteria.

Optimally, freshly passed feces should be transported immediately to the laboratory for microscopic examination or culture. Rectal swabs are convenient and practical, particularly for epidemiological investigations; however, the yield of enteric bacterial pathogens from cultures of this type of specimen is less than from cultures of freshly passed feces. Acidification, which occurs during storage of feces, is detrimental to the survival of enteric pathogens; therefore, specimens for culture of *Salmonella* and *Shigella* that require storage overnight or longer or that must be shipped should be placed into buffered glycerol–saline transport medium. Specimens for culture of *Campylobacter* should be refrigerated. Rectal swabs may be transported in Stuart medium.

3. Collection and Transport for Intestinal Parasites

a. Number of Specimens

Three separately collected diarrheal or saline-purged (from the patient without diarrhea) specimens should be examined to rule out infection

Table 1–7. Etiologic Agents of Gastroenteritis

Cause of disease			Symptoms			
Organism	Mechanism	Incubation	Fever	Vomiting	Diarrhea	Circumstances
Staphylococcus aureus	Preformed toxins	2–4 hr	0	+++	++	Poorly refrigerated pastries, meats, custard, salads, sandwiches
Clostridium perfringens	Enterotoxins following colonization	12–16 hr	0	±	+++	Preparation of large quantities of food; partially cooked meat, gravy, stew
Clostridium difficile	Exotoxin cytotoxin produced by increased numbers of antibiotic-resistant strains	—	+	±	+++	Antibiotic-associated diarrhea and colitis
Bacillus cereus	Enterotoxin (LT) following colonization	10–12 hr	0	±	+++	Improper holding conditions of sausage, vegetable dishes, cream pastry, meat or vegetable soup, meat and meat products
Bacillus cereus	Preformed toxin (?)	1–5 hr	0	+++	±	Consumption of rice
Vibrio cholerae	Enterotoxin (LT) following colonization	24–120 hr	0	+++	+++	Contaminated food or water; travel to endemic areas (Asia, India-Pakistan-Bangladesh subcontinent, Middle East)
Escherichia coli (ETEC)	Enterotoxin (LT and or ST) following colonization	1–36 hr	±	+	+++	Contaminated foor or water; travel to endemic areas (Mexico, Kenya, Bangladesh)
Escherichia coli (EIEC)	Invasion and multiplication (*Shigella*-like)	24–48 hr	+++	±	+++ (bloody)	
Escherichia coli (EPEC)	(?) (*Salmonella*-like)	24–48 hr	+++	±	+++	Infantile or intractable diarrhea
Shigella	Invasion and multiplication	24–120 hr	+++	±	+++	Predominantly in young children;

					(bloody)	contaminated food or water; infected food handler
Salmonella	Mucosal and lamina propria invasion	6–48 hr	++	++	++	Insufficiently cooked meat, poultry, or egg products; contamination by cutting surfaces and utensils; infected food handler
Campylobacter jejuni	Invasion	48–240 hr	+	±	+++ (bloody)	Not well defined
Vibrio, halophilic species	Invasion	10–20 hr	±	++	+++ (often bloody)	Seafood, especially shellfish
Yersinia enterocolitica	Lamina propria invasion, mesenteric lymphadenitis; enterotoxin (ST)	10 hr	+	±	+++ (often bloody)	Not well defined
Aeromonas hydrophila	Enterotoxin (?)	24–48 hr	±	+	+++	Not well defined
Parvovirus (Norwalk-like virus)	Lamina propria inflammation, shortened microvilli	24–48 hr	+	++	++	
Rotavirus	Shortened, blunted microvilli; patchy irregularities in small bowel mucosa	24–72 hr	±	++	++	Predominantly in infants and young children (6 mos. to 2 yrs.)
Protozoa						
Entamoeba histolytica	Cytolytic enzymes, ingestion of red cells; intestinal ulcer	1–14 weeks	+	0	+++	Cysts in contaminated water or food; food handlers; sexually transmitted
Dientamoeba fragilis	Occasionally ingests red cells	1–2 weeks	0	±	++	Trophozoites in contaminated water and food, food handlers; sexual transmission
Giardia lamblia	Adhesive disk allows organism to attach to absorptive surface of intestinal cells, blocking absorption of fats	1–2 weeks	0	0	++++	Cysts in contaminted water or food; food handlers; sexual transmission

Table 1–7 (*continued*)

Cause of disease			Symptoms			
Organism	Mechanism	Incubation	Fever	Vomiting	Diarrhea	Circumstances
	and vitamins, "malabsorption syndrome"					
Balantidium coli	Cytolytic enzymes, intestinal ulcer	1–2 weeks	+	0	++++	Cysts in contaminated water or food, food handlers, close association with hogs
Isospora hominis *Isospora belli*	Sporozoites enter intestinal epithelial cells, villous atrophy, "malabsorption syndrome"	7–16 days	+	0	+++	Oocysts in contaminated water or food, food handlers
Cryptosporidium sp.	Sporozoites invade the microvillus of small intestinal epithelial cells, villous atrophy, infiltration of lamina propria of jejunum and ileum, "malabsorption syndrome"	5–7 days	+	+	++++	Oocysts in feces of animals (especially calves and lambs), food and water, sputum, direct human to human; sexual transmission
Plasmodium vivax *Plasmodium ovale* *Plasmodium malariae*	Hemolysis, hemoglobinemia, bilirubinemia may be produced by all four species, capillary plugging especially by *P. falciparum*	10–17 days	++++	±	±	Anopheles mosquito transmission in endemic areas of the world, blood transfusion, narcotic addicts using contaminated needle and syringe ("New York or mainliner Anopheles," congenital disease)
Plasmodium falciparum			++++	++	++	
Nematodes						
Trichinella spiralis	Young worms migrating into intestinal wall	1–3 days	++++	++++	++++	Poorly cooked meat (pork, bear, walrus, seal)
Trichuris trichiura	Heavy infections with pene-	3–10 days	+	++	+++	Ingestion of embryonated eggs

	tration of intestinal mucosa may produce anemia, rectal prolapse, and secondary bacterial infections					found in contaminated soil, hands, food, or drink
Ancylostoma duodenate *Necator americanus*	In heavy hookworm infections, attachment and maturation of the worms in the intestinal mucosa	2–6 weeks	±	0	++++	Infection occurs when skin penetrating filariform larvae are contacted in soil contaminated with feces
Strongyloides stercoralis	Heavy infections with penetration of intestinal mucosa may produce anemia, chronic dysentery, and the danger of autoinfection and secondary bacterial infection as filariform larvae penetrate the intestinal wall to carry enterics to blood stream	28 days	±	++	++++	Primary infection occurs when skin penetrating filariform larvae are contacted in soil contaminated with feces; autoinfection can occur in the intestinal tract as rhabditiform larvae become filariform and penetrate to blood stream; sexual transmission
Capillaria philippinensis	Heavy infections with penetration of intestinal mucosa may produce emaciation, cachexia, abdominal distention, "malabsorption syndrome"; there is the suggestion that autoinfection can occur as with *Strongloides stercoralis*	1–2 weeks	+	+	++++	Primary infection results from ingestion of uncooked fish, massive infection would appear to be the result of autoinfection
Anasakis sp.	Invasion of intestinal mucosa by larval worms produces "eosinophilic gastroenteritis" and eosinophilic granuloma	1–3 days	±	+	±	Ingestion of uncooked marine fish harboring larval worms that are unable to develop further in human host

Table 1–7 (*continued*)

Cause of disease			Symptoms			
Organism	Mechanism	Incubation	Fever	Vomiting	Diarrhea	Circumstances
Cestodes						
Diphyllobothrium latum	Toxic secretions probably from degenerating proglottids, mucosal irritation, especially in instances where more than one worm is present, vomiting of segments	3–5 weeks	0	++++	±	Ingestion of uncooked freshwater fish
Hymenolepis nana	In heavy infections, an enteritis is produced as worms invade the intestinal mucosa; autoinfection resulting in massive worm burden is possible	10–12 days	0	++++	±	Primary infection occurs when eggs are ingested from contaminated hands, food, water, or insects, no intermediate host is required, eggs are immediately infective when passed in feces, can also hatch in intestine producing autoinfection and increased worm burden
Trematodes						
Schistosoma mansoni *Schistosoma japonicum*	These intestinal blood flukes take residence in the mesenteric veins (*S. mansoni*—mesenteric, *S. japonicum*—superior-mesenteric), where the walls of the intestine are then invaded with eggs, producing intense cellular infiltration leading to fibrosis and granulomas	7–8 weeks	+	0	++++	Skin penetrating cercariae from freshwater snails develop into adult egg producing worms; swimming, bathing, or working in such water.

Fasciola hepatica *Fasciola gigantica*	Toxic metabolic products produce inflammation of biliary tract and can lead to biliary obstruction	10–12 weeks	+	0	++++	Ingestion of aquatic plants (watercress) or contaminated water harboring encysted metacercariae from freshwater snails; cattle often serve as reservoir hosts.
Fasciolopsis buski	Areas of inflammation and ulceration occur at sites where the fluke attaches and elaborates toxic metabolic products	25–30 days	±	±	++	Ingestion of aquatic plants (raw water nuts) or contaminated water harboring encysted metacercariae from freshwater snails; hogs often serve as reservoir hosts
Small trematodes *Heterophyes heterophyes*	The intestinal flukes (*Heterophyes, Metagonimus, Echinostoma,* and *Gastrodiscoides*) produce lesions and pathology similar to the above for *Fasciolopsis buski;* the liver flukes (*Clonorchis, Opisthorchis* and *Dicrocoelium*) produce inflammation, etc., similar to the above for *Fasciola hepatica*	7–10 days	±	0	++	*Heterophyes, Metagonimus, Echinostoma, Clonorchis,* and *Opisthorchis* metacercariae are harbored by freshwater fish; ingestion of these uncooked fish produces infection. *Gastrodiscoides* metacercariae are encysted on aquatic vegetation, which when eaten raw produces infection. *Dicrocoelium* metacercariae are harbored by an ant, *Formica fusca,* which when ingested produced infection.
Metagonimus yokogawl		7–10 days	±	0	++	
Echinostoma ilocanum		10–14 days	±	0	±	
Gastrodiscoides hominis		10–14 days	±	0	±±	
Clonorchis sinensis		3–4 weeks	±	0	++	
Opisthorchis felineus		3–4 weeks	±	0	++	
Opisthorchis viverrini		3–4 weeks		0	++	
Dicrocoelium dentriticum		1–2 weeks		+	+++	

Table 1–7 (*continued*)

Cause of disease			Symptoms			
Organism	Mechanism	Incubation	Fever	Vomiting	Diarrhea	Circumstances
Acanthocephalids *Moniliformis moniliformis*	Local trauma and inflammation at the site of attachment to the intestinal wall and possible perforation of the intestine	3–4 weeks	0	±	++++	Ingestion of beetles or cockroaches harboring an intermediate larval worm; usual definitive hosts are rats, mice, and hogs
Arthropods a. Intestinal myiasis (larval flies)						
Musca Gasterophilus Sarcophaga Chrysomya Calliphora Fannia Stomoxys Muscina Piophila Drosophila Tubifera	Local trauma and inflammation, possibly toxic metabolic products elaborated by the feeding larvae	1–2 weeks	0	±	++++	Contaminated food or drinking water containing eggs or developing larvae of the flies
b. Intestinal canthariasis (larval beetles)						
Blaps Tenebrio Tribolium	As above for flies	1–2 weeks	0	±	++++	Contaminated drinking water, food (especially meal and flour—so-called "meal worms")

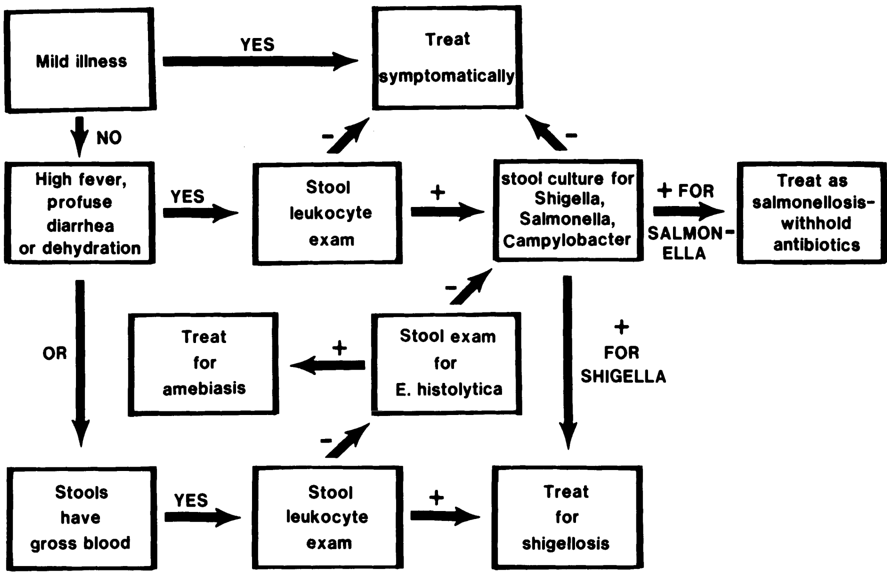


Figure 1–4. An algorithm for management of patients with acute diarrhea. (Modified from DuPont, H. L. Enteropathogenic organisms: New etiologic agents and concepts of disease. *Med. Clin. N. Amer.* **62:**945, 1978.)

due to intestinal parasites. Organisms, particularly *Giardia lamblia,* may only be present intermittently in feces. A negative report of examination of a single specimen of feces has limited value.

b. Precautions

Specimens should be free of urine, toilet water, and soil. Patients should not have taken antidiarrheal compounds, antacids or oily laxatives or have undergone barium studies of the intestinal tract for at least a week prior to examination.

c. Preparation for Collection

Unless contraindicated by the presence of diarrhea or another condition, purged specimens are desirable. The patient should receive a saline purgative, such as magnesium sulfate (Epsom salts) or buffered phosphosoda (Fleet's Phospho-Soda) on arising in the morning, followed by breakfast, and then collect the specimen at home or come to a specimen collection area (Figure 1–5). The area is equipped with a toilet containing a reusable plastic bowl (Speci-Pan, Scientific Products), measuring 7 cm in depth and 15 cm in diameter and having a tightly fitted lid to prevent leakage. The patient is instructed as to how to use the facilities, asked whether the saline purgative was taken, and questioned about recent foreign

Figure 1–5. Stool collection facility with toilet containing a reusable plastic bowl.

travel. After passing the specimen and sealing the container, the patient calls the technician by pulling the cord on the wall near the hand rail and passes the container through the window.

d. Storage and Transport of Specimens

When collection of a fresh specimen is not possible and storage for 1 or more days or mailing of the specimen is necessary, the laboratory should provide the patient with a wide-mouth, leakproof jar containing a preservative, polyvinyl alcohol (PVA) fixative (see Section II,B,5,b). *N.B.* Components include mercuric chloride and glacial acetic acid; therefore, container must bear POISON label. Expiration date must also be included.

H. Intraabdominal Infections

1. Etiology

Escherichia coli
Proteus
Klebsiella
Pseudomonas aeruginosa

Streptococcus, enterococcal group D
Staphylococcus aureus
Mycobacterium tuberculosis
Bacteroides fragilis
Bacteroides melaninogenicus
Bacteroides, other
Peptostreptococcus
Peptococcus
Clostridium
Eubacterium

2. Specimen Collection

Pus or other fluid should be aspirated into a syringe for transport directly to the laboratory or for injection into an anaerobic transport container.

I. Genital Tract Infections

1. Etiology

a. Sexually Transmitted Diseases (STD)

Neisseria gonorrhoeae
Chlamydia trachomatis
Ureaplasma urealyticum
Trichomonas vaginalis
Candida albicans
Herpes simplex virus
Treponema pallidum
Sarcoptes scabiei
Phthirus pubis
Gardnerella vaginalis (?)
Haemophilus ducreyi
Giardia lamblia
Entamoeba histolytica
Strongyloides stercoralis
Cryptosporidium

b. Female Genital Tract Infections Other than STD (Endomyometritis, Salpingitis, Pelvic Abscess, Bartholin Abscess, Vaginal Cuff Abscess, Wound Infection)

Bacteroides melaninogenicus
Bacteroides fragilis

Bacteroides, other
Fusobacterium
Peptococcus
Peptostreptococcus
Clostridium
Eubacterium
Escherichia coli
Enterobacteriaceae, other
Pseudomonas aeruginosa
Streptococcus
Staphylococcus aureus
Neisseria gonorrhoeae

c. Prostatitis

Escherichia coli
Klebsiella
Enterobacteriaceae, other
Pseudomonas aeruginosa
Streptococcus, enterococcal group D
Staphylococcus epidermidis (?)
Streptococcus, viridans group (?)
Corynebacterium (?)

2. Specimen Collection and Transport

a. STD

A swab of urethral exudate, distal urethral lumen, vagina, cervix, or anal crypts may be used for cultures for *Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasma urealyticum, Candida albicans,* herpes simplex virus, and *Haemophilus ducreyi.* Such swabs should be transported in an appropriate medium (e.g., modified Stuart's for bacteria, fungi, and viruses or sucrose–phosphate for chlamydiae and ureaplasmas) or should be used for immediate inoculation of Thayer–Martin medium for culture of *N. gonorrhoeae.* Swabs of the same sites listed above can be used to prepare smears for microscopic examination (wet preparations for fungi and *T. vaginalis* and Gram-stained smears for *N. gonorrhoeae* and *H. ducreyi*).

Swabs should be processed within 6 hr of specimen collection.[12] Specimens for isolation of herpes simplex virus should be refrigerated (not frozen) during storage, while those for *C. trachomatis* and *U. urealyticum* should be frozen for storage overnight or longer. Should Transgrow or JEMBEC (see p. 22) be inoculated for isolation of *N. gonorrhoeae,* it is

imperative that either of these devices be incubated as soon as possible and for a minimum of 18 hr if the cultures must be mailed to a reference laboratory. Failure to do so will result in a significant decrease in the isolation rate of *N. gonorrhoeae.*

In patients suspected of having primary or secondary syphilis, skin lesions should be cleaned thoroughly with saline-moistened gauze pads, dried, and abraded to produce a serous exudate for microscopic examination. *Skin lesions are infectious and must be handled with rubber gloves.* Exudate is applied directly to a glass coverslip, which in inverted onto a glass slide; the coverslip edges are sealed to prevent dessication. Slides are examined by darkfield microscopy; alternatively, a direct fluorescent antibody stain can be used.

b. Other Female Genital Tract Infections

The microbiological diagnosis of endometritis, salpingitis, and peritonitis (pelvic inflammatory disease, or PID) is complicated by the lack of direct access to the infected site or abscess. Because of its normally heavy and diverse indigenous microflora (Table 1–1), cultures of the endocervix should be limited to those for *Neisseria gonorrhoeae,* which is a frequent cause of PID, and group A β-hemolytic streptococci, which are rarely a cause of postpartum endomyometritis. Anaerobic bacterial cultures should not be done because of their normally heavy presence in this site. Material for Gram-stained smears and aerobic and anaerobic cultures should be obtained by culdocentesis, laparoscopy, or at the time of surgery if performed.

The microbial etiology of nonspecific vaginosis (NSV) remains unresolved but is most likely a mixed anaerobic bacterial infection. Although *Gardnerella vaginalis* is usually associated with NSV, it may be present in vaginal cultures of 30 to 40% of asymptomatic women. The diagnosis of NSV may be established without culture by the following findings in or characteristics of vaginal secretions: (1) thin, homogeneous, milk-like consistency; (2) the presence of clue cells microscopically; (3) pH >4.5; and (4) the release of a fishy or amine odor upon the addition of 10% KOH.[1]

J. Ocular Infections[21]

1. Etiology

a. Conjunctivitis

Bacteria

Streptococcus pneumoniae
Haemophilus influenzae

Staphylococcus aureus
Neisseria gonorrhoeae
Enterobacteriaceae
Streptococcus pyogenes
Pseudomonas aeruginosa
Bacillus
Actinomyces
Clostridium
Bifidobacterium
Eubacterium
Lactobacillus
Peptostreptococcus
Peptococcus
Propionibacterium
Chlamydia trachomatis

Viruses
Herpes simplex
Varicella-zoster
Adenoviruses
Poxviruses
Measles
Rubella

b. Corneal Infections

Bacteria
Streptococcus pneumoniae
Staphylococcus aureus
Staphylococcus epidermidis
Pseudomonas aeruginosa
Streptococcus pyogenes
Moraxella lacunata
Enterobacteriaceae
Bacillus
Corynebacterium
Haemophilus influenzae
Nocardia
Mycobacterium fortuitum-chelonei complex
Actinomyces
Peptococcus
Peptostreptococcus
Propionibacterium

Viruses
Herpes simplex

- Varicella–zoster
- Adenoviruses

Fungi
- *Aspergillus*
- *Penicillium*
- *Candida*
- *Cephalosporium*
- *Fusarium*
- *Blastomyces*
- *Sporothrix*
- *Curvularia*
- *Allescheria*

c. Intraocular Infections

Bacteria
- As for corneal infections
- *Bacteroides*
- *Fusobacterium*
- *Lactobacillus*
- *Veillonella*
- *Mycobacterium tuberculosis*

Spirochetes
- *Treponema pallidum*

Parasites
- *Toxoplasma gondii*
- *Onchocerca volvulus*

Viruses
- Herpes simplex
- Varicella–zoster
- Cytomegalovirus
- Poxviruses
- Adenoviruses
- Mumps
- Measles
- Epstein-Barr

Fungi
- Endogenous
  - *Aspergillus*
  - *Candida albicans*
  - *Cryptococcus neoformans*
  - *Blastomyces dermatitidis*
  - *Coccidioides immitis*
  - *Histoplasma capsulatum*

Zygomycetes
Sporothrix schenckii
Posttraumatic or postoperative
Cephalosporium
Paecilomyces
Candida
Aspergillus
Volutella
Cladosporium
Penicillium
Trichosporum
Sporothrix schenckii

2. Specimen Collection[21]

a. Conjunctivitis

(1) A swab moistened with broth should be used to obtain material from the conjunctiva and to inoculate directly to media suitable for cultures of bacteria and fungi or tissue cultures suitable for cultures of chlamydiae and viruses.
(2) The conjunctiva should be scraped with a sterile platinum spatula to prepare Gram- and Giemsa-stained smears for microscopic examination of the predominant cellular response and the group of the microorganism present. Herpesviruses and adenoviruses may be detected by staining smears with fluorescein-labeled specific conjugates.

b. Corneal Infections

(1) The conjunctiva is swabbed and cultured as described above.
(2) The cornea is anesthetized with 0.5% proparacaine hydrochloride.
(3) The base and margin of the corneal ulcer is scraped under slit lamp or operating scope visualization with a sterile platinum spatula.
(4) Scrapings are used to
 (a) Prepare slides for
  i. Gram stain
  ii. Giemsa stain
  iii. Potassium hydroxide (KOH) wet mount
  iv. Acid-fast stain or Gomori methenamine silver stain when indicated.
 (b) Direct inoculation ("C-streaks" on agar)
  i. Blood agar plates (1 for aerobic, 1 for anaerobic incubation)
  ii. Chocolate agar (for incubation in 3 to 10% CO_2)
  iii. Supplemented thioglycollate broth

iv. Sabouraud's dextrose agar
v. Inhibitory mold agar (IMA)
vi. Lowenstein-Jensen medium.
(c) Transfer to swab in transport medium for viral cultures.

c. Intraocular Infections

(1) The conjunctiva is swabbed and cultured as described above.
(2) Intraocular fluid
(a) Prepare slides for
i. Gram stain
ii. Giemsa stain
iii. Acid-fast stain when indicated
(b) Cultures
As for corneal scrapings above.
(3) Blood cultures for bacteria and fungi.
(4) Serum for viral, fungal, and *Toxoplasma gondii* serology.

K. Tissue Specimens

Tissue collected at the time of surgery is obtained at some risk and considerable expense to the patient. Moreover, once the incision has been closed, additional material is not available. Tissue, therefore, requires the utmost in care and processing.

1. Etiology

In general, etiology comprises virtually unlimited bacterial, mycobacterial, fungal, and parasitic species. Viruses, particularly herpes simplex, are of special concern in brain tissue. Other viruses, *Chlamydia trachomatis, Mycoplasma pneumoniae,* and *Pneumocystis carinii* are of special concern in lung tissue.

Biopsies of certain sites may be performed to help establish the diagnosis of parasitic diseases.

a. Rectum—amebiasis, schistosomiasis (*Schistosoma mansoni* and *S. japonicum*).
b. Bladder—schistosomiasis (*S. haematobium*).
c. Bone marrow—visceral leishmaniasis or kala-azar (*Leishmania donovani*).
d. Muscle—trichinosis, cysticercosis.
e. Skin—cutaneous leishmaniasis or Oriental sore (*L. tropica*), mucocutaneous leishmaniasis (*L. braziliensis*), onchocerciasis.
f. Lymph nodes, spleen, liver—leishmaniasis, trypanosomiasis, toxoplasmosis, filariasis, schistosomiasis.

2. Specimen Collection[6]

Specimens should be obtained from several parts of a large lesion or from different lesions if more than one is present to ensure adequate sampling. A generous portion of an abscess wall, as well as of pus, should be obtained when an abscess is surgically removed. Portions of several lymph nodes, if multiple, should be removed when enlarged nodes are found. When culture of splenic material is requested, all tissue that is not required for histopathological examination should be submitted for microbiological examination. Tissue from wounds for quantitative smear and culture (see Ch. 3) must be taken after thorough cleansing and debridement. Finally, special attention must be paid to patients undergoing abdominal exploration for fever of unknown origin (FUO); these cases require communication among the internist, surgeon, and microbiologist.

3. Specimen Transport

a. Sterile screw-capped, leakproof containers for tissue.
b. Anaerobic transport containers for pus and other fluids.
c. Expedite handling!

L. Postmortem Microbiology

1. Significance

Bacteriological examination of autopsy tissue is of limited value except in selected circumstances.[8,9,25] The reasons are that there is an indigenous postmortem tissue flora that does not appear to be related to the postmortem transmigration of flora. The frequency of positive bacterial cultures correlates poorly with clinical or autopsy evidence of infectious disease, and there is generally a substantial lack of correlation between results of ante- and postmortem cultures.[37] The results of ante- and postmortem cultures of heart blood have been reported to correlate in 48% to 83% of cases. Cultures performed on a single postmortem tissue sample are rarely of value; however, postmortem cultures of multiple tissues *may* be of value in some cases, especially if these represent cases with well-recognized diseases due to a single organism (e.g., *Brucella, Mycobacterium tuberculosis,* a deep mycotic infection).

2. Special Technique for Collection and Processing of Tissue and Body Fluids[8]

a. Lungs, Liver, Spleen, and Kidney

(1) Obtain a 6 cm cube of tissue with one intact serosal or capsular surface.
(2) Prepare three smears of abscess fluid for Gram, acid-fast, and Gomori

methenamine silver stains, respectively. Remove aliquot of pus and portion of wall of abscess for culture. If abscess was entered unexpectedly, mark the specimen request form to indicate that the specimen has been contaminated.

b. Pleural, Pericardial, and Peritoneal Fluids

(1) Aspirate the fluid percutaneously with a syringe attached to a 15- or 18-gauge needle after cleansing the skin with an antiseptic.
(2) Aspirate pericardial fluid either through the chest wall or through the pericardium after the chest cavity has been opened. The pericardial surface should be seared prior to aspiration.
(3) Aspirate peritoneal fluids through the body wall whenever possible; however, loculated fluids may not be encountered until the body is opened. On such cases, the surface of the loculated fluid wall should be seared prior to aspiration.

c. Urine

Aspirate urine through the anterior superior portion of the bladder after searing its surface.

d. Lymph Nodes

These cannot usually be removed aseptically but are most likely to be cultured for mycobacteria and fungi following decontamination procedures.

e. Blood

Aspirate blood from the right atrium with an 18-gauge needle attached to a 20 ml syringe after searing the epicardial surface. Other techniques include aspiration by direct cardiac puncture through the unopened chest with an 18-gauge spinal needle attached to a syringe.

M. Integumentary Infections

1. Etiology

a. Primary

Staphylococcus aureus
Streptococcus pyogenes (group A)
Gram-negative bacilli
Clostridia
Bacteroidaceae

Anaerobic cocci
Pasteurella multocida
Corynebacterium diphtheriae
Bacillus anthracis
Francisella tularensis
Erysipelothrix rhusiopathiae
Treponema pallidum
Nonsyphilitic treponemes
Mycobacteria
 M. leprae
 M. tuberculosis
 M. marinum
 M. ulcerans
Fungi
 dermatophytes
 yeasts
 filamentous fungi
Viruses
 poxviruses
 herpesviruses
Parasites
 nematodes (larva or migrans)
 filariae
 anthropods

b. Secondary to Systemic Disease

Staphylococcus aureus
Pseudomonas aeruginosa
Neisseria meningitidis
Haemophilus influenzae
Neisseria gonorrhoeae
Streptococcus pyogenes (group A)
Mycobacteria
Fungi
Rickettsiae
Viruses
Parasites

2. Specimen Collection

a. General

Material from an open lesion is usually collected on a swab; however, that from a previously unopened purulent lesion (e.g., pustule, furuncle,

carbuncle), an area of cellulitis, petechiae, eruptions, or bullae is obtained preferably by aspiration with a #25 needle attached to a syringe. In some instances, it may be necessary to inject a small amount of saline (bacteriostat-free) into the area to obtain material for microscopic examination and culture. In other instances, the diagnosis is best established with a biopsy of the lesion. Either a punch biopsy or tissue fluid expressed from a skin incision 2 mm deep is examined microscopically for acid-fast bacilli in cases of suspected leprosy.

b. Dematomycoses

(1) Skin

Lesions should be washed with soap and water to free them of dirt, cosmetics, or ointments. Scales, scrapings from part of an active border of the lesion, or tops of vesicles are taken for microscopic examination and/or culture. Plastic cover slips (diSPo, Scientific Products) have a surface charge so that scales or scrapings adhere to them. The slides can then be placed over a drop of KOH on a microscope slide for microscopic examination.

(2) Nails

Material from infections beneath the nail plate should be obtained by scraping under the nail plate or by boring a hole through the nail plate with a scalpel blade. Infections on the surface of the nail plate are easily scraped to obtain material for examination.

(3) Hair

Scalp lesions are scraped with a scalpel blade to remove fragments of hair for examination. A Wood's lamp may be used to detect and remove hairs infected with *Microsporum* species.

3. Specimen Transport

Specimens should be placed in a sterile petri dish or into a dry paper envelope. Refrigeration should be avoided, as should exposure to moisture.

References

1. Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C. S., Eschenbach, D., and Holmes, K. K. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiological characteristics. *Am. J. Med.* 74:14, 1983.
2. Auckenthaler, R., Ilstrup, D. M., and Washington, J. A., II. Comparison of

recovery of organisms from blood cultures diluted 10% (volume/volume) and 20% (volume/volume). *J. Clin. Microbiol.* **15**:860, 1982.

3. Barry, A. L., Fay, G. D., and Sauer, R. L. Efficiency of a transport medium for the recovery of aerobic and anaerobic bacteria from applicator swabs. *Appl. Microbiol.* **24**:31, 1972.
4. Bartlett, J. G., Faling, L. J., and Willey, S. Quantitative tracheal bacteriologic and cytologic studies in patients with long-term tracheostomies. *Chest* **74**:635,1978.
5. Bille, J. Stockman, L., Roberts, G. D., Horstmeier, C. D., and Ilstrup, D. M. Evaluation of a lysis-centrifugation system for recovery of yeasts and filamentous fungi. *J. Clin. Microbiol.* **18**:469,1983.
6. Brewer, N. S., and Weed, L. A. Diagnostic tissue microbiology methods. *Human Pathol.* **7**:141, 1976.
7. Collee, J. G., Watt, B., Brown, R., and Johnstone, S. The recovery of anaerobic bacteria from swabs. *J. Hyg. (Camb.)* **72**:339, 1974.
8. Dolan, C. T. Autopsy microbiology. *In* Ludwig, J. (ed.), *Current Methods of Autopsy Practice,* 2nd ed. Philadelphia, Saunders, 1979, p. 138.
9. Dolan, C. T., Brown, A. L., and Ritts, R. E., Jr. Microbiological examination of postmortem tissues. *Arch. Pathol.* **92**:206, 1971.
10. Dunn, C., and Martin, W. J. Comparison of media for isolation of salmonellae and shigellae from fecal specimens. *Appl. Microbiol.* **22**:17, 1971.
11. Fairley, F. K., Carson, N. E., Gutch, R. C., Leighton, P., Grounds, A. D., Laird, E. C., McCallum, P. H. G., Sleeman, R. L., and O'Keefe, C. M. Site of infection in acute urinary-tract infection in general practice. *Lancet* **2**:615, 1971.
12. Hall, C. B., and Douglas, R. G., Jr. Clinically useful method for the isolation of respiratory syncytial virus. *J. Infect. Dis.* **131**:1, 1975.
13. Hall, M. M., Ilstrup, D. M., and Washington, J. A., II. Comparison of three blood culture media with tryptic soy broth. *J. Clin. Microbiol.* **8**:299, 1978.
14. Hall, M. M., Mueske, C. A., Ilstrup, D. M., and Washington, J. A., II. Evaluation of a biphasic medium for blood cultures. *J. Clin. Microbiol.* **10**:673, 1979.
15. Hallander, H. O., Flodström, A., and Holmberg, K. Influence of the collection and transport of specimens on the recovery of bacteria from peritonsillar abscesses. *J. Clin. Microbiol.* **2**:504, 1975.
16. Henry, N. K., McLimans, C. A., Wright, A. J., Thompson, R. L., Wilson, W. R., and Washington, J. A. II. Microbiological and clinical evaluation of the Isolator lysis-centrifugation blood culture tube. *J. Clin. Microbiol.* **17**:864, 1983.
17. Henry, N. K., Grewell, C. M., McLimans, C. A., and Washington, J. A., II. Comparison of the Roche SeptiChek blood culture bottle with a brain heart infusion biphasic medium bottle and with a tryptic soy broth bottle. *J. Clin. Microbiol.* **19**:315, 1984.
18. Henry, N. K., Grewell, C. M., VanGrevenhof, P. E., Ilstrup, D. M., and Washington, J.A. II. Comparison of lysis-centrifugation with a biphasic blood culture medium for the recovery of aerobic and facultatively anaerobic bacteria. *J. Clin. Microbiol.* **20**:413, 1984.
19. Ilstrup, D. M., and Washington, J. A., II. Effects of atmosphere of incubation on recovery of bacteria and yeasts from blood cultures in tryptic soy broth. *Diagn. Microbiol. Infect. Dis.* **1**:215, 1983.
20. Johnson, S. E., Klein, G. C., Schmid, G. P., Bowen, G. S., Feeley, J. C., and

Schulze, T. Lyme disease: a selective medium for isolation of the suspected etiologic agent, a spirochete. *J. Clin. Microbiol.* **19**:81, 1984.

21. Jones, D. B., Liesegang, T. J., and Robinson, N. M. *Cumitech 13: Laboratory Diagnosis of Ocular Infections* (Washington, J. A., II. coordinating ed.). Washington, D.C., American Society for Microbiology, 1981.
22. Kellogg, D. S., Jr., Holmes, K. K., and Hill, G. A. *Cumitech 4: Laboratory Diagnosis of Gonorrhea* (Marcus, S., and Sherris, J. C., coordinating ed.). Washington, D.C., American Society for Microbiology, 1976.
23. Kelly, R. Cultivation of *Borrelia hermsi. Science* **173**:443, 1971.
24. Klein, J. O. Diagnostic lung puncture in the pneumonia of infants and children. *Pediatrics* **44**:486, 1969.
25. Koneman, E. W., Minckler, T. M., Shires, D. B., and DeJongh, D. S. Postmortem bacteriology. II. Selection of cases for culture. *Am. J. Clin. Pathol.* **55**:17, 1971.
26. Koneman, E. W., and Davis, M. A. Postmortem bacteriology. III. Clinical significance of microorganisms recovered at autopsy. *Am. J. Clin. Pathol.* **61**:28, 1974.
27. Levine, N. S., Lindberg, R. B., Mason, A. D., and Pruit, B. A., Jr. The quantitative swab culture and smear: A quick, simple method for determining the number of viable aerobic bacteria on open wounds. *J. Trauma* **16**:89, 1976.
28. Mackowiak, P. A., Jones, S. R., and Smith, J. W. Diagnostic value of sinus-tract cultures in chronic osteomyelitis. *J. Am. Med. Assoc.* **239**:2772, 1978.
29. Reimer, L. G., Reller, L. B., Mirrett, S., Wang, W.-L.L., and Cox, R. L. Controlled evaluation of hypertonic sucrose medium at a 1:5 ratio of blood to broth for detection of bacteremia and fungemia in supplemented peptone broth. *J. Clin. Microbiol.* **17**:1045, 1983.
30. Reller, L. B., Murray, P. R., and MacLowry, J. D. *Cumitech 1A: Blood Cultures II* (Washington, J. A., II. coordinating ed.) Washington, D.C., American Society for Microbiology, 1982.
31. Roberts, G. D., Horstmeier, C., Hall, M., Washington, J. A., II. Recovery of yeast from vented blood culture bottles. *J. Clin. Microbiol.* **2**:18, 1975.
32. Ross, P. W. The isolation of *Streptococcus pyogenes* from throat swabs. *J. Med. Microbiol.* **10**:69, 1977.
33. Smith, T. F., Weed, L. A., Pettersen, G. R., and Segura, J. W. Recovery of *Chlamydia* and genital *Mycoplasma* transported in sucrose phosphate buffer and urease color test medium. *Health Lab. Sci.* **14**:30, 1977.
34. Stamey, T. A. *Pathogenesis and Treatment of Urinary Tract Infections.* Baltimore, Williams and Wilkins, 1972.
35. Washington, J. A., II. Microbiology of trauma. *In* Day, S. B. (ed.), *Trauma: Clinical and Biological Aspects.* New York, Plenum, 1975, p. 123.
36. Washington, J. A., II (ed.). *The Detection of Septicemia.* West Palm Beach, FL, CRC Press, 1978.
37. Wilson, W. R., Dolan, C. T., Washington, J. A., II, Brown, A. L., and Ritts, R. E., Jr. Clinical significance of postmortem cultures. *Arch. Pathol.* **94**:244, 1972.
38. Wimberley, N., Faling, L. J., and Bartlett, J. G. A fiberoptic bronchoscopy technique to obtain uncontaminated lower airway secretions for bacterial culture. *Am. Rev. Resp. Dis.* **119**:337, 1979.
39. Wood, W. W., Oldstone, M., and Schultz, R. A reevaluation of blood culture as an autopsy procedure. *Am. J. Clin. Pathol.* **43**:241, 1965.

2

Direct Examination of Specimens

Glenn D. Roberts, Ph.D. Pauline K. W. Yu, M.S.
John A. Washington II, M.D.

I. General Considerations

Direct examination of clinical material can often provide the etiological diagnosis of an infectious process (Table 2–1) and the opportunity to initiate appropriate therapy before the results of cultures become available.

For microscopic examination, it is sufficient for the laboratory to have a compound binocular microscope with low power (10×, 1.6 mm), high dry (40×, 4 mm), and oil immersion (100×, 1.25 mm) achromatic objectives; 8 or 10× wide field oculars, mechanical stage, substage condenser; and a good light source. For examining wet mount preparations, however, increased image contrast is desirable and can be obtained with a darkfield condenser or, preferably, with a special condenser and objectives for phase contrast microscopy.

Exciter and barrier filters, darkfield condenser, and an ultraviolet light source must be added to the standard microscope for fluorescence microscopy. Fluorescent antibody techniques can be used to identify the presence of certain microorganisms in clinical specimens or in culture (Ch. 4.3) or to detect antibodies to certain microorganisms in serum (Ch. 4.3). The details of fluorescent antibody techniques are given elsewhere (Ch. 4.3).

Inasmuch as direct microscopic examination of specimens is integral to the diagnosis of parasitic diseases, specific techniques for examining specimens and identification of parasites observed microscopically will be deferred to Chapter 11.

Table 2–1. Summary of Rapid Tests for Detection of Microorganisms and Microbial Components or Products

Specimen	Test or stain[a]	Application
Blood	CIE, LA, COA, FA	*Haemophilus influenzae, Neisseria meningitidis, Streptococcus agalactiae* (group B), *S. pneumoniae*
	LA	*Cryptococcus neoformans*
	Giemsa	Plasmodia, hemoflagellates, microfilariae
	EIA, RIA	Hepatitis A and B viruses
Cerebrospinal fluid	Gram	Bacterial meningitis
	CIE, LA, FA, COA	*H. influenzae, N. meningitidis, S. agalactiae, S. pneumoniae, C. neoformans,* California virus (antibody)
	Limulus lysate	Gram-negative bacterial meningitis
	GLC	
	Lactate	Bacterial vs. other meningitis (?)
	Carbohydrates, fatty acids	Bacterial meningitis
	Acid-fast	Mycobacteria
Brain tissue	Gram	Bacterial abscess
	Acid-fast	Nocardial abscess
	FA	Herpes simplex virus, rabies virus
Body fluids, exudates, pus	Gram	Bacteria
	Acid-fast	*Nocardia,* mycobacteria
	KOH	Fungi
	CIE, LA, FA	*H. influenzae, N. meningitidis, S. pneumoniae*
	GLC	*Bacteroides fragilis*
Ocular material	Gram	Bacteria
	Giemsa	Cytology, fungal elements, *Chlamydia trachomatis*
	Acid-fast	*Nocardia,* mycobacteria
	FA	Adenovirus, herpes simplex virus, varicella-zoster virus
	Limulus lysate	Gram-negative bacterial endophthalmitis
Respiratory secretions	FA	*Bordetella pertussis, Legionella;* adenovirus, respiratory syncytial virus
	FA, LA	*Streptococcus pyogenes* (group A)
	Gram	Screening of sputum for acceptability for bacterial culture; examination for bacteria
	Acid-fast	*Nocardia,* mycobacteria

Table 2–1. (*continued*)

Specimen	Test or stain[a]	Application
	KOH	Fungi
	CIE, Quellung, COA	*S. pneumoniae*
Lung biopsy	Gram	Bacteria
	Acid-fast	*Nocardia*
	KOH	Fungi
	Toluidine blue, Giemsa, or GMS	*Pneumocystis carinii*
	FA	*Legionella*
Urethral exudate	Gram, EIA	*Neisseria gonorrhoeae*
Cervical or vaginal exudate	KOH	Yeasts, nonspecific vaginitis
	Wet mount	*Trichomonas vaginalis,* clue cells
	pH (>5.0)	Nonspecific vaginitis
	H & E, Giemsa, Papanicolaou, FA	Herpes simplex virus
	Dark field, FA	*Treponema pallidum*
	EIA	*Neisseria gonorrhoeae*
	FA	*Chlamydia trachomatis*
Urine	Gram, wet mount	Screen for significant bacteriuria
	Leukocyte esterase/nitrite	
	Bioluminescence	
	Colorimetry	
	CIE, LA	*H. influenzae, N. meningitidis, S. agalactiae, S. pneumoniae*
	EIA, RIA	*Legionella*
Feces	Wet mount	*Campylobacter jejuni*
	Methylene blue	Leukocytes
	Eosin wet mount, trichrome	Parasites
	EM, CIE	Rotavirus

[a] CIE, counterimmunoelectrophoresis; COA, coagglutination; EIA, enzyme immunoassay; EM, electron microscopy; FA, fluorescent antibody; GLC, gas-liquid chromatography; GMS, Gomori methenamine silver; H & E, hematoxylin and eosin; KOH, potassium hydroxide; LA, latex agglutination; RIA, radioimmunoassay.

II. Specific Indications

A. Central Nervous System

1. Acute Bacterial Meningitis

Gram-stained smears of cerebrospinal fluid (CSF) in previously untreated acute bacterial meningitis due to *Haemophilus influenzae* (Figure 2–1a, see color insert in the center of the book for Figure 2–1), *Neisseria menin-*

gitidis (Figure 2–1b), or *Streptococcus pneumoniae* (Figure 2–1c) will be positive in 60 to 80% of cases; however, this figure can be reduced by approximately 15% in partially treated cases. Immunologic testing (p. 270) of CSF to detect these species' antigens is particularly helpful in partially treated cases. The presence of nonsporulating gram-positive bacilli should suggest the possibility of *Listeria monocytogenes* (Figure 2–1d), although meningitis due to *Propionibacterium* and *Corynebacterium* (Figure 2–1e) occurs rarely. More often, *Corynebacterium* and *Proponibacterium* represent contaminants.

2. Chronic Meningitis

CSF from patients with chronic meningitis may be examined for the presence of *Cryptococcus neoformans* in an India ink or nigrosin wet mount preparation (Figure 2–2); however, since less than 50% of cases of cryptococcal meningitis are detected by an India ink or nigrosin preparation, the latex agglutination test for cryptococcal antigen is preferred. Acid-fast stains of CSF are rarely positive in cases with tuberculous meningitis and should therefore be selectively requested.

3. Brain Abscess

Gram-stained smears of pus from an abscess are particularly helpful in distinguishing between staphylococcal (Figure 2–1f) and anaerobic bacterial (Figure 2–1g) infections.

4. Encephalitis

Brain biopsy material can be examined directly by electron microscopy or with immunofluorescence for herpes simplex virus (Ch. 10).

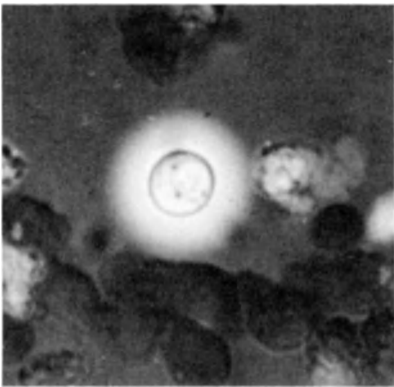


Figure 2–2. Encapsulated *Cryptococcus neoformans* in India ink preparation of cerebrospinal fluid (×2000).

B. Wound, Abscess, Cellulitis

Gram-stained smears of wound exudates, abscesses, and aspirates of areas of cellulitis can help to differentiate staphylococcal (Figure 2–1f), streptococcal (Figure 2–1h), clostridial (Figure 2–1i), and mixed anaerobic bacterial (Figure 2–1g) infections. The presence of pleomorphic gram-negative bacilli (Figure 2–1j) should suggest infection with *Bacteroides*, while that of spindle-shaped gram-negative bacilli with pointed ends (Figure 2–1k) should suggest infection with *Fusobacterium nucleatum*. Sulfur granules (Figure 2–3, see color insert in the center of the book) consisting of masses of gram-positive bacilli should suggest infection with *Actinomyces*. Lactobacilli may form highly pleomorphic morphology in Gram-stained smears (Figure 2–1l).

C. Upper Respiratory Tract

Gram-stained smears of sinus and middle ear aspirates can provide an early indication as to the etiology of sinusitis and otitis media.

In the hands of trained observers, the mean sensitivity, specificity, and predictive value of the Gram stain in providing an early diagnosis of group A streptococcal pharyngitis has been reported as 73%, 96%, and 71%, respectively, when the criteria for a positive result included the presence of gram-positive cocci typical of streptococci (Figure 2–1m) associated with polymorphonuclear leukocytes, which are characterized by loss of cytoplasmic integrity and cellular outlines.[6] Because of substantial differences in the test's sensitivity and predictive value among observers in this study, caution has been advised until the Gram-stained smear has been endorsed as an ancillary technique in diagnosing streptococcal pharyngitis.[2] Immunologic tests, including coagglutination and latex agglutination, have become available commercially for detection of group A streptococci. Although such tests are generally quite specific, their sensitivity is usually directly related to the number of group A streptococci in specimens, so that small numbers of organisms may go undetected.

Emphasis was placed in the past on the value of the Gram-stained smear of exudate from the edge of a diphtheritic membrane in making the presumptive diagnosis of diphtheria, especially when metachromatic granules (Figure 2–4, see color insert in the center of the book) could be demonstrated by the methylene blue stain (p. 89) in any coryneform bacteria present. Few microbiologists today have sufficient experience with diphtheria to make a presumptive diagnosis reliably by microscopy except possibly with immunofluorescence.[12] Immunofluorescence is a rapid and reliable technique for establishing the diagnosis of pertussis.

D. Lower Respiratory Tract

1. Sputum

a. Gram Stain

Gram-stained smears of sputum should be performed routinely and examined with low power (×100) magnification to determine whether the specimen is acceptable for bacterial culture (Figure 2–5, Table 3–6).[12] If acceptable, the smear should be examined under oil immersion (×1000) to try to establish the etiology of the pneumonia. The sensitivity and specificity of the Gram-stained smear in identifying pneumococci in sputum is 62% and 85%, respectively, when the diagnostic criteria include a preponderance of, or >10 lancet-shaped, gram-positive diplococci per oil immersion (×1000) field.[13] The Gram-stained smear can suggest the etiology of pneumonias due to other groups of bacteria (Figure 2–1n,o).

b. Acid-Fast Stain

Sputum from patients suspected of having mycobacterial disease should be stained with a fluorochrome (p. 85) or carbol fuchsin (p. 390) stain and examined microscopically. Both stains have been shown to have equivalent sensitivity (52%) and specificity (99.9%) in our laboratory; however, the fluorochrome stain is more efficient for routine purposes,

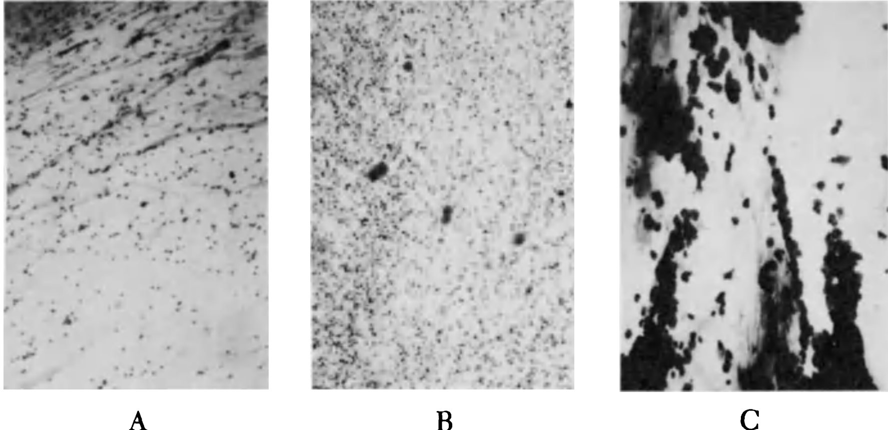


Figure 2–5. Smears of representative sputum specimens (Gram stain, ×100): (a) leukocytes >25/lpf, epithelial cells <10/lpf; (b) leukocytes >25/lpf, epithelial cells <25/lpf; (c) leukocytes <10/lpf, epithelial cells >25/lpf. Specimens represented in (a) and (b) are acceptable for bacterial culture, whereas that in (c) is not. (Reproduced with permission from Murray and Washington.[12])

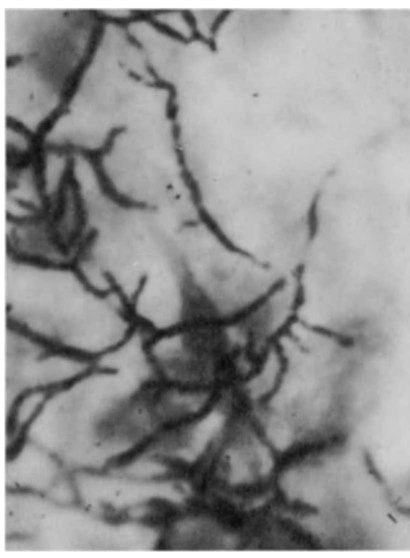


Figure 2–6. Acid-fast stained smear of *Nocardia asteroides* in sputum demonstrating filaments, some of which are breaking into coccobacillary forms (×2000).

since it is possible to scan a much larger area in the same time with the 25× objective than is possible with the 95× objective, which is required for examining smears stained with carbol fuchsin. The decolorizer in the carbol fuchsin method should be modified when staining *Nocardia* (p. 412, Figure 2–6).

c. Potassium Hydroxide (KOH) Wet Mount Preparation

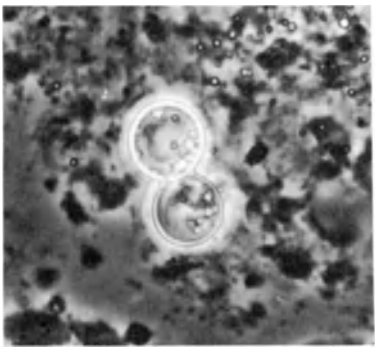
Lower respiratory secretions from patients with suspected fungal diseases should be examined in a KOH preparation (p. 90) with phase contrast microscopy for the presence of fungal elements (Fig. 2–7).[14] Examination (KOH) of respiratory secretions on a routine basis, however, is usually unrewarding.

2. Transtracheal Aspirate (TTA)

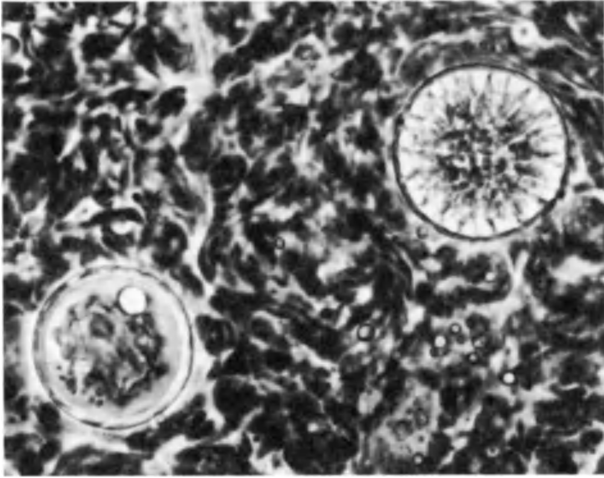
All TTAs should be Gram-stained routinely to determine whether the infection is uni- or polymicrobial, of which the latter is most often associated with anaerobic bacteria. Such material may, when indicated, be appropriately stained for mycobacteria, *Legionella* sp., and *Pneumocystis carinii.*

3. Open Lung Biopsy

Open lung biopsies are usually performed in immunosuppressed hosts with pneumonias of unknown etiology. Such specimens must be examined



(a)



(b)

Figure 2–7. Phase contrast microscopy of clinical specimens. (a) *Blastomyces dermatitidis* in sputum. Characteristic yeast form has budding cell attached by broad base. Also note "double contoured" appearance of cell wall (×2000). (Reproduced by permission from Roberts.[14]) (b) *Coccidioides immitis* in sputum. Large thick-walled spherules with few endospores scattered within interior of spherule or cleavage furrows developing along periphery to form endospores (×2000). (Reproduced by permission from Roberts.[14]) (c) *Cryptococcus neoformans* in sputum. Spherical yeast cell is surrounded by large capsule with small bud arising from parent cell (×2000). (d) Dermatophyte in skin scraping. Septate hyphae intertwine among squamous cells (×2000). (Reproduced by permission from Roberts.[14]) (e) *Candida albicans* in urine. Hyphae and budding yeasts appear among epithelial cells (×2000). (Reproduced by permission from Roberts.[14]) (f) *Mucor* sp. in pus from skin lesion. The large, branching, ribbonlike aseptate hyphae are indicative of a zygomycete (×2400). (Reproduced by permission from Roberts.[14]) (g) *Aspergillus fumigatus* with dichotomous septate hyphae in sputum (×2000). (h) *Nocardia asteroides* in sputum. Small branching filaments are interpositioned among leukocytes (×2000).

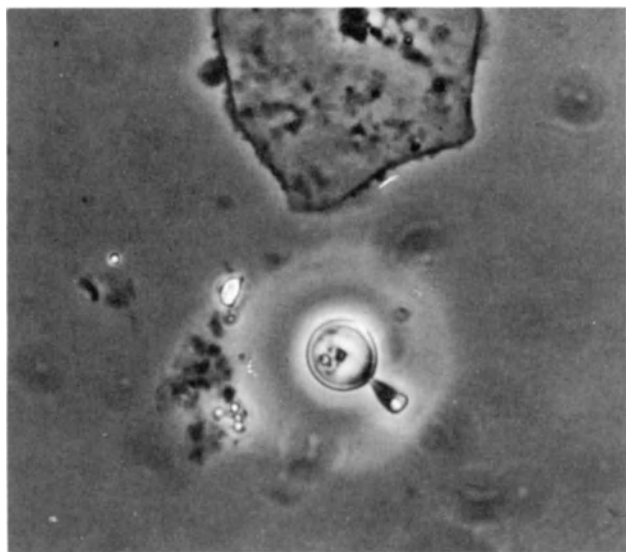


Figure 2–7c

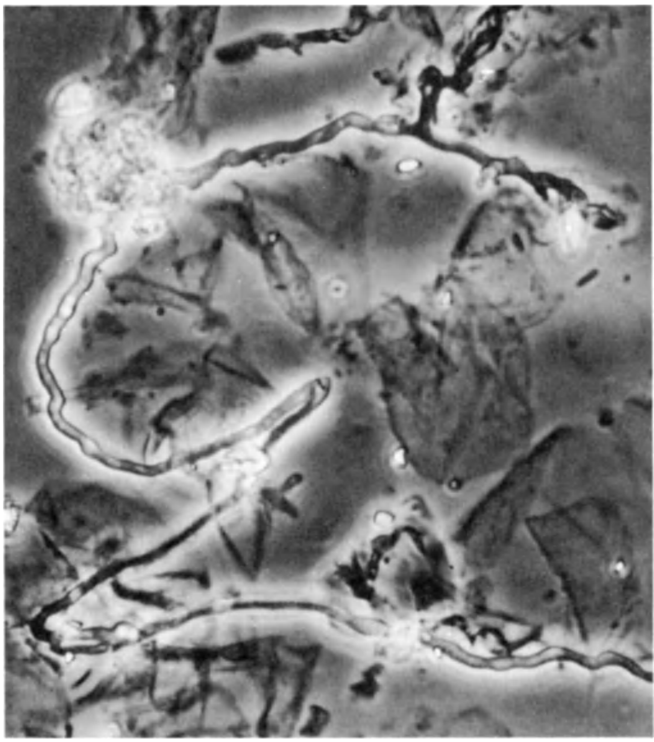


Figure 2–7d

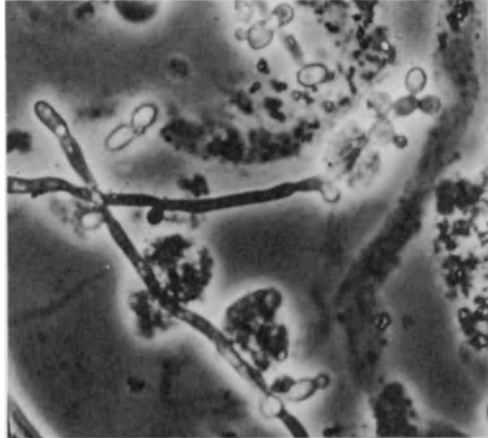


Figure 2–7e

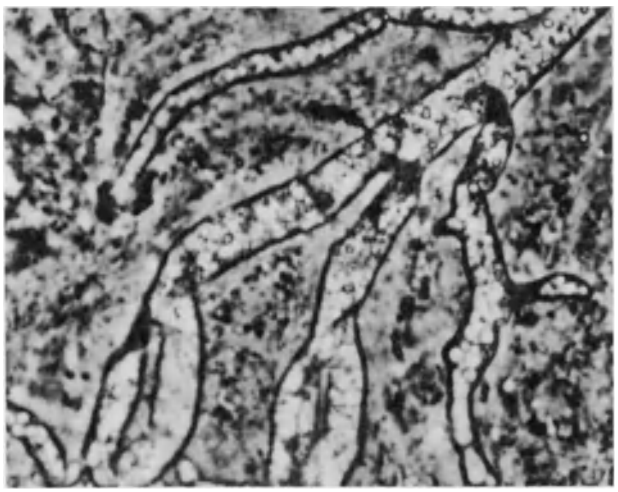


Figure 2–7f

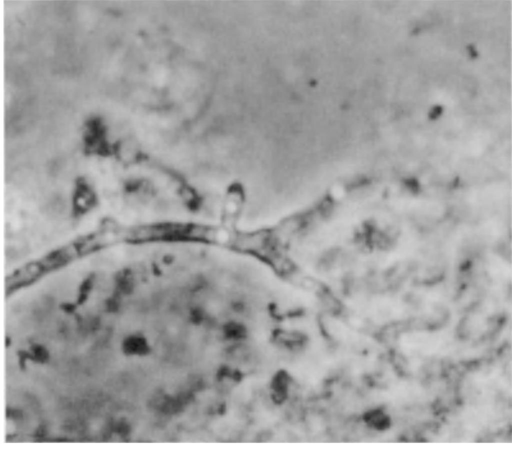


Figure 2–7g

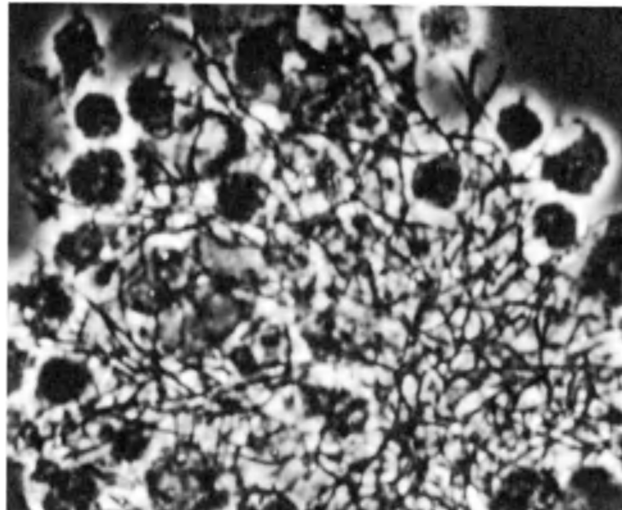


Figure 2–7h

for a diverse group of microorganisms, including bacteria, mycobacteria, fungi, viruses, and parasites. The following stains should be made of impression smears (touch preparations): Gram, acid-fast, fluorescent antibody (p. 262) for *Legionella* sp. (Figure 2–8, see color insert in the center of the book), and Gomori methenamine silver (GMS), toluidine blue (p. 91), or Giemsa (p. 87) for *Pneumocystis carinii* (Figure 2–9, see color insert in the center of the book). A KOH preparation and an acid-fast stain are performed with the homogenized tissue suspension.

Frozen and permanently fixed sections of the biopsy must be examined to determine the type of inflammatory process (purulent, granulomatous, interstitial) present and to suggest which special stains and cultures should be done. Hematoxylin and eosin (H & E) stained sections demonstrate the basic histopathology of the tissue, as well as intranuclear inclusions of herpesviruses and the broad nonseptate filaments of zygomycetes. Gomori methenamine silver stains fungi and *Pneumocystis carinii* cysts. Although the GMS stain may demonstrate yeasts resembling *Histoplasma capsulatum* or spherules and endospores of *Coccidioides immitis* that may not grow in cultures, the reverse may occur. Also, structures resembling fungi but lacking definitive characteristics may be seen in sections so that cultures are necessary to confirm the diagnosis. Acid-fast stains of tissue may provide an early indication of mycobacterial infection; however, such stains are positive in only 30 to 40% of culturally proved cases. The Brown-Hopps[4] tissue Gram stain demonstrates bacteria, including *Nocardia.* Subject to the clinical, epidemiological, serological, and histopathological information available, the Dieterle silver impregnation stain[19] can be helpful in establishing the diagnosis of legionellosis, how-

ever, its use has been largely supplanted by the more specific fluorescent antibody stain (p. 262).

E. Genitourinary Tract

1. Urine

a. Clean Voided Midstream Urine Specimens

The presence of ≥ 2 bacteria per oil immersion field ($\times 1000$) in a Gram-stained smear of a drop of properly collected and transported, well-mixed urine signifies (90% predictive positive value) the presence of at least 10^5 CFU/ml, while <2 bacteria per oil immersion field is usually associated (99% predictive negative value) with bacteriuria of $<10^5$ CFU/ml[20] (Figure 2–10, see color insert in the center of the book). The presence of leukocyte esterase and nitrite (Chemstrip LN™, Boehringer Mannheim, Indianapolis, IN), bioluminescence (Lumac™ Bacteriuria Screening Kit, 3M, St. Paul, MN), and colorimetry (Bacteriuria Detection Device, Marion Laboratories, Kansas City, MO) have been used for rapid detection of significant bacteriuria. Problems with the sensitivity and/or specificity of such devices have limited their use as culture substitutes.

Acid-fast stains may be helpful in suggesting the diagnosis of renal or disseminated tuberculosis; however, a positive smear must be interpreted cautiously due to the presence of nontuberculous mycobacteria in the urethra of some normal, healthy people.

2. Sexually Transmitted Diseases

a. Gonorrhea

The finding of intracellular, gram-negative diplococci (Figure 2–1p) in smears of male urethral exudates is virtually diagnostic of gonorrhea; however, smear-negative exudates require culture since approximately 10% of males with culturally proved gonorrhea have negative smears.[7] Cervical smears have been reported as positive in 66 to 77% of culturally proved cases and in as many as 18% of culture-negative cases.[7] The latter group (false positives) may be due to inadequate sampling, nonviable gonococci, gonococci inhibited by vancomycin in Thayer-Martin medium, and, more often, bacteria resembling gonococci (overdecolorized staphylococci, other *Neisseria* sp., *Veillonella*). Gram-stained smears of cervical (or vaginal) exudates are, therefore, not recommended for the diagnosis of gonorrhea in low-risk populations.

b. Chancroid

The finding of tangled chains or long parallel rows of gram-negative coccobacilli in smears of exudate from the undermined border of the ulcer is presumptive evidence of the presence of *Haemophilus ducreyi.*[3]

c. Vulvovaginitis

(1) Candidosis

Hyphae and budding yeasts may be seen in 40 to 80% of KOH preparations (Figure 2–7e) and in 70 to 100% of Gram-stained smears (Figure 2–1q)[8]; however, because yeasts may be present in the vagina of as many as 26% of asymptomatic women,[17] care must be taken to interpret the microbiological findings in the light of the clinical signs and symptoms.

(2) Trichomoniasis

The diagnosis of trichomoniasis is usually made by finding trichomonads with characteristic motility in wet mount preparations of vaginal secretions (Figure 2–11).

(3) Nonspecific Vaginosis (NSV)

The diagnosis of NSV is based on the presence of any three of the following four criteria: vaginal pH >4.5, characteristic thin discharge, release of a fishy amine odor upon addition of a drop of 10% KOH to a drop of vaginal discharge, and the presence of clue cells[1] (Figure 2–12, see color insert).

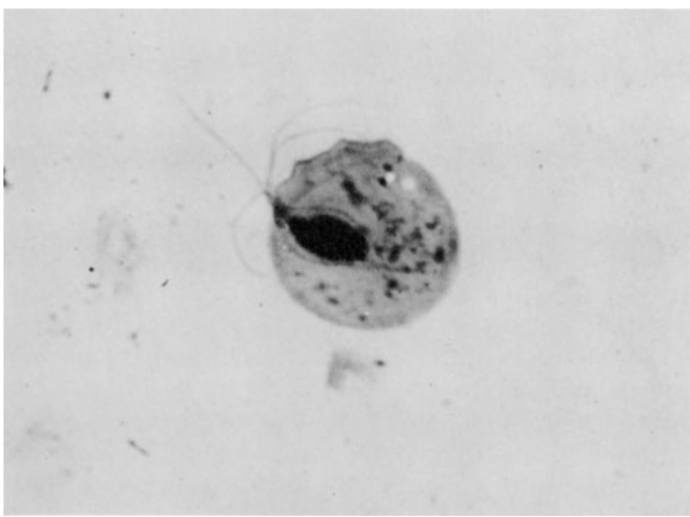


Figure 2–11. *Trichomonas vaginalis* in wet mount preparation of vaginal secretions.

d. Chlamydia Trachomatis

The diagnosis of chlamydial genital infections can be made by finding extracellular elementary bodies in direct fluorescent-antibody stained urethral and cervical smears (Ch. 8).[16]

F. Gastroenteritis

Exclusive of finding intestinal parasites in eosin–saline wet mount and stained preparations of feces (Chapter 11), direct examination of feces from patients with diarrhea is of limited utility with three possible exceptions. These are:

1. Methylene Blue Preparation

The presence of numerous leukocytes is associated with colitis due to invasion by intestinal pathogens, including *Salmonella, Shigella,* and *Campylobacter.* The absence of leukocytes is usually associated with noninvasive and usually toxigenic intestinal pathogens, such as *Vibrio cholerae* and toxigenic *Escherichia coli.*

2. Phase Microscopy of Wet Mount Preparations

Campylobacters may often be recognized in feces of patients in the acute phase of illness as curved or spiral rods with a unique, corkscrew type of motility.

3. Immunoassay

Rotaviruses in feces which are a frequent cause of diarrhea in infants during the winter months, can be rapidly detected, with immunoassay techniques (p. 584).

G. Ocular Infections

Gram- and Giemsa-stained smears of material from patients with corneal ulcers and endophthalmitis are used to detect bacteria and fungi and to guide initial therapy. Giemsa-stained (p. 87) smears of conjunctival scrapings may also be useful in detecting *Chlamydia trachomatis* in conjunctival scrapings from patients with inclusion conjunctivitis, although the sensitivity of the stain in detecting the organisms in adults and infants is 40% and 90%, respectively. When indicated, KOH, Gomori methena-

mine silver, and acid-fast stains may also be used. Fluorescent antibody stains may be performed to identify adenovirus and herpes simplex virus (Ch. 10) in ocular material.

III. Stains and Wet Mount Preparations

Auramine–Rhodamine (Truant) Stain[18]

Reagents:

Fluorescent dye solution

Auramine O	1.5 g
Rhodamine B	0.75 g
Glycerol	75 ml
Phenol (melted)	10 ml
Distilled water	50 ml

Dissolve the phenol in 25 ml of water; then add the dyes and the remaining water and glycerol, and mix well. Clarify by filtration. Store at 4°C or room temperature. Expiration date is 1 year.

Decolorizer:

HCl, concentrated	0.5 ml
Ethyl alcohol, 70%	100 ml

Add the acid to the alcohol, and mix. Store in a screw-capped bottle.

Counterstain:

Potassium permanganate	0.5 g
Distilled water	100 ml

Procedure:

1. Dry and flame the smears.
2. Flood the slides with stain for 15 min.
3. Rinse well with distilled water.
4. Decolorize with acid–alcohol for 2 min and rinse well with distilled water.
5. Flood the slides with counterstain for 2 min and rinse well with distilled water.
6. Examine with fluorescence microscope using 25× objective.

Interpretation:
Acid-fast bacilli appear bright yellow against a dark background.

Capsule Stain

Reagents:

Nigrosin, granular (Harleco)	10 g
Formalin, 10%	100 ml

Dissolve the mixture in a boiling water bath for 30 min. Replace the amount of any formalin lost by evaporation. Filter twice through double layers of Whatman no. 1 filter paper. India ink (Pelikan) may be used instead of nigrosin; however, nigrosin is preferred because it is free of discernible particulate matter.

Procedure:

1. Mix a drop of the specimen with a drop of nigrosin solution on a glass microscope slide.
2. Apply a coverslip.
3. Scan microscopically under low-power magnification with reduced illumination. Examine any structures observed under high-power magnification.

Interpretation:
Encapsulated yeasts (i.e., *Cryptococcus neoformans*) demonstrate a halo about the cell (Figure 2–2). *N.B.* Structures, such as red blood cells, leukocytes, and nonencapsulated yeasts, and artifacts, such as starch granules, may resemble cryptococci and require careful examination for differentiation.

Flagella Stain (Leifson)[9,10]*

Reagents:

Stock Solutions

1. NaCl	1.5 g
Distilled water	100 ml

* Not applicable to direct staining of specimens.

2. Tannic acid	3 g
Distilled water	100 ml
3. Pararosaniline acetate	0.9 g
Pararosaniline hydrochloride	0.3 g
Ethyl alcohol, 95%	100 ml

Leave alcoholic dye solution at room temperature to ensure complete solution. Divide dye in four aliquots and store at −10°C until use.

Complete Stain:
Mix equal volumes of the three stock solutions, shake well, and adjust pH between 4.8 and 5.2. Store stain in a screw-capped bottle at 4°C. Precipitate will settle at the bottom of bottle during storage, and it should not be disturbed. If stored at room temperature, stain is satisfactory only for several days. Expiration date is 6 months when stored at 4°C and indefinitely when kept frozen.

Procedure:

1. With bacterial growth from an overnight culture (35°C) on sheep blood agar, prepare a barely turbid suspension in distilled water. Alternatively, use growth from an overnight culture (22°C) on a nutrient agar slant if the organism is not actively motile.
2. Apply one drop of suspension to a well-heated clean glass microscope slide. Allow drop to run down length of slide.
3. Air dry the smear. Do not heat fix.
4. Apply 1 ml of clear supernatant stain solution to the slide. Avoid carryover of any precipitate.
5. A precipitate (metallic sheen) will begin to form on the slide as alcohol evaporates from the stain solution.
6. Staining is complete when metallic sheen forms over the entire surface of the slide. This usually takes between 8 to 15 min, depending on the age of the stain.
7. Wash slide with water, drain and allow to air dry.
8. Examine microscopically under oil immersion (1000×) for the presence, location, and numbers of flagella.

Giemsa Stain

Reagents:

Stock Giemsa stain solution (Harleco)
Phosphate buffer, M/15, pH 7.0

Methyl alcohol, absolute
Dilute stock solution 1:20 in buffer (prepare daily)

Procedure:

For blood and tissue parasites, see p. 32
For *Pneumocystis carinii*[15]:

1. Prepare impression smears by pressing the cut surface of lung tissue against sterile glass microscope slides.
2. Air dry the smears for 30 min.
3. Fix in methyl alcohol for 1 min.
4. Air dry.
5. Stain for 30 min.
6. Rinse briefly in buffer.
7. Air dry.
8. Examine microscopically.

For *Chlamydia trachomatis* in conjunctival scrapings, the procedure is the same as that described above except that the slide is placed in methyl alcohol immediately after the scrapings have been smeared on the slide.

Interpretation:
Trophozoites demonstrate a red nucleus and blue cytoplasm. The cyst wall is not stained. Cysts and trophozoites may occur in clusters.

Gram Stain

Reagents:

Crystal violet

Crystal violet, 90% dye content	10 g
Methyl alcohol, absolute	500 ml

Gram's iodine solution

I_2 crystals	6 g
KI	12 g
Distilled water	1800 ml

Decolorizer

Acetone	400 ml
Ethyl alcohol, 95%	1200 ml

Counterstain

Safranin, 99% dye content	10 g
Distilled water	1000 ml

Procedure:

1. Thoroughly flame one side of a clean glass slide to be used for the smear and allow to cool before smearing.
2. Using freshwater to emulsify a colony or portion thereof, prepare a thin smear on the previously flamed side of the slide and allow to air dry.
3. Fix the smear by passing the slide through a flame several times.
4. Flood the slide with crystal violet solution.
5. After at least 10 sec, pour off the crystal violet and gently wash the slide in water.
6. Flood the slide with iodine solution.
7. After at least 10 sec, rinse off the iodine solution with water.
8. Decolorize with acetone–alcohol and wash off immediately with water.
9. Flood with safranin solution for 10 sec, and rinse off with water.
10. Blot the slide dry with clean unused filter paper.
11. Examine the smear under oil immersion (1000×)

Interpretation:
Gram-positive bacteria stain blue or purple while gram-negative bacteria stain red or pink.

Methylene Blue Stain (Albert Stain)

Reagents:

Methylene blue, certified	0.3 g
Ethyl alcohol, 95%	30.0 ml
Distilled water	100 ml

Dissolve methylene blue in ethyl alcohol. Add distilled water.

Procedure:

1. On a flamed microscope slide, apply material taken from the edge of the pseudomembrane or, preferably, emulsify a portion of an isolated colony from a Loeffler agar slant in a loopful of water.
2. Air dry and heat fix smear.
3. Stain slide with methylene blue for one minute.
4. Rinse with tap water and blot dry.

Interpretation:
Granules of *Corynebacterium diphtheriae* stain dark blue or metachromatically bright pink (Figure 2–4), while bacteria other than *C. diphtheriae* stain pale blue and do not display granules.

Potassium Hydroxide (KOH) Preparation

Reagents:

KOH	10 g
Glycerin	10 ml
Distilled water	80 ml

Procedure:

1. Into a drop of KOH solution place a small portion of the material to be examined (skin scrapings, hair).
2. Press coverslip down on sample.
3. Warm slide slightly to dissolve keratinized cells.
4. Examine under high dry or oil immersion objective.

Interpretation:
Characteristic fungal elements are readily recognized, especially when viewed with phase contrast microscopy (Figure 2–7).

Spore Stain*

Reagents:

Malachite green	5 g
Distilled water	100 ml
Safranin	1 g
Distilled water	100 ml

Procedure:

1. Flood an air-dried, heat-fixed smear with malachite green solution for 5 to 10 min.
2. Rinse with water.
3. Counterstain with safranin for 30 sec.
4. Rinse with water and blot dry.

* Not applicable to direct staining of specimens.

5. Examine microscopically under oil immersion (1000×) for the presence and location of spores.

Interpretation:
Spores are stained green while the bacterial cells are stained pink (Figure 2–13, see color insert in the center of the book).

Toluidine Blue O Stain[5]

Reagents:

Sulfation reagent

Ether	35 ml
H_2SO_4, concentrated	25 ml
Distilled water	10 ml

Mix ether and water vigorously in a separatory funnel for approximately 1 min. Allow to stand until the aqueous and ether phases separate, and discard aqueous phase. Place 25 ml of ether (water-saturated) into a flask in ice water. Slowly add while mixing the H_2SO_4. Transfer the reagent to a Coplin jar and seal the lid with vaseline. Stir the reagent with a glass rod before using. Expiration date is 1 week.

Toluidine blue

Toluidine blue O	0.3 g
HCl, concentrated	2 ml
Ethanol, absolute	140 ml
Distilled water	60 ml

Mix dye in water, add HCl, and then alcohol.

Isopropyl alcohol, 99%
Xylene

Procedure:

1. Prepare impression smears (p. 43).
2. Place slides in sulfation reagent for 5 min.
3. Rinse slide in tap water.
4. Stain for 3 min.
5. Dehydrate in two changes of isopropyl alcohol.
6. Clear in two changes of xylene.
7. Apply coverslip with mounting medium (e.g., Permount).
8. Examine microscopically.

Interpretation:
Pneumocystis carinii cysts are stained purple, while tissue remnants and debris are stained blue (Figure 2–9). Trophozoites are not discernible.

References

1. Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C. S., Eschenbach, D., and Holmes, K. K. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *Am. J. Med.* **74**:14, 1983.
2. Bisno, A. L. The diagnosis of streptococcal pharyngitis. *Ann. Intern. Med.* **90**:426, 1979.
3. Borchardt, K. A., and Hoke, A. W. Simplified laboratory technique for diagnosis of chancroid. *Arch. Derm.* **102**:188, 1970.
4. Brown, R. C., and Hopps, H. C. Staining of bacteria in tissue sections. *Am. J. Clin. Pathol.* **60**:234, 1973.
5. Chalvardjian, A. M., and Grawe, L. A. A new procedure for the identification of *Pneumocystis carinii* cysts in tissue sections and smears. *J. Clin. Pathol.* **16**:383, 1963.
6. Crawford, G., Brancato, F., and Holmes, K. K. Streptococcal pharyngitis: Diagnosis by Gram stain. *Ann. Intern. Med.* **90**:293, 1979.
7. Dans, P. E., and Judson, F. The establishment of a venereal disease clinic: II. An appraisal of current diagnostic methods in uncomplicated urogenital and rectal gonorrhea. *J. Am. Vener. Dis. Assoc.* **1**:107, 1975.
8. Felman, Y. M., and Nikitias, J. A. Trichomoniasis, candidiasis, and *Corynebacterium vaginalis* vaginitis. *N.Y. State J. Med.* **79**:1563, 1979.
9. Leifson, E. Staining, shape, and arrangement of bacterial flagella. *J. Bacteriol.* **62**:377, 1951.
10. Leifson, E. *Atlas of Bacterial Flagellation.* New York, Academic Press, 1960.
11. McCracken, A. W., and Mauney, C. U. Identification of *Corynebacterium diphtheriae* by immunofluorescence during a diphtheria epidemic. *J. Clin. Pathol.* **24**:641, 1971.
12. Murray, P. R., and Washington, J. A., II. Microscopic and bacteriologic analysis of sputum. *Mayo Clin. Proc.* **50**:339, 1975.
13. Rein, M. F., Gwaltney, J. M., O'Brien, W. M., Jennings, R. H., and Mandell, G. L. Accuracy of Gram's stain in identifying pneumococci in sputum. *J. Am. Med. Assoc.* **239**:2671, 1978.
14. Roberts, G. D. Detection of fungi in clinical specimens by phase-contrast microscopy. *J. Clin. Microbiol.* **2**:261, 1975.
15. Smith, J. W., and Bartlett, M. S. Diagnosis of Pneumocystis pneumonia. *Lab. Med.* **10**:430, 1979.
16. Tam, M. R., Stamm, W. E., Handsfield, H. H., Stephens, R., Kuo, C.-C., Holmes, K. K., Ditzenberger, K., Krieger, M., and Nowinski, R. C. Culture-independent diagnosis of *Chlamydia trachomatis* using monoclonal antibodies. *N. Engl. J. Med.* **310**:1146, 1984.
17. Tashjian, J. H., Coulam, C. B., and Washington, J. A., II. Vaginal flora in asymptomatic women. *Mayo Clin. Proc.* **51**:557, 1976.
18. Truant, J. P., Brett, W. A., and Thomas, W., Jr. Fluorescence microscopy of

tubercle bacilli stained with auramine and rhodamine. *Henry Ford Hosp. Med. Bull.* **10**:287, 1962.

19. Van Orden, A., and Green, P. Modification of the Dieterle spirochete stain. *J. Histotechnol.* **1**:51, 1977.
20. Washington, J. A., II, White, C. M., Laganiere, M., and Smith, L. H. Detection of significant bacteriuria by microscopic examination of urine. *Lab. Medicine* **12**:294, 1981.

3

Initial Processing for Cultures of Specimens

John A. Washington II, M.D.

Specimens must be processed expeditiously and in a manner that provides the greatest opportunity for the prompt isolation of the most probable cause of the infection.

Work should be organized in the laboratory so as to provide information that is important for the patient's management as soon as it becomes available[19]; however, it is important for clinicians to be aware of the fact that not all laboratories perform all tests and that specimens submitted for the diagnosis of mycobacterial, fungal, viral, or parasitic infection are often sent to a reference laboratory. In such instances, access to information regarding work in progress is limited, and it is likely to take longer to obtain reports.

Processing of specimens for examination for parasites will be described in Chapter 11.

I. Blood Cultures[22]

A. Procedures

Blood cultures should be examined once, and preferably twice, within the first 6 to 18 hr of incubation for the presence of colonies, turbidity, hemolysis, and/or gas formation. This step is essential before monitoring cultures radiometrically (BACTEC, Johnston Laboratories, Cockeysville, MD) to prevent cross-contamination from an obviously positive culture. Broth cultures should be examined for evidence of growth at least once daily thereafter for a minimum of 7 days, although detectable growth

of certain fastidious bacteria, such as *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Haemophilus aphrophilus*, and some yeasts may require a second week of observation. Culture media inoculated with lysis-centrifugation (Isolator™) concentrate should be incubated and examined according to the manufacturer's directions, although we have found that 72 hr of incubation of the blood and chocolate agar plates (Table 3–1) is sufficient for detection of aerobic and facultatively anaerobic bacteria. Cultures for *Brucella* should be incubated at 35°C in an atmosphere of air with added CO_2 and examined for a minimum of 2 weeks. Cultures for fungi should be incubated at 25 to 30°C (optimal for *Cryptococcus neoformans* and filamentous fungi) in air and examined for a minimum of 2 weeks.

The aerobic or vented bottles should be subcultured onto chocolate blood agar routinely after the first 6 to 18 hr of incubation. The value of this procedure in the radiometric (BACTEC) system is controversial; however, it is suggested that routine subculture be performed until it can be documented that the procedure lacks clinical value in the patient population under study.[22] Routine subcultures of anaerobic or unvented bottles are unnecessary, except in cultures of pediatric patient populations at risk for *Haemophilus influenzae* bacteremia.[8] Routine subcultures in this instance are also made onto chocolate blood agar. All chocolate blood agar plates are incubated for 48 hr at 35°C in an atmosphere of air with added CO_2. Biphasic media bottles (including Roche SeptiChek) are subcultured at the time of each examination by tipping them so that the blood-broth mixture flows over the agar surface or surfaces.

Positive cultures are examined microscopically and the patient's physician immediately notified as to the organism's Gram-stain morphology. If the Gram-stained smear suggests a pure culture of gram-negative bacilli and there is no growth on any subculture media, suspensions may be

Table 3–1. Subculture Media for Suspected Positive Blood Cultures

	Subculture environment				
	Capneic			Anaerobic	
Bacteria	Blood agar	EMB	Chocolate agar	Schaedler's broth	Blood agar
Gram-positive					
Cocci	X			X	X
Bacilli	X			X	X
Gram-negative					
Cocci	X		X	X	X
Bacilli	X	X	X	X	X

prepared and adjusted to the appropriate turbidity from centrifuged sediment of the broth for identification and antimicrobial susceptibility tests. If the Gram-stained smear suggests a pure culture of staphylococci, an aliquot of the broth can be inoculated into plasma for the tube coagulase test. Isolated colonies in subculture media, including media inoculated with lysis-centrifugation concentrate, can be identified and tested for antibiotic susceptibility according to established procedures in the laboratory. Positive broth cultures must be subcultured onto general purpose and differential media for isolation of aerobic, facultatively anaerobic, and anaerobic bacteria (Table 3–1).

II. Cultures of Normally Sterile Body Fluids, Tissue, Wounds, and Abscesses

A. Procedures

1. Fluids

Media* and incubation conditions for cultures of bacteria, mycobacteria, and fungi are listed in Table 3–2. All normally sterile fluids may be inoculated directly into appropriate media. Provided there is sufficient volume (>2 ml), cerebrospinal fluid is filtered through a 0.45 μm sterile, disposable filter, which is then placed "upstream" side down on the surface of the appropriate agar medium. The filter should be moved every 24 to 48 hr with sterile forceps to another location on the agar so that colonies forming beneath it can be detected. Alternatively, fluid may be centrifuged at a minimum of 1500 $\times$ g for 15 min and the sediment used for preparing both smears and cultures.

Fluids submitted in an anaerobic transport medium are withdrawn with a sterile needle and syringe which are then used to inoculate media directly for the isolation of aerobic, facultatively anaerobic, and anaerobic bacteria, and, if requested, for mycobacteria or fungi. Fluid remaining in the syringe is transferred into a sterile test tube, and inoculated with a sterile Pasteur pipette into the bottom of a tube containing thioglycollate medium supplemented with rabbit serum. If plates and tubes for anaerobic culture cannot be incubated immediately under anaerobic conditions, they should be stored for not more than 2 hr in a holding jar that is constantly flushed with CO_2.

There are three basic approaches to anaerobic incubation: anaerobic jar, anaerobic chamber, and roll tube. Although the three are equivalent

* The media listed are those used in this laboratory; however, acceptable alternatives are often available[9] and supplemental media may be inoculated in certain instances.

Table 3–2. Procedures for Cultures of Normally Sterile Body Fluids, Tissue, Wounds, and Abscesses

Category	Medium or cell culture	Incubation: Temp. (°C)	Incubation: Atmosphere	Incubation: Duration (days)	Comments	Microscopic examination of suspected positive culture
Bacteria						
Aerobic and facultatively anaerobic	Blood agar	35	5–10% CO_2	2	Cerebrospinal fluid is inoculated directly into supplemented MHB and thioglycollate medium; filter is applied directly to chocolate blood agar only. Material from wounds or abscesses is not inoculated onto chocolate blood agar or supplemented MHB.	Gram-stained smear
	Chocolate blood agar					
	Eosin-methylene blue (EMB) agar					
	Colistin-nalidixic acid (CNA) blood agar					
	Mueller-Hinton broth (MHB) with Supplement C			5		
	Thioglycollate medium with rabbit serum			5		
Brucella	Blood heart infusion agar	35	5–10% CO_2	28		Gram-stained smear
	Wisconsin ("W") agar					FA-stained smear
Anaerobic	Blood agar	35	5–10% CO_2	2		
	Rabbit blood agar		Anaerobic	7	Tissue is inoculated onto Rabbit blood agar only	Gram-stained smear, FA-stained smear for *Bacteroides fragilis*
	Rabbit blood agar with gentamicin and vancomycin (RGV)					
	Phenylethyl alcohol (PEA) blood agar					
	Thioglycollate medium with vitamin K, hemin, and rabbit serum					

Actinomyces	Brain heart infusion agar (BHIA) Thioglycollate medium with vitamin K, hemin, and rabbit serum	35	Anaerobic	21		Gram-stained smear
Mycobacteria	Lowenstein–Jensen medium Middlebrook 7H10 medium Selective 7H11 (Mitchison) medium (S7H11)	35	5–10% CO_2	60		Acid-fast stained smear
Fungi	Inhibitory mold agar (IMA) Brain heart infusion blood agar with chloramphenicol and gentamicin (BHIA-3) Brain heart infusion blood agar with chloramphenicol, gentamicin, and cycloheximide (BHIA-4) Sabouraud dextrose agar (SAB)	30	Air	30		KOH wet mount, India ink wet mount
Viruses	MRC-5, CMK	35	Air	14	Tissue is inoculated directly into 2 MRC-5 cultures. Fluids are inoculated (0.1 ml/culture) directly into cell cultures; maintenance medium is replaced the next day.	Examination at 125× for cytopathic effects

in terms of providing an atmosphere that is suitable for the recovery of clinically significant anaerobic bacteria, each has its advantages and disadvantages. The anaerobic jar (GasPak, BBL Microbiology Systems) is the most widely used method because of its convenience and economy. A convenient alternative for laboratories processing small numbers of specimens is a disposable transparent isolation unit (Bio-Bag™, Environmental Chamber, Marion Scientific, Kansas City, MO), designed to provide an anaerobic atmosphere for a petri dish. Anaerobic cultures in our laboratory are initially incubated in jars that are opened on the following day inside an anaerobic chamber. The jars are removed from the chamber, and the cultures remain inside the chamber for further incubation.

All inocula for bacterial cultures (except of urine) on solid media should be streaked for isolation as depicted in Figure 3–1. The relative numbers of colonies of each species isolated may be reported semiquantitatively as follows[2]:

	Colonies in streak areas		
Grade	Primary	#2	#3
1+	<10	—	—
2+	>10	<5	—
3+	>10	>5	<5
4+	>10	>5	>5

For laboratories preferring to use the description of "many," "moderate," or "few," grade 4+ would correspond to "many," grades 2+ and 3+ to "moderate," and grade 1+ to "few."

Inocula (0.5 ml) for fungal cultures are streaked on solid media as depicted in Figure 3–2.

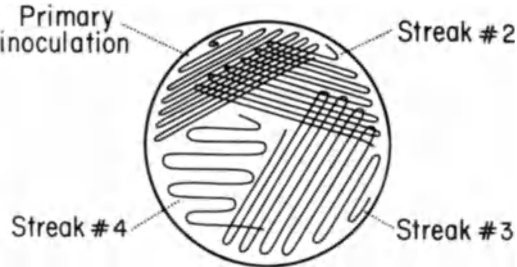


Figure 3–1. Method of streaking plates for bacterial cultures (except of urine). The inoculating loop is flamed each time direction of streaking is changed.

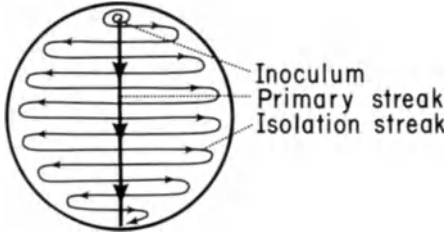


Figure 3–2. Streak plate method for quantitative purposes. Inoculum is deposited with a pipet or calibrated loop.

2. Tissues

a. Nonquantitative ("Routine") Cultures

Tissue specimens are homogenized whenever possible in a Stomacher Lab-Blender (Model no. 80, Tekmar Co., Cincinnati, OH); otherwise, they are minced with sterile scissors and then ground in a sterile mortar with a sterile abrasive (Alundum) and pestle. Sterile nutrient broth is added to the tissue to make a 10 to 20% suspension, which is then placed into a sterile dropper bottle and inoculated heavily onto appropriate media for culture of bacteria, mycobacteria, and fungi, or into cell cultures for detection of viruses (Table 3–2). Excess suspension should be stored at 5°C for 4 weeks in case cultures must be repeated or additional cultures are indicated on the basis of the specimen's histopathology.

b. Quantitative Bacterial Cultures[3,10,12,15]

(1) Weigh sterile petri dish.
(2) Weigh tissue in petri dish.
(3) Mince tissue with sterile scissors and then grind with sterile pestle in sterile mortar containing 1 ml of nutrient broth per gram of tissue.
(4) Dilute the suspension as follows:
 0.9 ml nutrient broth + 0.1 ml suspension (10^{-1})
 0.9 ml nutrient broth + 0.1 ml 10^{-1} suspension (10^{-2})
 0.9 ml nutrient broth + 0.1 ml 10^{-2} suspension (10^{-3})
(5) Inoculate 0.1 ml of each of the diluted suspensions onto blood agar (optional: EMB and CNA) and into thioglycollate medium with rabbit serum. Spread the inoculum evenly over the surface of the agar with a sterile bent ("hockey stick") glass rod.
(6) Incubate blood agar plates at 35°C for 24 hr.
(7) Quantitate growth as follows:

$$\text{Organisms/gram of tissue} = \frac{N \times D \times F \times 10}{W}$$

where

N = number of colonies on agar plate selected for enumeration
D = reciprocal of dilution inoculated onto agar
V = volume of broth used to suspend tissue
W = weight of tissue in grams
F = dilution factor, $(V+W)/W$
10 = constant factor since 0.1 ml of suspension is used as inoculum.

C. Quantitative Stained Smears for Bacteria[3,10,15]

(1) Transfer 0.01 ml of undiluted suspension [see 2,b,(3) above] onto a clean glass microscope slide and spread over an area not exceeding 15 mm in diameter.
(2) Dry in an oven (75°C) for 15 min.
(3) Gram stain.
(4) Examine all fields under oil immersion (×1000). Finding one or more bacteria in *any* field (*not* per oil immersion field) denotes the presence of at least 10^5 bacteria/gram of tissue.

3. Wounds and Abscesses

Media and incubation conditions are listed in Table 3–2, except that neither chocolate blood agar nor supplemented Mueller-Hinton broth is inoculated, and specimens that are known to contain bacteria and require culture for mycobacteria must undergo decontamination (see p. 108).

4. Intravascular Catheter Tips[13]

a. Remove and discard the swab and transport catheter segment to the laboratory in a swab-transport medium device (e.g., Culturette, Marion Scientific Corporation) to prevent desiccation.
b. Transfer catheter segment to surface of blood agar and, with sterile forceps, roll the catheter back and forth across the agar at least four times.
c. Incubate blood agar at 35°C in 5–10% CO_2 for 2 days.
d. Report growth as follows: ≥15 CFU/agar surface = positive; <15 CFU/agar surface = negative. A positive culture result correlates significantly with infection (vs. contamination of the catheter surface).

B. Examination

All cultures, except those for mycobacteria, are examined daily. Cultures for mycobacteria are examined twice a week for the first 4 weeks and

then once a week. If any of the media exhibit evidence of microbial growth, microscopic examination is made and appropriate tests initiated for identification. Bacteria isolated from normally sterile body fluids and tissues should ordinarily undergo antimicrobial susceptibility testing. (See p. 283 for indications for antimicrobial susceptibility testing.) *Haemophilus influenzae* should be tested for β-lactamase production (see p. 305).

III. Cultures of Specimens from the Upper Respiratory Tract

A. Procedures

Nearly all specimens from this area arrive on swabs which, with the exception of those for viral cultures, can be used directly to inoculate the appropriate media (Table 3–3). Swabs for viral cultures are vigorously agitated (extracted) in a serum-free medium containing antibiotics to suppress bacterial and fungal overgrowth (see p. 821). Aliquots of this medium are then used to inoculate cell cultures (Table 3–3).

Cultures of pharyngeal swabs for bacteria other than group A streptococci should be performed only by specific request so that the appropriate media can be inoculated. Agar media should be inoculated by rolling the swab over the area of primary inoculation and then streaking the agar surface for isolation (Figure 3–1). When culturing for group A streptococci, it is recommended that several stabs be made with an inoculating loop in the areas of primary inoculation and streaks #3 and #4. This step is taken to ensure detection of rare strains of group A streptococci which elaborate only the oxygen-labile hemolysin (streptolysin O) and which will not produce hemolysis below the agar surface.

For group A streptococci, the blood agar plate should be incubated at 35°C in air. Incubation in an atmosphere with added CO_2 or under anaerobic conditions increases the frequency of isolation of β-hemolytic streptococci but not specifically of group A β-hemolytic streptococci.[11,17]

Group A streptococci are detected microscopically in some laboratories by staining the sediment of a 2 to 4 hr broth culture of a pharyngeal swab with a fluorescein-labeled anti-group A streptococcal conjugate. Although this procedure provides rapid results and is highly sensitive and specific, it is expensive in terms of equipment, reagents, and technical requirement. Other immunologic tests (coagglutination, latex agglutination, enzyme immunoassay) are becoming available commercially. Since none of these tests entails a preincubation step for swabs, their ability to detect small numbers of group A streptococci may be less than 90% (relative to culture).

Table 3–3. Procedures for Culturing Specimens from the Upper Respiratory Tract

				Incubation				Examination	
Category	Specimen	Specimen preparation	Medium or cell culture	Temp. (°C)	Atmosphere	Duration	Other	Frequency	Method or specific characteristic sought[a]
Bacteria									
Streptococcus, group A	Pharyngeal or nasopharyngeal swab	Inoculate medium	1. Todd-Hewitt broth	35	Air	2–4 hr	—	—	FA-stained smear of centrifuged specimen
			2. Extraction reagent	—	—	—	—	—	CoA, LA, or EIA
			3. Blood agar	35	Air	1 day	Extend to 48 hr to detect delayed hemolysis, small numbers of colonies, or carrier state	Daily	β-Hemolytic colonies
Bordetella pertussis	Nasopharyngeal aspirate or swab	1. Prepare smear on microscope slide		—	—	—	—	—	FA-stained smear
		2. Inoculate medium	Charcoal or Bordet-Gengou agar	35	5–10% CO_2	4 days	—	Daily	Smooth, gray colonies

Corynebacterium diphtheriae	Pharyngeal swab or swab of cutaneous lesions	Inoculate media	Cystine tellurite blood agar	35	Air	2 days	—	Daily	Grayish-black colonies; toxigenicity
Neisseria gonorrhoeae or *N. meningitidis*	Pharyngeal or nasopharyngeal swab	Inoculate medium	Modified Thayer-Martin	35	5–10% CO_2	3 days	—	Daily	Oxidase-positive colonies
Fungi	Oral or pharyngeal swab	Inoculate medium	See Table 3–2	30	Air	30 days	—	Daily	Colonies
Mycoplasma pneumoniae	Pharyngeal swab	Inoculate media	1. Complete *Mycoplasma* agar	35	Air	30 days	—	2 × / week	
			2. Diphasic broth	35	Air	30 days	Subculture at 4–6 day intervals × 2 onto *Mycoplasma* selective agar. Incubate subcultures in air at 35°C for 10 days	2 × / week	Colonies (observed at 50 × and 125 × magnification)
Viruses	Pharyngeal swab	Extract in serum-free medium containing antibiotics. Inoculate cell cultures	0.25 ml into MRC-5 and 0.3 ml into CMK	35	Air	14 days	No routine medium changes in cell cultures	3 × / week	Cytopathic effects (observed at 125 × magnification) or hemadsorption

[a] FA, fluorescent antibody; CMK, Cynomolgus monkey kidney cells; CoA, coagglutination; LA, latex agglutination; MRC, Medical Research Council cells; EIA, enzyme immunoassay.

Incubation conditions for other categories of cultures are listed in Table 3–3.

B. Examination

The frequency of examination of cultures and the methods or particular findings sought are listed in Table 3–3.

IV. Cultures of Specimens from the Lower Respiratory Tract

A. Procedures

1. Open Lung Biopsy

Such specimens should be processed as expeditiously as possible. From the freshly cut surface of the biopsy, impression smears are made for staining for *Legionella* (see p. 43) and *Pneumocystis carinii* (see p. 43). The tissue is then homogenized and a suspension prepared, as described in Section II,A,2,a (p. 101). The suspension can be used to prepare stained smears for bacteria, mycobacteria, or fungi and as inoculum for cultures on appropriate media or cell cultures (Table 3–3). Stained preparations of lung tissue concentrates, prepared by centrifuging (20 min, 800 $\times g$) the homogenate, are superior to stained impression smears for detection of *P. carinii* cysts.[25]

2. Other Specimens

Procedures for processing and culturing are described in Table 3–4. The Gram-stained smear serves as a screening test to determine the acceptability of sputum submitted for bacterial culture, so that the presence of ≥ 25 squamous epithelial cells (SEC) per low power field (100×) represents the criterion for rejection of the specimen for culture.[2,5] Failure to restrict bacterial cultures to microscopically acceptable specimens will limit the value of cultures. Because of the selective isolation procedures for mycobacteria, fungi, chlamydiae, mycoplasmas, and viruses, screening procedures are not used to determine the acceptability of sputum for culture of those organisms. Sputum should not be cultured for anaerobic bacteria.

The value of bacterial, and particularly anaerobic bacterial, cultures of bronchoscopy specimens is limited unless the specimen is obtained through a telescoping distally occluded double catheter and is cultured quantitatively.[21,27]

Table 3–4. Procedures for Cultures of Specimens from the Lower Respiratory Tract[a]

Category	Specimen	Processing	Media or cell cultures	Incubation		
				Temp. (°C)	Atmosphere	Duration (days)
Bacteria						
Aerobic and facultatively anaerobic	Sputum	Examine Gram-stained smear at 100×: (1) >25 SEC—do not culture, request another specimen	—	—	—	—
		(2) <25 SEC—examine at 1000×, report findings, culture	Blood agar, chocolate blood agar, EMB, and *excluding sputum* thioglycollate plus rabbit serum	35	5–10% CO_2	2
	TTA, pleural fluid, tissue or abscess	—		35	5–10% CO_2	5
Anaerobic	TTA, pleural fluid, tissue or abscess	—	Brucella blood agar, RGV, PEA, thioglycollate plus rabbit serum	35	Anaerobic	7
Legionella	Sputum, TTA, pleural fluid, bronchial washing, tissue	Pretreat sputum (0.5 ml) KCl-HCl, pH 2.2, solution (4.5 ml) for 4 min	BCYE, BVPA	35	3% CO_2	10
Nocardia asteroides	Sputum, TTA, bronchial washings, tissue, abscess	—	BHIA	35	Air	14

Table 3–4 (*continued*)

Category	Specimen	Processing	Media or cell cultures	Incubation		
				Temp. (°C)	Atmosphere	Duration (days)
Mycobacteria	Sputum, induced sputum, TTA, bronchial washings, gastric washings, tissue	Decontaminate by adding equal volume 2% NaOH, mixing, and treating as follows: sputum—20 min; bronchial and gastric washings—15 min. Neutralize with 1.25 *N* HCl containing bromcresol purple just to point at which solution becomes uniformly yellow; add few drops of 2% NaOH so that solution becomes uniformly blue. Centrifuge ($2000 \times g$) and discard all but 5 ml of supernatant. Inoculate 0.5 ml of sediment onto each agar plate and 0.25 ml onto each agar slant. Plates are sealed in polyethylene, CO_2-permeable bags.	Lowenstein-Jensen, S7H11	35	5–10% CO_2 (1st 4 weeks, then air)	60
Fungi	Sputum, induced sputum, TTA, bronchial washings, tissue, mucous plugs	—	IMA, Sabouraud agar, BHIA, BHIA-4	30	Air	30

Chlamydia trachomatis	Pharyngeal swab, tissue	Extract swab or prepare suspension of tissue in 2SP medium. Inoculate each of 4 vials containing McCoy cells with 0.1 ml of specimen in 2SP, centrifuge (700 × *g*) for 1 hr, add cycloheximide, incubate. Contaminated cultures should be repeated by treating specimen with streptomycin–vancomycin solution (0.1 ml/0.5 ml specimen), centrifuging (300 × *g*) for 5 min, and inoculating McCoy cells as described above.	McCoy cells	35	Air	2–3
Mycoplasma pneumoniae	Pharyngeal swab, sputum, tissue	See Table 3–3 for processing and incubation conditions				
Viruses	Pharyngeal swab, sputum, tissue	See Table 3–3 for processing and incubation conditions				

[a] TTA, transtracheal aspirate; SEC, squamous epithelial cells; media are as listed in Table 3–2.

B. Examination

The frequency of examination of cultures is as shown in Table 3–3.

Bacteria isolated from sputum deemed acceptable for culture on the basis of the initial microscopic examination (Table 3–4) should be identified, regardless of numbers, with the possible exception of viridans streptococci, corynebacteria, neisseriae, and coagulase-negative *Staphylococcus,* which can be reported as "usual oropharyngeal flora." Should initial microscopic screening of sputum not be done and all sputum specimens received be cultured (a practice that should be discouraged), it is suggested that identification be restricted to colonies present in numbers equivalent to grades 3+ or 4+ (see p. 100). In general, yeasts recovered from fungal cultures of respiratory secretions need not be identified; however, they should be tested for urease production to rule out the presence of *Cryptococcus neoformans*[16] (see Ch. 7).

V. Cultures of Urine

A. Procedures

Urine should be agitated on a mechanical (Vortex) mixer prior to culture. The media or cell cultures and incubation conditions are listed in Table 3–5. Cultures for aerobic and facultatively anaerobic bacteria, as well as for fungi, should be streaked for isolation as depicted in Figure 3–2 so that the growth of colonies can be reported quantitatively. Alternatively, and less practically, quantitation of bacteria may be achieved by mixing 0.1 ml of a 1:10 dilution of urine in either 10 or 20 ml of molten (50°C) nutrient agar, which is then poured into an empty petri dish on a flat surface and allowed to solidify before incubation.

B. Examination

Colonies of bacteria and yeasts are enumerated and multiplied by the appropriate dilution factor (× 1000) to provide the final colony count. Precise enumeration is unnecessary and exceeds the accuracy of the procedure; therefore, it is sufficient to report colony counts for each type or species of microorganism isolated in ranges, e.g., 10^4–10^5 colonies/ml (Table 3–5). Although bacteriuria of 10^5 colony-forming units (CFU)/ml is generally regarded as being clinically significant, lesser numbers (10^4 CFU/ml) should be similarly regarded provided the urine specimen has been properly collected and transported to the laboratory. Recent evidence suggests that in acute dysuria and frequency in women, a criterion of $\geq 10^2$ CFU/ml, usually of Enterobacteriaceae, provides the best

Table 3–5. Procedures for Cultures of Urine

Category	Type of urine specimen	Specimen preparation	Media or cell cultures	Incubation			Comments
				Temp. (°C)	Atmosphere	Duration (days)	
Bacteria							
Aerobic and facultatively anaerobic	Midstream, catheterized, or SPA	Mix well on mechanical mixer. Inoculate each agar medium by rapidly dipping a 0.001 ml calibrated platinum loop vertically into urine. Streak agar surface as shown in Figure 3–2.	Blood agar EMB	35	5–10% CO_2	1	Growth must be reported quantitatively: 1–10 colonies = $<10^4$ col/ml; 10–100 colonies = 10^4–10^5 col/ml. $>$ 100 colonies = $> 10^5$ col/ml. Report $< 10^4$ col/ml as "organisms present" but do not identify routinely.[a]
			Add thioglycollate plus rabbit serum for SPA	35	5–10% CO_2	7	
Anaerobic	SPA	—	See Table 3–2 for media and incubation conditions				
Mycobacteria	Midstream or catheterized	Decontaminate for 20 min as described in Table 3–4. When gram-negative bacillus is known to be present on basis of prior bacterial culture, decontaminate for 25 min.	See Table 3–2 for media and incubation conditions				

Table 3–5. (*continued*)

Category	Type of urine specimen	Specimen preparation	Media or cell cultures	Incubation			Comments
				Temp. (°C)	Atmosphere	Duration (days)	
Leptospira	Midstream (freshly voided)	Dilute 1 ml of urine in 9 ml of sterile broth. Filter (0.45 μm pore size) 5 ml. Inoculate 3 tubes containing EMJH semisolid medium with 0.1 ml unfiltered diluted urine, 0.1 ml and 0.5 ml of filtered diluted urine, respectively.	EMJH	30	Air	30	Examine weekly for macroscopic evidence of growth (1–3 cm below medium surface) and by dark field microscopy for presence of leptospires.
Fungi	Midstream or catheterized	As for aerobic and facultatively anaerobic bacteria above.	See Table 3–2 for media and incubation conditions				Report growth of yeasts quantitatively[6,28] as described above for aerobic and facultatively anaerobic bacteria.
Viruses	Midstream or catheterized	Add 0.1 ml of penicillin–streptomycin solution and 0.1 ml of nystatin solution per ml of urine.	MRC-5	35	Air	14	Examine by IFA for cytomegalovirus-infected cells.

[a] For exceptions, see text on p. 110.

separation of infected from uninfected patients[24] but that this criterion should not be extrapolated to populations that have a substantially greater likelihood of contamination during voiding or a lower prevalence of infection.[20] Low (10^3–10^4 CFU/ml) but clinically significant colony counts may be seen in urine from patients who have chronic pyelonephritis, urinary calculi, or obstructive uropathy or who are receiving antimicrobial therapy. Unless urine specimens from women with acute dysuria and frequency are identified as such, so that at least 0.01 ml can be cultured, it would seem appropriate to limit identification to species present in pure culture in numbers exceeding 10^4 CFU/ml and to those present in mixed culture in numbers exceeding 10^5 CFU/ml.[2] Renal candidosis is usually associated with candiduria exceeding 10^4 CFU/ml.[6,28]

VI. Cultures of Feces

Clinical laboratory methods for determining the etiology of gastroenteritis (Table 1–7) are limited to immunoassay for rotaviruses (Chapter 10), microscopic examination for intestinal parasites (Chapter 11), cytotoxic assay and possibly, culture for *Clostridium difficile* (Chapter 5), and cultures for *Campylobacter jejuni, Salmonella, Shigella,* and *Yersinia enterocolitica.* Although it is not technically difficult, culture for vibrios can probably be performed on a selective basis, except in coastal areas where disease due to vibrios is more likely to occur. Tissue culture and animal inoculations had provided the only means of detection of toxigenic and invasive strains of *Escherichia coli* until the recent development of simple and rapid immunologic tests for the heat-labile toxin (LT).[4,23] Certain serotypes of *E. coli* cause gastroenteritis by toxigenicity, invasiveness, or as yet undefined mechanisms of pathogenicity, so that it seems likely that serological methods will become available in the future, either for determining the presence of antibody in serum or for identifying the presence of these strains in feces. In the meantime, laboratories are advised *not* to screen cultures of feces for the classical "enteropathogenic" serotypes of *E. coli.* Rotaviruses and parvovirus-like agents cannot be cultured and are usually detected by immunoassay or electron microscopy.

A. Procedures

Procedures for cultures of salmonellae, shigellae, vibrios, *Campylobacter jejuni, Yersinia enterocolitica, Clostridium difficile,* fungi, and viruses are listed in Table 3–6. Although definitive evidence is lacking for an etiological role of fungi, and particularly *Candida,* in producing gastroenteritis, fungi that invade the gastrointestinal tract may be isolated from cultures of feces. Similarly, cultures of feces for viruses may be indicated

Table 3–6. Procedures for Cultures of Fecal Material

Test request	Media[a]	Temp (°C)	ATM	Duration (d)	Subculture	What to look for
Salmonella, Shigella	BA	35	Air	3		General distribution of flora, preponderance of *Bacillus,* yeasts, filamentous fungi
	EMB					Clear, colorless colonies
	HE					Blue-green colonies with black centers
	Sel-F				Within 24 hr subculture 1 drop onto HE (incubate for 48 hr)	—
	C-5	42	5% O_2 (CO_2-N_2)	2		Gray, flat, nonhemolytic watery (opaque in older cultures) colonies
Clostridium difficile	CCFA	35	Anaerobe	7		Yellow colonies
	PBS	(send to Virology)	—	—		
Salmonella outbreak investigation	BS	35	Air	2		Black colonies
Yersinia enterocolitica	CIN	25	Air	3		Translucent pink-red colonies
	PBS	5	Air	21	0.1 ml onto CIN once weekly	
Vibrios	TCBS	35	Air	3		Yellow or blue-green colonies

[a] BA, blood agar; EMB, eosin-methylene blue agar (Levine); HE, Hektoen enteric agar; Sel-F, selenite F enrichment broth; C-5, modified Skirrow's selective medium for *Campylobacter jejuni;* CIN, cefsulodin-irgasan-novobiocin selective medium for *Yersinia enterocolitica;* CCFA, cycloserine-cefoxitin-fructose–egg yolk agar; BS, bismuth sulfite; PBS, 0.1 M phosphate-buffered saline, pH 7.6; TCBS, thiosulfate-citrate–bile salts–sucrose agar.

in patients with respiratory, central nervous system, or exanthematous diseases suspected of being of viral origin. Toxigenic strains of *Clostridium difficile* may be demonstrated by adding 0.1 ml of a supernatant of feces to a cell culture (e.g., MRC-5, diploid fibroblast cells) and examining it in 4 to 24 hr for evidence of cytotoxicity (p. 363) or by isolating the organism on selective media from feces in anaerobic cultures (Table 3–6).

B. Examination

Organisms producing the characteristics listed in Table 3–6 are subcultured for identification. Prior antimicrobial therapy may alter the distribution of normal bacterial flora on the blood agar and differential agar media. Occasionally, there is overgrowth by a bacterial species or by yeasts, particularly *Candida;* however, it seems likely that this occurrence per se is not clinically significant. It appears, therefore, to be unnecessary to report the presence or overgrowth of bacteria other than those listed in Table 3–6.

VII. Cultures of Genital Tract Infections

A. Procedures

1. Sexually Transmitted Diseases (STD)

Procedures for cultures of specimens from the genital tract for microorganisms associated with sexually transmitted diseases are listed in Table 3–7. Modifications of Thayer-Martin medium (i.e., Martin-Lester or Martin-Lewis) are available, either as an agar slant in a screwcapped bottle containing CO_2 (Transgrow) or as a layer of agar in a rectangular plastic dish to which a CO_2-generating tablet can be added before placing it in a Ziploc plastic bag for incubation (JEMBEC). Transgrow and JEMBEC should be incubated prior to shipment to the laboratory.

2. Female Genital Tract Infections Other than STD

Because of the quantity and complexity of the microbial flora indigenous to the vagina (Table 1–1), cultures of specimens from this site should generally be limited to the isolation and identification of microorganisms associated with STD (Table 3–7).

Exceptions: Specimens obtained at the time of pelvic surgery, those

Table 3–7. Procedures for Cultures of Microorganisms Associated with Sexually Transmitted Diseases

Category[a]	Specimen preparation	Medium or cell culture	Incubation: Temp. (°C)	Incubation: Atmosphere	Incubation: Duration (days)	Characteristic sought
Neisseria gonorrhoeae	—	Thayer-Martin agar[b]	35	5–10% CO_2	4	Oxidase-positive colonies of gram-negative diplococci
Chlamydia trachomatis	Extract swab in 2SP transport medium. See Table 3–4 or processing.	McCoy cells	35	Air	2–3	IFA-stained cells or iodine-stained inclusions
Ureaplasma urealyticum	Extract swab in 2SP transport medium. Transfer 0.2 ml to 1.8 ml U-9 broth.	U-9 broth	35	Air	5	Shift to alkaline pH
Trichomonas vaginalis	Make fresh wet preparation. Culture is optional supplemental procedure.	Lash's casein hydrolysate-serum medium	35	Air	2	Examine for motile trichomonads
Candida albicans	—	Inhibitory mold agar	30	Air	30	Colonies of yeast
Herpes simplex virus	Extract in serum-free medium containing antibiotics. See Table 3–3 for processing.	MRC-5, CMK	35	Air	14	IFA-stained HSV-infected cells

Gardnerella vaginalis	—	Human blood in agar bilayer containing Tween 80 (HBT)	35	5–10% CO_2	2	Small, white, β-hemolytic, catalase-negative colonies of nonsporulating gram-positive bacilli
Haemophilus ducreyi	—	GC medium base plus 1% hemoglobin, 1% IsoVitaleX, 5–10% fetal calf serum, and 3 μg/ml vancomycin	35	5–10% CO_2	7	Nonmucoid, yellow-gray, translucent colonies

[a] Listed in approximate decreasing order of frequency.
[b] Martin-Lester or Martin-Lewis agar media are acceptable alternatives.

obtained by laparoscopy or culdocentesis, and those obtained by needle aspiration of an abscess should be transported to the laboratory either in a stoppered syringe or in an anaerobic transport vial (p. 21) and then cultured for aerobic, facultatively anaerobic, and anaerobic bacteria (Table 3–2). It may also be indicated to culture material obtained by laparoscopy from the fallopian tubes of patients with acute salpingitis for *Chlamydia trachomatis.*[14]

Toxic shock syndrome (TSS) is caused by lysogenized strains of *Staphylococcus aureus* that produce a toxin known as enterotoxin F or pyrogenic exotoxin C and are most frequently associated with vaginal infections in menstruating tampon users but are occasionally associated with infections (e.g., surgical wound, cutaneous and subcutaneous lesions, abscesses) outside the genital tract in males or females. Strains of *S. aureus* producing the TSS-associated toxin can be isolated from vaginal cultures of approximately 90% of women with TSS. Since *S. aureus* is present in the vaginal flora of fewer than 10% of asymptomatic healthy women, its isolation from vaginal cultures supports the clinical diagnosis of TSS and facilitates administration of appropriate antimicrobial therapy based on in vitro susceptibility test results. A simple and practical test to confirm the presence of TSS-associated toxin is not yet available.

Interest in culturing vaginal or rectal swabs from ante- or postpartum women for group B streptococci has been stimulated by the high morbidity and mortality associated with group B streptococcal sepsis in neonates. However, the epidemiology of both the carrier state and the disease, as well as the methods of treatment of women who are carriers of the organism, remain quite complex. Antepartum vaginal or rectal cultures for group B streptococci are, therefore, not routinely recommended. When cultures for group B streptococci are requested for epidemiological purposes, isolation of the bacterium is both expedited and increased by incubating the swab at 35°C for 18 to 72 hr in Todd-Hewitt broth containing polymyxin (1 μg/ml), crystal violet (0.1 μg/ml), and nalidixic acid (15 μg/ml), subculturing the broth if it becomes turbid onto blood agar, and then identifying any streptococci isolated.[7]

B. Examination

Cultures are examined for the characteristics listed in Table 3–7. Group A and group B streptococci should be reported because of their potential importance epidemiologically and etiologically in puerperal (group A) and neonatal (group B) sepsis. Otherwise, no useful purpose is served in identifying bacteria known to be indigenous to the vagina when they are isolated from cultures of vaginal or cervical swabs. In fact, reporting such bacteria as anything other than "usual vaginal flora" is potentially

misleading because of the connotation of clinical significance accorded by specific identification. Microorganisms listed in Table 3–7 require definitive identification. Isolates cultured from specimens obtained at surgery or by laparoscopy or culdoscopy should be identified and undergo antimicrobial susceptibility testing (for indications for testing aerobic and facultatively anaerobic bacteria, see Ch. 4.4; for anaerobic bacteria, see Ch. 5).

VIII. Cultures of Integumentary Infections

Cultures for bacteria, mycobacteria, and viruses are processed as shown in Table 3–2, while those for dermatophytes are inoculated onto Mycobiotic (Difco Laboratories) or Mycosel (BBL Microbiology Systems) agar, incubated at 30°C in air, and examined twice weekly for 4 weeks.

IX. Cultures of Material for Epidemiological Purposes

Cultures of fluids or devices to determine sterility or contamination of a product are often requested for epidemiological purposes. Special care must be taken to prevent contamination of such products during processing in the laboratory. All techniques must be aseptic and should preferably be carried out in a biological safety cabinet. A record should be kept of the product's identification, brand name, lot and/or serial number, product code, whether or not the packaging or container was opened prior to its receipt in the laboratory, and any other identifying characteristics.

A. Large Volume Parenteral Fluids and Medications

The procedures described below are based on recommendations made by the National Coordinating Committee on Large Volume Parenterals[18] for fluids and medications suspected of being contaminated.

1. Documentation

a. Record the product's name, its manufacturer, lot number, additives, date, and hour of preparation or administration.
b. Record appearance of fluid, container, intravenous administration sets or catheters with particular attention to abnormal findings, defects, cracks, leaks, etc.

2. Laboratory Tests

a. Intravenous (IV) Administration Set without Final In-Line Filter

(1) Clamp line as close to the container as possible.
(2) Replace needle with new sterile one.
(3) Remove IV set from the bottle or bag.
(4) Insert the needle into a blood culture bottle, open the clamp on the line, allow half the fluid in the IV set to drain into a blood culture bottle; allow remaining fluid to drain into a second blood culture bottle.
(5) Process each bottle as described for blood cultures (see p. 29) except that both are transiently vented and one of the bottles is incubated at 22 to 25°C.

b. Parenteral Fluid or Medication Remaining in Bottle

(1) Remove two separate and equal aliquots of approximately half (up to 40 ml) the remaining fluid and inoculate each into a blood culture bottle, and process as described above [Section IX,A,2,a, (5) and on p. 29].
(2) Remove the remaining fluid (up to 40 ml)
 (a) Transfer 4 ml to a sterile tube.
 (b) Prepare a direct Gram-stained smear of a drop of fluid, examine microscopically (1000×), and report as follows:

Findings	Estimated colony-forming units (CFU/ml)
No organisms seen	If present, $<10^5$
Occasional organism seen	Probably 10^5, $\leq 10^6$
Several organisms in most fields	$>10^6$

 (c) Perform semiquantitative cultures by inoculating 0.01 ml of fluid (as depicted in Figure 3–2) onto two blood agar, two chocolate blood agar, and two eosin-methylene blue (EMB) agar plates; incubate one of each pair of media at 22 to 25°C and the other at 35°C for 48 hr; and report number of CFU/ml (number of CFU on agar × 100).
 (d) Centrifuge (8000 × *g*) remaining fluid for 10 min; prepare Gram-stained smear of a drop of sediment examine microscopically (1000×), and report presence of bacteria.

(e) Culture remaining sediment onto two chocolate blood agar plates and into two tubes of brain heart infusion broth; incubate one of each pair of media at 30°C and the other at 35°C for 48 hr and report any growth.

B. Units of Blood or Blood Products

1. Documentation

As in Section IX,A,1 above.

2. Laboratory Tests

a. Remove 20 ml of fluid from the unit and inoculate half into each of two blood culture bottles.
b. Process each bottle as described in Section IX.A.2.(5) above.

C. Bags with Little Residual Blood, Plasma, Platelet, or Other Blood Component

1. Documentation

As in Section IX,A,1 above.

2. Laboratory Tests

a. Remove 2 ml of component from the tubing with a needle and syringe.
b. Inoculate 1 ml into each of two tubes containing enriched brain heart infusion broth; incubate one at 22 to 25°C and the other at 35°C for 10 days.
c. Report any growth.

References

1. Barry, A. L., Smith, P. B., and Turck, M. *Cumitech 2: Laboratory Diagnosis of Urinary Tract Infections* (Gavan, T. L., coordinating ed.). Washington, D.C., American Society for Microbiology, 1975.
2. Bartlett, J. G., Brewer, N. S., and Ryan, K. J. *Cumitech 7: Laboratory Diagnosis of Lower Respiratory Tract Infections* (Washington, J. A., II, coordinating ed.). Washington, D.C., American Society for Microbiology, 1978.
3. Cooney, W. P., III, Fitzgerald, R. H. Jr., Dobyns, J. H., and Washington, J. A., II. Quantitative wound cultures in upper extremity trauma. *J. Trauma* 22:112, 1982.

4. Czerkinsky, C. C., and Svennerholm, A.-M. Ganglioside GM1 enzyme-linked immunospot assay for simple identification of heat-labile enterotoxin-producing *Escherichia coli*. *J. Clin. Microbiol.* **17**:965, 1983.
5. Geckler, R. W., Gremillion, D. H., McAllister, C. K., and Ellenbogen, C. Microscopic and bacteriological comparison of paired sputa and transtracheal aspirates. *J. Clin. Microbiol.* **6**:396, 1977.
6. Goldberg, P. K., Kozinn, P. J., Wise, G. J., Nouri, N., and Brooks, R. B. Incidence and significance of candiduria. *J. Am. Med. Assoc.* **241**:582, 1979.
7. Gray, B. M., Pass, M. A., and Dillon, H. C., Jr. Laboratory and field evaluation of selective media for isolation of group B streptococci. *J. Clin. Microbiol.* **9**:466, 1979.
8. Henry, N. K., and Washington, J. A., II. Initial detection of bacteremia by subculture of unvented tryptic soy broth culture bottles. *Diagn. Microbiol. Infect. Dis.* **2**:107, 1984.
9. Isenberg, H. D., Schoenknecht, F. D., and von Graevenitz, A. *Cumitech 9: Collection and Processing of Bacteriological Specimens* (Rubin, S. J., coordinating ed.). Washington, D.C., American Society for Microbiology, 1979.
10. Krizek, J. J., and Robson, M. C. Evolution of quantitative bacteriology in wound management. *Am. J. Surg.* **130**:579, 1975.
11. Libertin, C. R., Wold, A. D., and Washington, J. A., II. Effects of trimethoprim-sulfamethoxazole and incubation atmosphere on isolation of group A streptococci. *J. Clin. Microbiol.* **18**:680, 1983.
12. Loebl, E. C., Marvin, J. A., Heck, E. L., Curreri, P. W., and Baxter, C. R. The method of quantitative burn-wound biopsy cultures and its routine use in the care of the burned patient. *Am. J. Clin. Pathol.* **61**:20, 1974.
13. Maki, D. G., Weise, C. E., and Sarafin, H. W. A semiquantitative culture method for identifying intravenous-catheter related infection. *N. Engl. J. Med.* **296**:1305, 1977.
14. Mardh, P.-A., Ripa, T., Svensson, L., and Westrom, L. *Chlamydia trachomatis* infections in patients with acute salpingitis. *N. Engl. J. Med.* **296**:1377, 1977.
15. Marshall, K. A., Edgerton, M. T., Rodeheaver, G. T., Magee, C. M., and Edlich, R. F. Quantitative microbiology: Its application to hand injuries. *Am. J. Surg.* **131**:730, 1976.
16. Murray, P. R., Van Scoy, R. E., and Roberts, G. D. Should yeasts in respiratory secretions be identified? *Mayo Clin. Proc.* **52**:42, 1977.
17. Murray, P. R., Wold, A. D., Schreck, C. A., and Washington, J. A., II. Effects of selective media and atmosphere of incubation on the isolation of group A streptococci. *J. Clin. Microbiol.* **4**:54, 1976.
18. National Coordinating Committee on Large Volume Parenterals. Recommended procedures for in-use testing of large volume parenterals suspected of contamination or of producing a reaction in a patient. *Am. J. Hosp. Pharm.* **35**:678, 1978.
19. Neu, H. C. What should the clinician expect from the microbiology laboratory? *Ann. Intern. Med.* **89** (part 2):781, 1978.
20. Platt, R. Quantitative definition of bacteriuria. *Am. J. Med.* **75**(Suppl. July 28):44, 1983.
21. Pollock, H. M., Hawkins, E. L., Bonner, J. R., Sparkman, T., and Bass, J. B. Diagnosis of bacterial pulmonary infections with quantitative protected catheter cultures obtained during bronchoscopy. *J. Clin. Microbiol.* **17**:255, 1983.

22. Reller, L. B., Murray, P. R., and MacLowry, J. D. *Cumitech 1A: Blood Cultures II.* (Washington, J. A., II, coordinating ed.) Washington, D.C., American Society for Microbiology, 1982.
23. Rönnberg, B., and Wadström, T. Rapid detection by a coagglutination test of heat-labile enterotoxin in cell lysates from blood agar-grown *Escherichia coli. J. Clin. Microbiol.* **17**:1021, 1983.
24. Stamm, W. E., Counts, G. W., Running, K. R., Fihn, S. S., Turck, M., and Holmes, K. K. Diagnosis of coliform infection in acutely dysuric women. *N. Engl. J. Med.* **307**:463, 1982.
25. Thomson, R. B. Jr., Smith, T. F., and Wilson, W. R. Comparison of two methods used to prepare smears of mouse lung tissue for detection of *Pneumocystis carinii. J. Clin. Microbiol.* **16**:303, 1982.
26. Washington, J. A., II (ed.). *The Detection of Septicemia.* West Palm Beach, FL, CRC Press, 1978.
27. Wimberley, N., Faling, L. J., and Bartlett, J. G. A fiberoptic bronchoscopy technique to obtain uncontaminated lower airway secretions for bacterial culture. *Am. Rev. Resp. Dis.* **119**:337, 1979.
28. Wise, G. J., Goldberg, P., and Kozinn, P. J. Genitourinary candidiasis: Diagnosis and treatment. *J. Urol.* **116**:778, 1976.

4

Aerobic and Facultatively Anaerobic Bacteria

Section 4.1

General Classification of Bacteria

John A. Washington II, M.D.

Bacteria are identified whenever their presence is considered clinically significant. Isolates, therefore, from normally sterile sites require identification and antimicrobial susceptibility testing. Those without recognized clinical importance from sites normally harboring indigenous flora (Table 1–1) seldom require identification. Indeed, reporting normal flora may be highly misleading since specific identification by the laboratory would generally appear to ascribe clinical importance to any organisms reported. For this reason, reports of throat cultures can usually be limited to whether or not group A streptococci are present, and reports of stool cultures can be limited to whether or not recognized enteric pathogens are present. No useful purpose is served by identifying and reporting organisms indigenous to the oropharynx or gastrointestinal tract, despite the fact that their isolation from other sites may be clinically significant. By the same token, when cultures fail to yield recognized pathogens, the report should clearly state that cultures were negative for the specific pathogens sought (e.g., *Salmonella, Shigella, Yersinia,* and *Campylobacter jejuni* in feces) rather than simply to report the cultures were negative.

Bacteria are initially categorized according to their growth characteristics on primary isolation media (Table 4.1–1), including the atmosphere of incubation and media in which growth occurs, and their microscopic characteristics. Their identification is generally based on biochemical reactions or physiological properties (Figures 4.1–1 and 4.1–2) but may often be expedited by using immunological tests (Section 4.3). In some instances, as noted in Table 2–1, the specimen may be examined directly by using one of these same immunological tests and provide preliminary or definitive identification of organisms present in the specimen.

Table 4.1–1. Growth Characteristics of Bacteria on Initial Isolation Media

	Organism		
Characteristic	Gram-positive	Gram-negative	
Growth in air ($+5-10\%\ CO_2$) BA only	*Staphylococcus* *Streptococcus* *Corynebacterium* *Bacillus* *Listeria* *Erysipelothrix*	*Neisseria* *Pasteurella* *Brucella*	
BA + EMB or Mac	*Streptococcus,* group D	Enterobacteriaceae *Pseudomonas* *Aeromonas* *Vibrio*	*Acinetobacter* *Flavobacterium* *Pseudomonas*-like bacilli
CBAP only		*Haemophilus*	
Growth anaerobically only BA	*Peptostreptococcus* *Peptococcus* *Actinomyces* *Bifidobacterium* *Eubacterium* *Propionibacterium* *Clostridium*	*Veillonella* Bacteroidaceae	
Laked BA, K-V		Bacteroidaceae	
BA, PEA	Anaerobic cocci		
Egg-yolk–neomycin–Nagler agar	*Clostridium*		

Antimicrobial susceptibility testing of aerobic and facultatively anaerobic bacteria will be described in Section 4.4, while the identification and antimicrobial susceptibility testing of anaerobic bacteria will be discussed in Chapter 5.

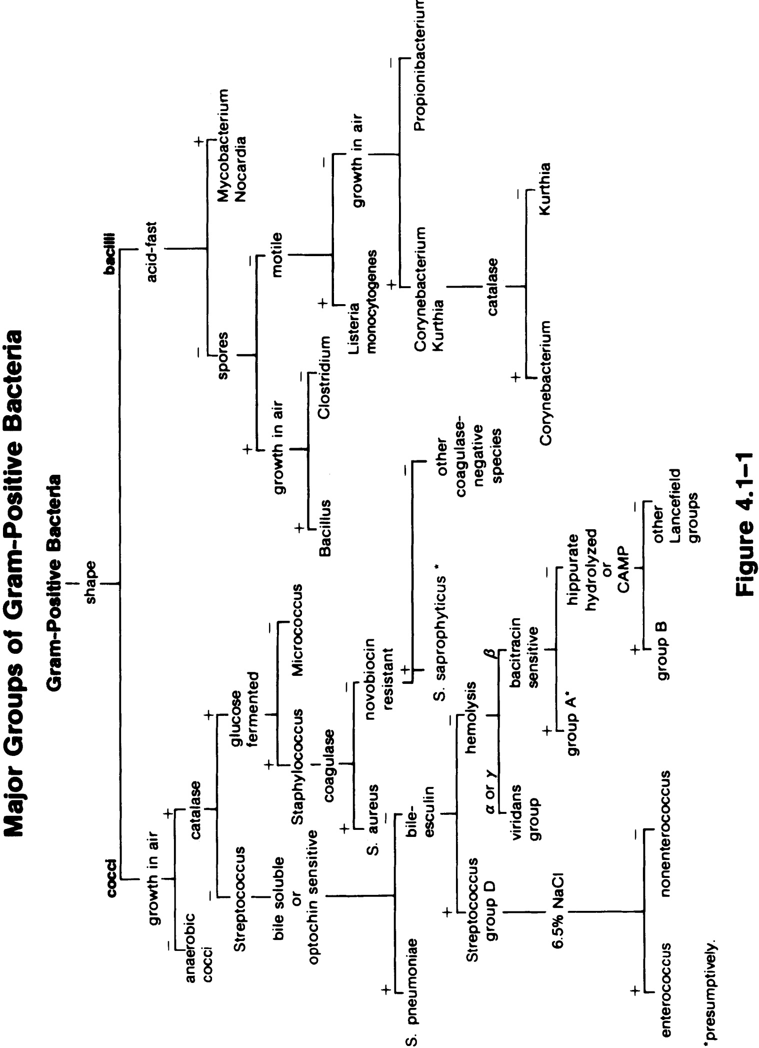
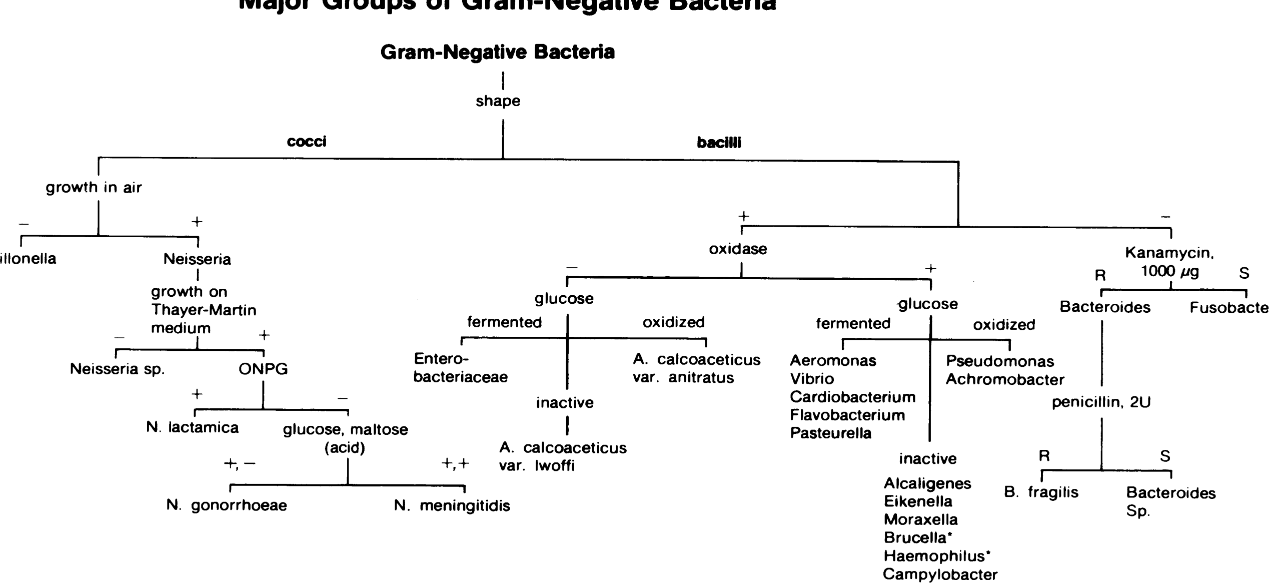


Figure 4.1-1



*Carbohydrate utilization not important for differentiation or speciation and can only be demonstrated in special media.

Figure 4.1–2

Section 4.2

Identification of Aerobic and Facultatively Anaerobic Bacteria

Pauline K. W. Yu, M.S. John A. Washington II, M.D.

I. Gram-Positive Cocci

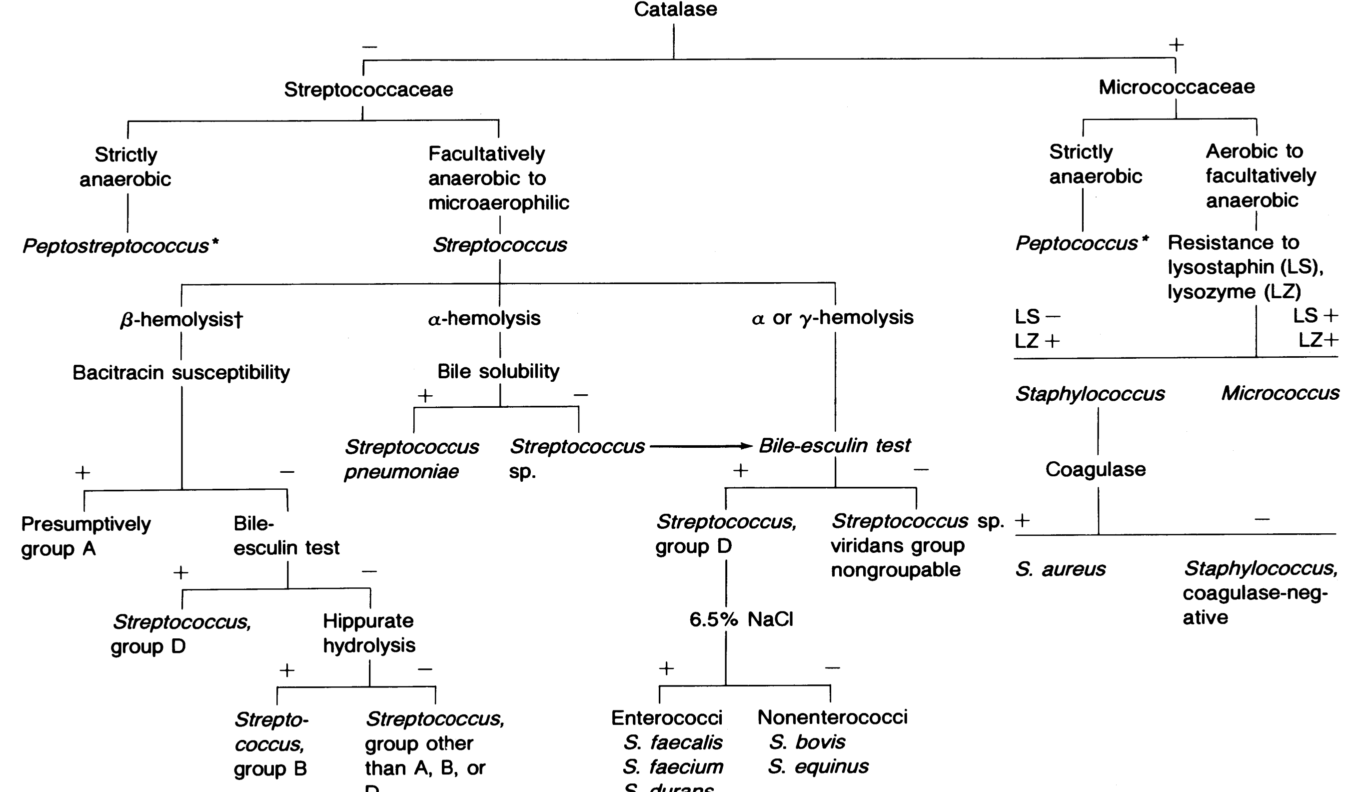
A. General Considerations

Aside from the members of the Family Enterobacteriaceae, gram-positive cocci are the most frequently isolated bacteria from a variety of clinical specimens. Figure 4.2–1 shows an abbreviated scheme for identifying gram-positive cocci. Unlike their aerobic to facultatively anaerobic counterparts, *Peptococcus* and *Peptostreptococcus* cannot be differentiated reliably by the catalase test because of variable reaction patterns. Bacterial cells of both Micrococcaceae and Streptococcaceae stain readily with crystal violet, but aged colonies can lose their ability to retain this dye and become gram-variable or even gram-negative. Microscopically, they exhibit a variety of forms, from single cells to pairs, clusters or chains, depending somewhat on the cultivating medium (Figure 2–1f).

B. Micrococcaceae

1. General Considerations

Staphylococci may now be separated into at least 14 species.[100] *Staphylococcus aureus,* which is the major pathogen, may colonize or produce clinically significant infection at local sites, spread contiguously to other sites, produce bacteremia with metastatic sites of infection, or infect im-



* Catalase reaction variable.
† Definitive Lancefield grouping is obtained by testing for precipitin reactions in group-specific antisera.

Figure 4.2–1. Abbreviated scheme for identification of gram-positive cocci.

planted prosthetic material.[133] Among the coagulase-negative staphylococci, *Staphylococcus epidermidis* is the most frequently isolated species from clinical material, followed distantly by *Staphylococcus saprophyticus*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis*. Although the coagulase-negative staphylococci are frequently isolated from the skin and ordinarily represent contaminants of little or no clinical significance, it is important to recognize that *S. epidermidis* is frequently associated with bacteremias in immunocompromised hosts and in patients with intravascular devices; with infections of bone, joints and wounds; and with catheter-related peritonitis. Thus, the isolation of a coagulase-negative *Staphylococcus* from a normally sterile tissue or fluid cannot be casually dismissed. *S. epidermidis* is usually recovered from multiple blood culture bottles when it is associated with endocarditis. Recently, *S. saprophyticus* has emerged as an important cause of lower urinary tract infection in young females.[90] Micrococci are usually recovered from soil, water, and skin, and are considered avirulent for man.

Most staphylococci, whether of hospital or community origin, produce a β-lactamase (penicillinase) and are, therefore, not amendable to therapy with penicillin. Resistance to the semisynthetic penicillinase-resistant penicillins, which has posed considerable problems in other parts of the world, is being encountered with increasing frequency in nosocomially acquired infections in the United States and particularly in large medical school tertiary-referral centers.

2. Identification of Staphylococcus

a. General Considerations

Staphylococci are gram-positive, strongly catalase positive, nonmotile, and oxidase negative. They reduce nitrate to nitrite. They are aerobic to facultatively anaerobic in their oxygen requirement. They have few stringent nutritional demands, and thrive very well on most conventional nonselective media.

b. Growth Characteristics

After 18 to 24 hr of incubation on sheep blood agar, staphylococcal colonies are 1 to 2 mm in diameter, convex, opaque, and butyrous. Colonies of coagulase-negative staphylococci range from white, gray-white, to yellow; occasional isolates from the upper respiratory tract tend to adhere to the agar surface and are difficult to emulsify. Most colonies of *Staphylococcus aureus* are β-hemolytic; some produce a golden yellow pigment that deepens in color when the culture is left at room temperature. Microscopically, staphylococcal cells are spherical, 0.5 to 1 μm in diameter,

usually occurring in grapelike clusters amidst single cells, pairs, and tetrads (Figure–1f). Short chains can also be found when a Gram-stained smear is prepared from a broth culture.

c. Speciation

In the clinical laboratory, speciation of clinically significant isolates of coagulase-negative staphylococci from blood or by special request can be achieved with the conventional tests of Kloos and Schleifer[101] or with a commercial kit like the API STAPH-IDENT™ system.[102] Isolates identified as *S. epidermidis* should be reported as such; other species can be reported as *Staphylococcus*, coagulase-negative. Urinary isolates of *S. saprophyticus* can be identified adequately by their resistance to novobiocin (p. 138).

d. Preliminary Test

Catalase Test

Purpose:
To separate staphylococci that are catalase positive from streptococci that are catalase negative.

Principle
The enzyme catalase decomposes hydrogen peroxide to water and oxygen. Bacteria thereby protect themselves from the lethal effect of hydrogen peroxide, which is accumulated as an end product of aerobic carbohydrate metabolism. Catalase is a hemoprotein. Catalytic decomposition of hydrogen peroxide involves the reduction of trivalent iron (Fe^{3+}) in catalase by hydrogen peroxide to its reduced form (Fe^{2+}) and the reoxidation of the latter by oxygen. The sum of these reactions is simply represented by the following equation:

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2\uparrow$$

Specimen:

1. Isolated colonies on agar plate.
2. Growth on blood-free agar slant.

Reagent:

3% hydrogen peroxide

Stock solution, 30% H_2O_2 (J. T. Baker Chemical Co.)	10 ml
Distilled water	90 ml

Stable for 6 months at 4°C in brown bottle. Aliquots used at the bench should be discarded weekly.

Procedure:

Agar Plate Test

1. Transfer a portion of an isolated colony, preferably from a blood-free medium since red cells possess catalase activity, to a flamed glass slide.
2. Add one drop of 3% H_2O_2 onto the colony.

Agar Slant Test
Add 1 ml of 3% H_2O_2 to bacterial growth on agar slant.

Interpretation:

Positive test: Evolution of gas bubbles.
Negative test: No gas bubbles.

Controls:
Positive: *Aeromonas sp.*
Negative: *Streptococcus pneumoniae*

---

e. Definitive Test

Coagulase Test[36,142]

Purpose:
To separate *Staphylococcus aureus,* which possesses coagulase activity, from coagulase-negative *Straphylococcus* species.

Principle:
S. aureus produces two forms of coagulase: bound and free. Bound coagulase, or "clumping factor," is bound to the bacterial cell wall. It acts directly on fibrinogen and alters it so that it precipitates on the staphylococci, causing them to clump when a bacterial suspension is mixed with plasma. The presence of bound coagulase correlates well with free coagulase, an extracellular protein enzyme that causes the formation of a clot when *S. aureus* colonies are incubated with plasma from an animal species.[36] The mechanism involves activation of a plasma coagulase-reacting factor (CRF), which is a modified or derived thrombin molecule, to form a coagulase–CRF complex. This complex, in turn, reacts with fibrinogen to produce the fibrin clot. The clotting process is independent of calcium ion.

Specimen:
Isolated colonies from sheep blood or colistin–nalidixic acid blood agar. The use of a mixed culture is not recommended since, when citrated plasma is used, organisms (e.g., *Pseudomonas aeruginosa*) that can utilize citrate will release free calcium ion to give a false positive test.

Reagent:
Reconstituted lyophilized rabbit plasma (BBL Microbiology Systems) is preferred over human plasma because the latter might contain factors inhibitory to coagulase activity. Once reconstituted, plasma can be used for 3 days if stored at 4°C or for 3 months if stored at −10°C.

Procedure:

Slide Test

1. Emulsify several colonies in one loopful of distilled water on a glass slide to give a smooth milky suspension.
2. Add one loopful of rabbit plasma and mix thoroughly.

Interpretation:
Positive test: Immediate clumping.
Negative test: No clumping.
Negative slide test should be confirmed by tube test.

Tube Test

1. Emulsify several colonies in 0.5 ml of rabbit plasma to give a milky suspension.
2. Incubate tube at 35°C for 4 hr and check for clot formation. If negative, reincubate overnight. The 4-hr reading is essential because fibrinolysin produced by occasional strains of *S. aureus* might lyse the clot within 16 to 18 hr of incubation and thus give a false negative test.

Interpretation:
Positive test: Complete clot formation, or a partial clot suspended in plasma.
Negative test: No clot formed.

Controls:
Positive: *S. aureus*
Negative: *S. epidermidis*

---

f. Alternative Tests

Alternatively, *S. aureus* can be differentiated from coagulase-negative staphylococci by a slide hemagglutination test.[34]

Staphyloslide™ Test

Purpose:
To separate *S. aureus* from coagulase-negative staphylococci.

Principle:
The Staphyloslide™ test is a slide hemagglutination test designed to detect a cell-wall polypeptide clumping factor found in most *S. aureus*. Fibrinogen, which has been coated on sheep erythrocytes, is converted by the clumping factor to insoluble fibrin, leading to clumping of the erythrocytes.

Specimen:
Isolated colonies on blood agar, or CNA agar.

Reagents:
Staphyloslide™ test kit (BBL Microbiology Systems) containing:

1. Sensitized sheep erythrocytes.
2. Negative control sheep erythrocytes.

Procedure:

1. Add one drop each of the well-mixed sensitized sheep erythrocytes and negative control sheep erythrocytes on separate areas of a glass slide.
2. Emulsify several colonies in each of the drops.
3. Rock the slide for 15 sec and read agglutination immediately.

Interpretation:

Positive test: A large amount of clumping of the sensitized sheep erthrocytes within 15 sec, while the negative control reagent remains homogeneous.

Negative test: No clumping in either reagent; however, if colonial morphology is consistent with *S. aureus*, perform tube coagulase test before reporting isolate as *Staphylococcus*, coagulase negative. The test is uninterpretable if clumping occurs with both reagents.

Controls:
Positive: *S. aureus*
Negative: *S. epidermidis*

Other alternative tests to the coagulase test, such as mannitol fermentation,[44,113] production of thermostable nuclease,[161] and deoxyribonucle-

ase[35,88] may require 4–48 hr for completion. However, they may be useful when the results of the coagulase test are equivocal.

Novobiocin Inhibition Test

Purpose:
To separate *Staphylococcus saprophyticus* from other coagulase-negative staphylococci.

Principle:
Several species of coagulase negative *Staphylococcus* are resistant to novobiocin; however, urinary isolates that exhibit resistance to this drug are most likely to be *S. saprophyticus.*

Specimen:
Colonies of coagulase-negative *Staphylococcus* present on sheep blood agar.

Medium:

1. Mueller-Hinton agar.
2. Physiologic saline or broth.

Reagent:
Novobiocin disk, 5 μg (BBL Microbiology Systems)

Procedure:

1. Suspend colonies in 2 ml of physiologic saline or broth to a density matching that of a 0.5 McFarland barium sulfate standard.
2. Swab bacterial suspension onto Mueller-Hinton agar plate as for disk diffusion test.
3. Apply novobiocin disk.
4. Incubate plate at 35°C for 18 hr.

Interpretation:
Zone diameter of inhibition $\leq$ 16 mm—*S. saprophyticus.*
Zone diameter of inhibition $>$16 mm—other coagulase-negative staphylococci.

Control:
Staphylococcus aureus ATCC 25923, with zone of inhibition—22 to 28 mm.

Table 4.2–1. Differentiation of *Staphylococcus* and *Micrococcus*[a]

Genus	Pattern	Resistance to: lysozyme (25 μg/ml)	Resistance to: lysostaphin (200 μg/ml)	Acid from glycerol-erythromycin medium (0.4 μg of erythromycin/ml)
Staphylococcus	Frequent	+	−, (±)	+
	Infrequent	+	−	−
Micrococcus	Frequent	+, (±)	+	−
	Infrequent	−	+, ±, −	−
	Infrequent	+	+	+

[a] Adapted from Schleifer and Kloos.[131]
+, no growth inhibition or acid production; −, complete growth inhibition or no acid production; (±) partial growth inhibition.

3. Identification of Micrococcus

Ordinarily, coagulase-negative Micrococcaceae are simply reported as coagulase-negative *Staphylococcus,* however, if the need to identify *Micrococcus* arises, the scheme of Schleifer and Kloos[131] (Table 4.2–1) is suggested. This scheme, which is based on the differential resistance of staphylococci and micrococci to lysozyme and lysostaphin and the ability of staphylococci to produce acid aerobically from glycerol in the presence of erythromycin, is preferred over the traditional oxidation–fermentation test of glucose, which does not always clearly differentiate the two genera.

C. Streptococcaceae

1. General Considerations

Streptococci produce a wide variety of diseases depending on their age of onset, site of initial infection, and the group of *Streptococcus* involved.[14]

Group A streptococci (*Streptococcus pyogenes*) most frequently cause an exudative pharyngitis which is often indistinguishable from that caused by viruses and which occurs predominantly in school age children during the late winter and early spring. Group A streptococci also frequently cause impetigo, pyoderma, otitis media, and sinusitis, and less frequently cause cellulitis and postoperative or traumatic wound sepsis. Nonsuppurative sequelae of pharyngitis and pyoderma particularly are acute rheumatic fever and acute glomerulonephritis.

Group B streptococci (*Streptococcus agalactiae*) are frequent causes of neonatal septicemia and meningitis. Recently, they have also emerged

as the major etiologic agent in neonatal osteomyelitis.[39] They are frequently present in the vagina of asymptomatic women; however, the relationship between the vaginal carrier state and neonatal disease remains unclear.

Group D streptococci, which are divided into enterococci and nonenterococci, are frequently associated with genitourinary, intraabdominal, and endocardial infections. Other Lancefield groups of streptococci, especially groups C and G, may be encountered as indigenous flora, particularly in the oropharynx, and infrequently cause disease in humans. Though extremely rare, group G streptococcal bacteremias have been reported in patients with underlying hematologic malignancies or solid tumors.[2]

The viridans streptococci comprise a heterogeneous number of species that represent indigenous flora, particularly in the upper respiratory, intestinal, and genital tracts. They are most frequently associated, usually in mixed culture, with intraabdominal, pelvic, and brain abscess; however, they also are the most frequent cause of subacute bacterial endocarditis.

Sometimes classified among the viridans streptococci, *Streptococcus* (formerly *Diplococcus*) *pneumoniae,* is a frequent cause of acute sinusitis and otitis media, acute lobar pneumonia, and acute bacterial meningitis. It may be present as indigenous flora in the oropharynx so that its mere isolation from respiratory secretions is not indicative of disease. Conversely, unless lower respiratory secretions are carefully collected, examined microscopically, and cultured, pneumococci may not be seen or isolated in the presence of disease.

All streptococci are susceptible to penicillin G with the notable exceptions of enterococci, rare strains of pneumococci, and occasional tolerant strains of viridans streptococci. Bactericidal activity against enterococci can only be achieved with combinations of penicillins and aminoglycosides. The occurrence of penicillin, or, more accurately, multiply resistant pneumococci has been confined to South Africa with only a single strain having been recognized to date in the United States.[155] Tolerance, an autolytic defect usually recognized by a minimum bactericidal concentration substantially in excess of that required for inhibition, has been recognized among occasional strains of viridans streptococci and staphylococci[146]; however, its clinical significance remains uncertain.

2. Identification of Streptococci

a. General Considerations

Streptococci are nonmotile gram-positive cocci. They are catalase negative, which differentiates them from staphylococci, and oxidase negative, which separates them from *Neisseria.* They ferment a variety of carbohy-

drates with production of acid and no gas. They are facultatively anaerobic in their oxygen requirement. Some strains, sometimes referred to as microaerophilic, prefer reduced O_2 tension and 5 to 10% CO_2 for cultivation. Although the majority of strains grow well on the usual enriched media, occasional strains are nutritionally deficient and require additional enrichment (e.g., pyridoxal) at least for primary isolation.[31]

Streptococcus pneumoniae is more complex in its nutritional requirements than other gram-positive cocci. Media containing meat extract or tryptic digest of soybean is usually used. Pneumococci are sensitive to acidic pH and thus do not survive long in glucose-containing medium. Some strains require 5 to 10% CO_2 for primary isolation.

Streptococci can be classified by different systems. Brown's original definition is based on the subsurface hemolytic activity of streptococci on horse blood. Taranta and Moody[143] described the different types of hemolysis that fitted in with Brown's definition. Alpha (α) hemolysis, which is due to the partial destruction of red cells, is characterized by a zone of greenish discoloration around the colonies. With β-hemolysis, colonies are surrounded by a clear zone of complete hemolysis, the diameter of which may vary with different serological groups of streptococci. Gamma (γ) hemolysis means no hemolysis in either surface or subsurface colonies. β-Hemolysis can sometimes be confused with what is known as the alpha-prime (α'), or wide-zone alpha, which is a narrow zone of α-hemolysis followed by a zone of β-hemolysis around the colonies.[143] Hemolytic reaction is not only a function of the type of mammalian erythrocytes used in the medium, but is also dependent on the composition of the basal medium and on the atmosphere of incubation. β-Hemolysis is related to the elaboration of two hemolysins: streptolysin O, which is oxygen-labile, and streptolysin S, which is oxygen-stable. To detect occasional strains of β-hemolytic streptococci that produce only streptolysin O, it is necessary to make several stabs in the agar during culturing to provide an environment of reduced oxygen tension. Inclusion of glucose in basal medium is not recommended, because the reduced pH of the medium resulting from bacterial metabolism will inactivate streptolysin S.

Another means of classifying streptococci is to group them according to their biologic characteristics: pyogenic, viridans, enterococcal, and lactic. Finally, streptococci may be classified serologically according to group-specific carbohydrate precipitin reactions.

Unfortunately, these systems of classification are not mutually exclusive. For example, while group A streptococci, or *Streptococcus pyogenes*, are β-hemolytic, enterococcal group D streptococci are usually not hemolytic, though some strains are β-hemolytic, and viridans streptococci are either α- or γ-hemolytic and usually not groupable serologically. All three systems of classification are, therefore, used in the clinical laboratory and in medicine.

b. Growth Characteristics

After 18 to 24 hr of incubation on sheep blood agar, colonies of group A streptococci are 0.5 to 1 mm in diameter, gray-white, raised, translucent, and punctate. Colonies are difficult to emulsify. The zone of β-hemolysis is usually several times the diameter of the colony. In contrast, group B streptococcal colonies are gray-white, less raised, very soft, and are surrounded by a very narrow rim of β-hemolysis. It should be noted that approximately 3% of group B streptococci which have been isolated from human infections and examined at the Centers for Disease Control are nonhemolytic (R. R. Facklam, personal communication). Group D streptococci are nonhemolytic to α-hemolytic except for *Streptococcus faecalis* subsp. *zymogenes,* which is β-hemolytic. Colonies are also gray-white and translucent. Older colonies tend to be much whiter. Colonies of viridans streptococci are raised, gray, and are mostly α-hemolytic and occasionally nonhemolytic.

Colonies of *Streptococcus pneumoniae* are grayish, glistening and smooth, about 0.5 to 1 mm in diameter after overnight incubation, and surrounded by a zone of green discoloration. Young colonies are raised and may resemble viridans streptococci; however, as the culture ages, colonies are lysed by an intracellular autolytic enzyme giving the colonies a depressed center or dimpled appearance. At least 84 serotypes of *S. pneumoniae* have been recognized. Colonies of type III pneumococci and occasionally other types are heavily encapsulated. They are usually larger and tend to be more mucoid.

c. Microscopic Appearance

Streptococcal cells are spherical, oval, or elongated, 0.5 to 1 μm in diameter. Aged cells can easily become gram-variable or even gram-negative in their staining characteristics. Short to long chains can be found, especially when a Gram-stained smear is prepared from a throat swab or broth culture (Figure 2–1h). Cells of group D streptococci are more elongated and occur mostly in pairs. Pneumococci usually occur in pairs and are typically lancet-shaped (Figure 2–1c). The paired cocci are pointed at their distal ends and more flattened at the sides adjacent to each other. However, short chains are not unusual findings. Pneumococci are often surrounded by a refractile capsule, which is especially prominent when a Gram-stained smear is prepared directly from cerebrospinal fluid or sputum.

d. Tests

(1) Group A Streptococci

The differentiation of group A from nongroup A β-hemolytic streptococci is of epidemiological, clinical, and therapeutic importance, primarily in

terms of the prevention of rheumatic fever and acute glomerulonephritis. Although nongroup A β-hemolytic streptococci have been implicated in rare foodborne epidemics of disease, antibiotic therapy of pharyngitis associated with them is not ordinarily recommended, since the prevalence of groups other than A in throat cultures of children with and without upper respiratory infection is equivalent[70] and investigators ascribing an etiological role to these other streptococcal groups in nonepidemic pharyngitis usually have failed to perform adequate mycoplasmal and viral tests in their study populations.

The proportion of β-hemolytic streptococci that are group A may vary geographically and does vary with age, ranging in our experience from 26 and 56% in adults and children, respectively, during the summer to 47 and 83% in these two age groups, respectively, during the winter months.[116] A close correlation between the isolation of β-hemolytic streptococci and the presence of group A streptococci, therefore, only exists in our pediatric population during the late winter and early spring months.

(*a*) *Presumptive Test*

Bacitracin Inhibition Test[50,53,108,115]

Purpose:
To differentiate group A streptococci from other groups of β-hemolytic streptococci.

Principle:
A low concentration of bacitracin will selectively inhibit the growth of group A streptococci. A bacitracin disk containing 0.04 units (not the high potency susceptibility testing disk) should be used for this purpose. The reliability of the test is inoculum dependent, and confluent but not excessive growth is important. Accurate determination of hemolysis is crucial since some α-hemolytic streptococci, including *Streptococcus pneumoniae,* are inhibited by the differential disk. Also, the test is designed for use with a pure culture and not directly on the primary isolation plate. While rare strains of group A streptococci are bacitracin resistant, approximately 5 to 10% of strains of nongroup A β-hemolytic streptococci (primarily in groups B, C, and G) are bacitracin susceptible. Inhibition by bacitracin, therefore, constitutes *presumptive* identification of group A streptococci and should be so reported.

Specimen:
Isolated colonies on sheep blood agar.

Media:

1. Sheep blood agar plate.
2. Trypticase soy broth, 2 ml tube.

Reagent:
Bacitracin differential disk (Taxo A, BBL Microbiology Systems; Bacto-Differentiation Disk, Difco Laboratories).

Procedure:

1. Inoculate one to two colonies into trypticase soy broth. Mix well.
2. Dip a cotton swab in the broth suspension, express excessive liquid from the swab against the side of the tube.
3. Streak the surface of the sheep blood agar plate.
4. Aseptically apply a bacitracin disk onto the center of the streaked area. Gently tamp the disk to ensure adequate contact with the agar surface.
5. Invert plate and incubate for 18 hr at 35°C.

Interpretation:
Positive test: Any zone of inhibition around the bacitracin disk.
Negative test: Uniform lawn of growth right up to the rim of the disk.

Controls:
Positive: Bacitracin susceptible group A *Streptococcus*
Negative: Bacitracin resistant group G *Streptococcus*

(*b*) *Definitive Tests*

Definitive identification requires either extraction of the group-specific carbohydrate antigen and a precipitin reaction with its homologous antiserum or immunofluorescence of cells with fluorescein-labeled group A specific antiserum (p. 257). The latter procedure may be used with a smear of either centrifuged sediment of a 2 to 4 hr Todd-Hewitt broth culture of a throat swab or of β-hemolytic colonies on blood agar. In those laboratories equipped for fluorescent microscopy, immunofluorescence is a rapid and accurate means of determining the presence of group A streptococci in throat swabs or in cultures thereof. There are several approaches to extraction for serological grouping of β-hemolytic streptococci[50]; however, we have found the autoclave technique to be most practical and accurate for batch processing of large numbers of strains. Another rapid approach to serological grouping of isolated colonies is to use coagglutination following extraction with a *Streptomyces albus*–lysozyme enzyme mixture.[25]

(2) Group B Streptococci
Because occasional strains of group B streptococci are nonhemolytic on blood agar, nonhemolytic streptococcal isolates from the blood or cerebrospinal fluid of infants should be tested to determine whether or not they belong to group B.

(*a*) *Presumptive Test*
Either of the tests described below is satisfactory and provides results which are equivalent in accuracy to that of serological grouping.

Hippurate Hydrolysis[53,87]

Purpose:
To differentiate group B streptococci from other β-hemolytic streptococci. This test is also useful in the identification of *Campylobacter jejuni* and *Gardnerella vaginalis*.

Principle:

The hydrolysis of sodium hippurate by hippuricase to sodium benzoate and glycine is represented by the following equation:

$$\text{Sodium hippurate} \xrightarrow{\text{hippuricase}} \text{sodium benzoate} + \text{glycine}$$

Subsequent addition of ninhydrin results in the oxidative deamination of the α-amino group in glycine to its corresponding aldehyde, with the release of carbon dioxide, ammonia, and hydrindantin, the reduced form of ninhydrin. Ammonia reacts with residual ninhydrin and hydrindantin to give a purple-colored complex. Some group D streptococci hydrolyze hippurate; however, since few of these are β-hemolytic on blood agar and their colonial morphology differs from that of group B streptococci, the accuracy of identifying group B streptococci by this method is very high, particularly when strains are bile-esculin (p. 148) negative.

Specimen:
Fresh isolated colonies on sheep blood agar.

Reagent:

1% Sodium hippurate

Sodium hippurate	1 g
Distilled water	100 ml

Dispense 0.4 ml in small screw-capped vials. Store at −10°C; expiration is 6 months.

Ninhydrin reagent

Ninhydrin	3.5 g
Acetone-butanol mixture, 1:1	100 ml

Store solution at room temperature; expiration is 12 months.

Procedure:

1. Inoculate sodium hippurate heavily with 18- to 24-hr-old colonies to produce a milky suspension.
2. Incubate suspension for 2 hr at 35°C.
3. Add 0.2 ml of ninhydrin reagent.
4. Mix well and incubate for additional 10 to 15 min.

Interpretation:

Positive test: Deep purple color indicates hippurate hydrolysis.
Negative test: No color change. A light inoculum or use of an old culture may give a gray to very slight purple color; this should be interpreted as a negative test.

Control:
Positive: Group B *Streptococcus*
Negative: Group A *Streptococcus*

CAMP* Test[30,32]

Purpose:
To differentiate group B streptococci from other β-hemolytic streptococci.

Principle:
Group B streptococci produce an extracellular "CAMP" factor that potentiates the lysis of sheep or ox red cells by staphylococcal β-lysin. This effect can be observed when the two organisms are placed in the proper orientation on sheep blood agar. A positive reaction will occur in 5 to 6 hr with CO_2 incubation, but 18 hr is usually required if plates are incubated without CO_2. Anaerobic incubation is not recommended because group A streptococci will also produce a positive reaction in this environment.

* CAMP is an acronym based on the first letters of the surnames of the authors who initially described the phenomenon: Christie, Atkins, and Munch-Petersen.

Specimen:

1. Isolated colonies of β-hemolytic streptococci on sheep blood agar.
2. β-Lysin–producing *Staphylococcus aureus* on sheep blood agar.

Medium:
Sheep blood agar plate.

Procedure:

1. Inoculate *S. aureus* along a line down the center of a sheep blood agar plate.
2. Inoculate a portion of a streptococcal colony along a thin 2 cm long line perpendicular to but not touching the *S. aureus* streak.
3. Incubate plate at 35°C for 18 hr.

Interpretation:

Positive test: A CAMP reaction is indicated by an arrowhead-shaped area of enhanced hemolysis at the junction between the streptococcal and staphylococcal streaks (Figure 4.2–2, see color insert in the center of the book).
Negative test: No enhanced hemolysis.

Control:
Group B *Streptococcus*
Group A *Streptococcus*

(*b*) *Definitive Tests*
Serological grouping
Fluorescent antibody (p. 257)
Counterimmunoelectrophoresis (p. 270)

(3) Group D Streptococci
The classification of group D streptococci is based on the scheme (Table 4.2–2) described by Shattock[136] with procedures outlined by Facklam.[48] Since penicillin is bactericidal to the nonenterococcal species of group

Table 4.2–2. Classification of Group D Streptococci

Group	Division	Species
Enterococcus	I	*S. faecalis*
	II	*S. faecium* *S. durans*
Nonenterococcus, group D	III	*S. bovis* *S. equinus*

D streptococci, *S. bovis* and *S. equinus,* but not to the enterococcal species, *S. faecalis* and *S. faecium,*[159] it is important to make this differentiation as quickly as possible with isolates from patients with group D streptococcal endocarditis.

(a) Presumptive Test

Bile-Esculin[49,52,141]

Purpose:
To differentiate group D streptococci from other streptococci.

Principle:
Group D streptococci can grow in the presence of 40% bile and subsequently hydrolyze esculin to esculetin and glucose. Esculetin diffuses into the agar and combines with ferric citrate in the medium to give a black complex. The reactions involved can be represented by the following equations:

$$\text{Esculin} \xrightarrow[\text{}]{\substack{\text{bacterial}\\ \text{enzyme}}} \text{esculetin} + \text{glucose}$$

$$\text{Esculetin} + Fe^{3+} \longrightarrow \text{black complex}$$

The concentration of bile in the medium is critical and a final concentration of less than 40% is less selective for group D streptococci so that many viridans streptococci will give a positive test. *S. mutans* may yield a positive bile-esculin reaction, but the colonies are small, hard, and tend to adhere to the agar surface. Otherwise, the test is highly specific and its accuracy is equivalent to that of serologic grouping.

Specimen:
Isolated colonies on blood agar.

Medium:
Bile-esculin agar (Difco Laboratories).

Procedure:

1. Inoculate one to two colonies on a bile-esculin agat slant or streak in a thin line on ⅛ of the surface of a bile-esculin agar plate.
2. Incubate at 35°C for 18 to 24 hr and for an additional day if the test is negative.

Interpretation:

Positive test: Blackening of agar slant or blackening around line of growth on the agar plate.
Negative test: No blackening of medium. Growth alone on bile-esculin agar does not constitute a positive test.

Controls:
Positive: group D *Streptococcus*
Negative: viridans *Streptococcus*

Alternatively, group D streptococci can be differentiated from other streptococci by a latex agglutination test.[104]

Latex Agglutination Test

Purpose:
To differentiate group D streptococci from other streptococci.

Principle:
Antibodies to group D streptococci that have been coated on latex particles bind to antigenic sites in group D streptococci to form visible clumps.

Specimen:
Isolated colonies on blood agar or CNA agar.

Reagent:
SeroSTAT Streptococcus Group D Test Kit (Scott Laboratories, Inc.)

Group D reagent
Group D control
GBS (glycine–buffered saline)–Polysorbate 80

Procedure:

1. Place one drop of GBS-Polysorbate 80 on two separate spots on a glass slide.
2. Emulsify 4–5 colonies in each drop.
3. Add one drop of well-mixed group D reagent and one drop of group D control to each of the two spots. Mix slightly.
4. Tilt the slide back and forth for up to 1 min and observe for agglutination.

Interpretation:

Positive test: Agglutination in group D reagent and no agglutination in group D control. Report as Group D *Streptococcus* if colonies are nonhemolytic; otherwise, perform bile-esculin test.
Negative test: No agglutination in either reagent. Report as viridans *Streptococcus* if colonies are α-hemolytic; otherwise, perform bile-esculin test.

Controls:
Positive: Group D *Streptococcus*
Negative: viridans *Streptococcus*

Salt Tolerance Test[49]

Purpose:

To differentiate enterococci from nonenterococci.

Principle:
Enterococci can withstand a higher salt concentration than nonenterococci. A modified 6.5% NaCl broth is usually used as the test medium. This broth also contains a small amount of glucose and bromcresol purple as the indicator for acid production. The salt tolerance test is inoculum dependent and too heavy an inoculum should be avoided.

Specimen:
Isolated colonies on sheep blood agar.

Medium:
Modified 6.5% NaCl broth.

Procedure:

1. Inoculate one to two colonies into 5 ml of modified NaCl broth.
2. Incubate tube at 35°C for 3 days. Check for growth daily.

Interpretation:

Positive test: Visible turbidity in broth, without or with acidification of the medium, which is indicated by a change in color of the medium from purple to yellow.
Negative test: No turbidity and no color change in NaCl broth.

Controls:
Positive: *Streptococcus faecalis*
Negative: viridans *Streptococcus*

(*b*) *Definitive Tests*

Definitive identification of group D streptococci requires serological grouping, while speciation may be obtained by performing physiological and biochemical tests, as described by Facklam.[48] For all practical purposes, however, it should suffice for the clinical laboratory to use the bile-esculin test or latex agglutination to identify this group of bacteria and then to separate the enterococcal and nonenterococcal divisions with the salt tolerance test when these are isolated from patients with endocarditis.

(4) Viridans Streptococci

The viridans *Streptococcus* group is characterized by giving either an α- or γ-hemolytic reaction on blood agar, and negative bile-esculin reaction (Figure 4.2–1). Serological separation is not fruitful because there is no well-defined group antigen that will differentiate among the physiological species. They can be speciated according to their physiological and biochemical characteristics, as reported by Facklam[51]; however, the need to speciate viridans streptococci seldom arises since they are almost uniformly susceptible to penicillin.

It is important to recognize that some strains are nutritionally deficient.[31] These will grow without difficulty in conventional blood culture media; however, they can seldom be subcultured unless the medium has been supplemented with 0.001% pyridoxal. Alternatively, they will grow as satellite colonies in the vicinity of a *Staphylococcus aureus* streak on an agar surface. Above all, it is essential to recognize their existence and not to disregard them mistakenly as nonviable bacteria that may have contaminated the isolation medium.

(5) Streptococcus pneumoniae

(*a*) *Presumptive Tests*

Of the three tests described below for presumptive identification of pneumococci, bile solubility is the most rapid and easy to perform.

Bile Solubility[72]

Purpose:
To differentiate *S. pneumoniae* that is bile soluble from other α-hemolytic streptococci that are not lysed by bile.

Principle:
Pneumococcal colonies are rapidly lysed by bile or a solution of a bile salt. Some bile salts are more active than others; sodium deoxycholate or sodium taurocholate are commonly used. Lysis depends on the presence of an intracellular autolytic enzyme. In a heat-killed culture or in aged culture, the autolysin is inactivated, and pneumococci will not lyse. The role of bile salt is to lower the surface tension between the bacterial cell membrane and the medium, thus accelerating the organism's natural autolytic process.

Specimen:
Young, viable, isolated colonies on blood agar.

Reagent:

10% Sodium deoxycholate

Sodium deoxycholate (BBL Microbiology Systems, Difco Laboratories)	10 g
Distilled water	90 ml

Expiration: 12 months at room temperature.

Procedure:

1. Place one to two drops of sodium deoxycholate solution onto colonies.
2. Allow deoxycholate solution to dry for approximately 5 min. Do not move the plate around because some α-hemolytic streptococcal colonies can be washed away.
3. Examine colonies.

Interpretation:

Positive test: Lysis of *S. pneumoniae* is indicated by flattening of the colonies. An imprint of the lysed colony may remain within the zone of α-hemolysis surrounding the original colony.
Negative test: Intact colonies.

Controls:

Positive: *S. pneumoniae*
Negative: Other viridans *Streptococcus*

Solubility in Sodium Dodecylsulfate (Dreft Test)

Purpose:
To differentiate *Streptococcus pneumoniae* from other α-hemolytic streptococci.

Principle:
A growing culture of *S. pneumoniae* will be lysed by the addition of 4% sodium dodecylsulfate (Dreft). The mechanism of lysis is similar to that described for bile solubility. The test is especially useful when the results of the bile solubility test are questionable. The broth culture to be tested should not be overly turbid. In such cases, lysis will occur only if a large volume of Dreft is used.

Specimen:
Isolated colonies on blood agar.

Reagent:

4% Dreft

Dreft	4 g
Distilled water	100 ml

Expiration: 12 months at room temperature

Procedure:

1. Inoculate one to two colonies into 5 ml of veal infusion broth to which 0.1 ml of horse serum has been added.
2. Incubate broth at 35°C in 5–10% CO_2.
3. When visible turbidity has occurred (usually within 6 to 18 hr), divide the broth suspension into two portions.
4. To one portion add five drops of physiological saline; to the other add five drops of 4% Dreft. Mix well and let both tubes stand for 5 to 15 min at room temperature.

Interpretation:

Positive test: Lysis is indicated by clearing of the broth in the tube to which Dreft has been added.
Negative test: Turbidity of the broth to which Dreft has been added is equivalent to that of thesaline control.

Controls:

Positive: *S. pneumoniae*
Negative: Other *Streptococcus* sp.

(b) Alternative Presumptive Test
Though accurate, this test requires incubation overnight and is, therefore, not as rapid as those based on bile solubility.

Optochin Sensitivity[126]

Purpose:
To differentiate *S. pneumoniae* from other α-hemolytic streptococci.

Principle:
The Optochin sensitivity test is based on the differential susceptibility of *S. pneumoniae* and other α-hemolytic streptococci to ethylhydroxycupreine hydrochloride. *S. pneumoniae* is inhibited by ≤ 5 μg/ml of this agent. When contained within a paper disk and applied to an agar surface, as for the disk diffusion antibiotic susceptibility test, the inhibitory zones obtained with *S. pneumoniae* significantly exceed those obtained with other viridans streptococci.

Specimen:
Isolated colonies on blood agar.

Medium:
Sheep blood agar

Reagent:
Optochin disk (Taxo P disk, BBL Microbiology Systems; Bacto-Differentiation disk, Optochin, Difco Laboratories).

Procedure:

1. Streak an isolated colony on a quadrant of a sheep blood agar plate.
2. Aseptically apply an Optochin disk onto the center of streaked area. Gently tamp the disk to ensure adequate contact with the agar surface.
3. Invert plate and incubate for 18 hr at 35°C. Do not incubate in CO_2.[126]

Interpretation:
Zone diameter of inhibition ≥ 15 mm—*S. pneumoniae.* Zone diameter of inhibition <15 mm—viridans *Streptococcus* group. Occasional strains of *S. pneumoniae* are not inhibited by Optochin. Accurate measurement of zone of inhibition is essential because some viridans streptococci can give zone diameters of 10 to 12 mm.

Controls:

Positive: *S. pneumoniae*
Negative: Other viridans *Streptococcus*

(c) Definitive Test

Neufeld Quellung Reaction[3,106]

Purpose:
This test can be performed directly to detect the presence of pneumococci in specimens (e.g., cerebrospinal fluid, pleural fluid, sputum), to identify pneumococci in cultures, or to determine the specific capsular type of a pneumococcal isolate.

Principle:
The Quellung, or "capsular swelling," reaction is a reaction between the bacterial capsular polysaccharide and its homologous antiserum. When capsular antigen reacts with its homologous antiserum, the capsule appears microscopically as an enlarged halo around the organism because the antigen-antibody complex formed has a different refractive property than that of the fluid medium in which the organism is suspended.

Specimen:

1. Colonies growing on agar.
2. Broth culture.
3. Cerebrospinal or other body fluid or secretion.

Reagents:

1. *Pneumococcal antiserum:* polyvalent, pooled, or monovalent antiserum. (Statens Seruminstitut, Copenhagen, Denmark). Pooled and certain monovalent antisera are also available in the United States (Difco Laboratories). It should be noted that the nomenclatures of the Danish and American typing systems differ.
2. *Methylene blue solution,* 1%

Methylene blue	100 mg
Distilled water	10 ml

Store at room temperature. Expiration is 12 months.

Procedure:
For testing colonies on agar or for testing a broth culture:

1. Prepare a barely turbid bacterial suspension in 0.5 ml physiological saline or use 0.5 ml of broth culture.
2. Add 0.5 ml of methylene blue solution to the bacterial suspension or the broth culture, and mix well.

3. Place one drop of the stained bacterial suspension on a glass microscope slide and invert a cover glass over it (control slide).
4. Examine control slide microscopically (1000×) with oil immersion and reduced illumination. *S. pneumoniae* will be stained blue; the capsule surrounding the bacteria will appear as a small halo. If there are >100 organisms per field, dilute the bacterial suspension or broth culture with saline to avoid a possible false negative reaction due to antigen excess, especially when testing with homologous antiserum.
5. When concentration of bacteria is satisfactory (50 to 100 cells/oil immersion field), place one drop of suspension on another glass slide. Invert over it a cover glass to which has been added one drop of antiserum (test slide).
6. Examine the test slide in similar manner as the control slide. Several fields should be examined before calling the test negative. Repeat the test with all of the antisera to be tested until the capsular type is determined.

For testing cerebrospinal fluid, uncentrifuged fluid or centrifuged sediment can be used, depending on the number of organisms seen on Gram-stained smear. The remainder of the procedure is identical to that described above for the bacterial suspension.

Interpretation:
A positive Quellung reaction usually occurs within minutes. Pneumococci will be stained blue and are surrounded by a very refractive, enlarged, and well-delineated capsule. It is the sharpness of the defined capsule, rather than its size, that constitutes a positive reaction. In ambiguous situations the test slide should be compared with the control slide to determine if the Quellung reaction has occurred. Negative slides should be stored in a moist chamber and examined again after an hour.

Controls:

Positive: Type III *S. pneumoniae* against polyvalent (Omni) serum

---

(6) Aerococcus viridans

Aerococcus viridans is a gram-positive coccus that can sometimes cause confusion for the inexperienced microbiologist. It is an opportunist and only occasionally causes infection. On sheep blood agar, colonies are gray-white, slightly raised, and surrounded by a zone of greening. Microscopically, the cells are spherical, usually arranged in tetrads, and are slightly larger than staphylococcal cells. In broth culture they do not form chains. They are weakly catalase positive, give a positive bile-esculin reaction, and will tolerate up to 10% NaCl.[45]

II. Gram-Negative Cocci

A. General Considerations

The genus *Neisseria* comprises gram-negative cocci that include two known pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis,* and other saprophytic species that normally colonize the oropharynx, gastrointestinal, and genitourinary tracts.

Neisseria gonorrhoeae remains the most frequent cause of purulent urethritis, cervicitis, and salpingitis. It may cause opthalmia neonatorum, septic arthritis or tenosynovitis, pharyngitis, proctitis, perihepatitis, septicemia, and endocarditis. The sequelae in untreated males include chronic prostatitis, epididymitis, and urethral stricture. Although the majority of infected women are asymptomatic, the majority of men are symptomatic.

Gonococci have become progressively less susceptible to penicillin over the years, necessitating increased dosage and the concomitant use of probenicid for treatment. Strains producing β-lactamase have been recognized since 1976. Spectinomycin has been an effective antimicrobial against most of these penicillin resistant strains; non-β-lactamase–producing penicillin-resistant strains, as well as spectinomycin-resistant strains, have been recognized recently. Cefoxitin plus probenecid or cefotaxime is recommended for the treatment of infections due to such strains.

Neisseria meningitidis is a frequent cause of acute bacterial meningitis in children and in young adults. The incidence of the asymptomatic nasopharyngeal carrier state is approximately 5 to 10% in civilian populations but may be significantly higher in military populations and in household contacts of patients with meningococcal disease. Any isolate from an index case should be tested for its susceptibility to sulfonamides, so that household contacts can be given appropriate prophylaxis, i.e., sulfonamide or rifampin. Minocycline is less frequently used for prophylaxis because of the frequency of vestibular toxicity associated with this agent.

Neisseria meningitidis is occasionally associated with transient bacteremia but can cause a fulminant, often fatal, septicemia. Other diseases that may be caused by *N. meningitidis* include pneumonia, arthritis, and possibly urethritis.

Neisseria lactamica, which can be confused with *Neisseria meningitidis* in the laboratory, is part of the indigenous flora of the upper respiratory tract and is rarely associated with disease. Other species of *Neisseria* are also rarely of concern clinically. *Branhamella* (formerly *Neisseria*) *catarrhalis,* an organism indigenous to the upper respiratory tract, has been found to cause otitis media and, in patients with chronic lung disease or compromised immunity, pneumonia. Its isolation, therefore, from a specimen that has not been contaminated during collection with upper

respiratory tract flora (e.g., transtracheal or tympanic aspiration) should not be dismissed casually. *B. catarrhalis* frequently produces a β-lactamase.

B. Identification

1. General Considerations

Neisseria are catalase and oxidase positive gram-negative cocci. They range from aerobic, facultatively anaerobic, to anaerobic in their oxygen requirement. Differentiation of some *Neisseria* species is given in Table 4.2.–3. The anaerobic gram-negative coccus, *Veillonella,* is discussed under anaerobic bacteria (p. 353).

2. Growth Characteristics

Neisseria gonorrhoeae and *Neisseria meningitidis* are fastidious in their growth requirements. Enriched medium, e.g., chocolate agar, is preferred over blood agar, especially for primary isolation. Thayer-Martin medium, a modified chocolate agar containing antimicrobials, is especially useful for the selective isolation of gonococci and meningococci from sources contaminated with indigenous flora. Both gonococci and meningococci are sensitive to cold and drying; incubation with 5 to 10% CO_2 and high humidity is essential to recovery of most strains.

After 24 hr of incubation, gonococcal colonies are small, translucent, moist, raised, and about 0.5 mm in diameter. Grossly, meningococcal colonies resemble those of gonococci except that they tend to be slightly larger. Colonies of both pathogens are nonpigmented, while some saprophytic species are yellow to tan. Colonies of *B. catarrhalis* are nonpigmented or gray, opaque, and smooth.

3. Microscopic Appearance

Microscopically, neisseriae are gram-negative, coffee bean-shaped cocci about 1 μm in diameter. They can occur in masses but are most frequently in pairs with their adjacent sides flattened against each other (Figure 2–1p). Overly decolorized or aged gram-positive cocci and some coccoid gram-negative bacilli (e.g., *Moraxella* and *Acinetobacter*) can occasionally be confused with neisseriae.

The isolation from genital sites of oxidase positive, gram-negative diplococci on Thayer-Martin medium constitutes strong presumptive evidence of gonococcal infection.

Table 4.2–3. Differentiation of Some *Neisseria* Species and *Branhamella*

Tests	*N. gonorrhoeae*	*N. cinerea*	*N. meningitidis*	*N. lactamica*	*N. subflava*	*N. mucosa*	*B. catarrhalis*
Oxidase	+	+	+	+	+	+	+
Growth on							
T-M medium[a]	+	+	+	+	–	–	–
N.A. (25°C)[b]	–	+	–	– or +	+	+	+
Acid production from[c]							
Glucose	+	–	+	+	+	+	–
Maltose	–	–	+	+	+	+	–
Sucrose	–	–	–	–	– or +	+	–
β-Galactosidase (ONPG)[d]	–	–	–	+	–	–	–
Nitrate reduction	–	+	–	–	–	+	+
DNase	–	–	–	–	–	–	+

[a] T-M, Thayer-Martin.
[b] Nutrient agar.
[c] Incubate at least 48 hr.
[d] Examine after 0.5–2 hr of incubation.

4. Preliminary Test

Oxidase Test

Purpose:
To aid in the rapid presumptive or definitive identification of oxidase-positive groups of bacteria (e.g., *Neisseria, Haemophilus, Pseudomonas, Aeromonas*).

Principle:
Cytochrome oxidase is a heme-containing protein component in the respiratory chain of enzymes responsible for reactions taking place during oxidative phosphorylation. Cytochromes are found in bacteria that can use oxygen as a final electron acceptor in their energy metabolism and are therefore absent in strict anaerobes. In the oxidase test, a substrate, 1% tetramethyl-*p*-phenylenediamine dihydrochloride, is oxidized to a purple colored substance. Only fresh reagent should be used, because it is readily oxidized on standing in air. Also, a platinum inoculating loop, rather than a nichrome loop or loop containing an oxidizing substance, should be used to prevent false positive results.

Specimen:
Colonies growing on agar. Pigmented colonies from eosin–methylene blue agar are not suitable for testing.

Reagent:

N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride, (Eastman Organic Chemicals)	1 g
Distilled water	100 ml

Dispense in 5 ml amounts in brown dropper bottles, store at −10°C. Expiration 6 months. A new bottle of reagent should be thawed once or twice daily for routine use.

Procedure:
Three comparable methods (A–C) using the tetramethyl-*p*-phenylenediamine dihydrochloride reagent are described below. Also described is a commercially available test strip (D), containing dimethyl-*p*-phenylenediamine and α-naphthol, which is slightly less sensitive than methods A–C.

A. Kovacs' method

1. Place a piece of filter paper in a petri dish and saturate it with oxidase reagent.

2. With a platinum loop, pick a portion of a colony and rub gently onto the impregnated filter paper.

Interpretation:

Positive test: Colony deposit turns purple within seconds.
Negative test: No purple color develops.

B. Reagent overlay method

1. Place a piece of nonabsorbent paper in a petri dish.
2. Streak a portion of a colony onto the paper.
3. Overlay the colony with one drop of oxidase reagent.

Interpretation:

Positive test: Colony streak turns purple within 10 sec.
Negative test: No purple color develops.

C. Direct plate method
Add one drop of oxidase reagent directly onto colonies growing on chocolate blood agar. Sheep blood agar is not recommended because the intensely red medium renders reading of the test very difficult. Colonies producing oxidase will first turn lavender and then darken to purple within a few seconds. Oxidase reagent is toxic to bacteria; thus, colonies should be subcultured immediately to maintain viability. However, it does not interfere with the Gram-stain reaction of colonies.

D. Oxidase strip (PathoTec, General Diagnostics)
Streak a portion of a colony directly onto the reagent-impregnated section of the strip.

Interpretation:
(As for C above.)

Controls:

Positive: *Aeromonas* sp.
Negative: *Escherichia coli*

---

5. Definitive Tests

Carbohydrate Utilization Tests

Purpose:
To speciate neisseriae.

Principle:
The differential utilization of carbohydrates is a valuable aid in separating pathogenic *Neisseria* species from other saprophytic species. Utilization of a particular carbohydrate is indicated by color change of indicator due to acid production. The serum-free, agar slanted medium described by Flynn and Waitkins[62] has proved to be the most reliable in our laboratory. Neisseriae utilize carbohydrates oxidatively, rather than fermentatively, so that only small amounts of acid are formed.

Specimen:
Isolated colonies on chocolate or Thayer-Martin agar.

Medium:

a. Tubed and slanted Flynn and Waitkins medium containing glucose, maltose, and sucrose, respectively. A test for β-galactosidase (p. 163) may be used in lieu of one for lactose utilization.
b. Alternatively, CTA medium containing carbohydrates listed above in a. This medium provides accurate results when careful attention is given to its preparation.[137]

N.B. Media should be brought to room temperature prior to use.

Procedure:

a. Flynn-Waitkins medium[62]

(1) Inoculate the agar slants heavily. Replace caps on the tubes loosely.
(2) Incubate at 35°C in CO_2 incubator.
(3) Inspect tubes for acid production daily for at least 2 days.

Interpretation:

Positive test: Yellow slant indicates acidification of medium; acidity will gradually diffuse into butt of medium with prolonged incubation.
Negative test: medium remains orange-red.

Controls:
Glucose: *N. gonorrhoeae* +, *B. catarrhalis* −
Maltose: *N. meningitidis* +, *N. gonorrhoeae* −
Sucrose: *N. mucosa* +, *N. gonorrhoeae* −

b. CTA medium

(1) Inoculate each tube of medium heavily by stabbing the agar. Replace caps on the tubes tightly.
(2) Incubate at 35°C in 5–10% CO_2.
(3) Inspect tubes for acid production daily for at least 2 days.

Interpretation:

Positive test: Yellow color on agar surface and down stab line; acid will gradually diffuse into rest of the tube with prolonged incubation.
Negative test: Color of medium remains orange-red.

Controls:
Use the same controls as with the Flynn-Waitkins medium.

ONPG Test

Purpose:
The ONPG test detects the presence of β-galactosidase. It is useful for differentiating between *N. lactamica* and *N. meningitidis*.

Principle:
The ability of bacteria to ferment lactose depends on two enzymes: a lactose permease, which allows lactose to enter bacterial cells, and a galactosidase, which breaks down the carbohydrate to galactose and glucose. When organisms are deficient in permease, the lactose fermentation test will be negative; however, the presence of galactosidase can be detected by the ONPG test. In this test, a chromogenic substrate, ONPG (*o*-nitrophenyl-β-D-galactopyranoside) is hydrolyzed by bacterial enzyme to yield a yellow compound, *o*-nitrophenol according to the equation:

$$\underset{\text{(colorless)}}{\text{ONPG} + H_2O} \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \underset{\text{(yellow)}}{o\text{-nitrophenol}}$$

Specimen:
Preferably, but not necessarily, colonies to be tested should be growing on lactose-containing medium.

Reagent:
ONPG disk (Difco Laboratories).

Procedure:

1. Prepare a heavy bacterial suspension in 0.3 ml physiological saline.
2. Aseptically add an ONPG disk.
3. Incubate tube at 35°C for 2 hr.

Interpretation:

Positive reaction: Yellow color. A positive test can occur in as little as 10 min with a heavy inoculum.

Negative reaction: No color change within 2 hr.

Controls:

Positive: *N. lactamica*
Negative: *N. gonorrhoeae*

---

6. Alternative Tests

1. Rapid nongrowth carbohydrate-degradation test.[91]
2. Fluorescent antibody (Section 4.3).
3. Counterimmunoelectrophoresis (Section 4.3).

III. Gram-Positive Bacilli

A. General Considerations

Aerobic to microaerophilic gram-positive bacilli are frequently encountered in the microbiology laboratory. They belong to the families Corynebacteriaceae, Lactobacillaceae, and Bacillaceae. Most are ubiquitous in nature and are frequent inhabitants of water and soil. Some represent indigenous flora on the mucous membranes of humans and animals (Table 1–1). Since certain species are highly pathogenic, it is important for the laboratory to be able to recognize them, however infrequently they might occur.

The many species comprising the different genera of gram-positive bacilli precludes presentation of a simple scheme for easy tests which the clinical laboratory can use to differentiate among the genera of this group of bacteria (Table 4.2–4).

B. Corynebacterium

1. General Considerations

Members of this genus are widely distributed on the mucous membranes and skin of man and animals, in soil and on plants. They are frequently isolated from air samples taken in operating rooms. With the exception of a few species (*C. diphtheriae, C. ulcerans, C. pyogenes, C. haemolyticum,* and *C. minutissimum*), they are rarely pathogenic for man but frequently contaminate cultures. Usually nonpathogenic species, commonly referred to as diphtheroids, may cause infections of inplanted prosthetic materials or devices and have been associated with a variety of opportunistic infections.[89] Particularly noteworthy is a group of

Table 4.2–4. Differential Characteristics among Genera of Gram-Positive Bacilli

	Corynebacterium	*Listeria*	*Erysipelothrix*	*Lactobacillus*	*Bacillus*
Atmosphere requirement	Usually aerobic	Aerobic	Facultatively anaerobic	Microaerophilic	Aerobic
Colony size	0.5–1 mm; occasional strains form pinpoint colonies	0.5–1.0 mm	Smooth colonies: 0.5–1.0 mm. Rough colonies: >1 mm	⩽0.5 mm	2 to several mm
Colony appearance	White to gray-white; creamy to dry and rough textured	Gray, translucent, smooth	Transparent, circular, smooth; rough colonies have a matte surface and fimbriated edge	Gray-white, often rough	Gray, dull, rough; ground glass appearance
Hemolysis	Variable	Narrow rim of β-hemolysis	Green discoloration around colonies	Usually α-hemolysis	Large zone of β-hemolysis
Microscopic appearance	Pleomorphic, uneven staining gram-positive bacilli, punctuated by granules, tending to palisade or form X and V patterns; 0.5 to 1 by 2 to 3 μm	Even staining, short gram-positive rod; can palisade; 0.5 by 1–2 μm	Short gram-positive bacilli from smooth colonies, 0.2 to 0.4 by 0.5 to 2.5 μm; long and filamentous forms from rough colonies; easily decolorized	Long, slender gram-positive rods; can be pleomorphic, often occur in long chains and palisade; 0.3 by 2 to 3 μm	Large gram-positive to gram-variable bacilli, square ends, often with spores; 1 to 1.3 by 3 to 10 μm
Spores	−	−	−	−	+
Catalase	Usually +	+	−	−	+
Oxidase	−	−	−	−	Usually +
H_2S in TSI butt	−	−	3–4+	−	−
Motility	−	+	−	−	Usually +
Growth on Rogosa agar	−	−	−	Usually +	Occasionally +

diphtheroids known as JK, which have been recovered from patients with endocarditis, leukemia, or marrow transplant and are susceptible to vancomycin but resistant to most other antimicrobial agents.[127] Certainly, the repeated isolation of a *Corynebacterium* from normally sterile body fluids, such as blood or cerebrospinal fluid, should be regarded as strong evidence of infection due to one of these species.

Diphtheria, a preventable disease by immunization, is classically associated with toxigenic (tox^+) strains of *C. diphtheriae;* however, rare cases have been attributed to a closely related species or variant, *C. ulcerans.*[110] *C. diphtheriae* causes a primary lesion, usually in the pharynx but occasionally in the skin, characterized by a tightly adherent, grayish pseudomembrane containing the bacilli which multiply and produce a potent exotoxin. Ultimately, the exotoxin is carried in the circulation to other organs, and involvement of the heart, nervous system, and kidneys is particularly prominent. The diagnosis of diphtheria is first and foremost a clinical one since treatment must be administered immediately. Cultures should be performed to confirm the diagnosis and to identify carriers. *C. pyogenes* and *C. haemolyticum* have been isolated from patients with pharyngitis and have been associated with infections of the skin, lungs, and central nervous system. *C. minutissimum,* which is possibly related to *C. xerosis,* causes erythrasma, a mild, chronic, superficial infection of the skin in body folds and clefts.

2. Identification

a. Growth Characteristics

Corynebacteria are aerobic, non-acid-fast, non-spore-forming, and nonmotile gram-positive bacilli. Their lack of motility is especially useful for differentiating them from *Listeria.* They are oxidase negative and usually catalase positive. Speciation of corynebacteria is usually necessary only to rule out the presence of *C. diphtheriae.* The biochemical differential characteristics are shown on Table 4.2–5.

Corynebacterium species grow readily on routine bacteriological media. On sheep blood agar, colonies are 0.5 to 1 mm in diameter after overnight incubation. Colonies are convex, white to gray-white, creamy to dull-textured, and occasional strains have irregular edges. Some strains are very dry and form small pinpoint colonies. *C. pyogenes* and *C. haemolyticum* are β-hemolytic on blood agar.

b. Microscopic Morphology

Corynebacterium species are gram-positive bacilli that frequently occur in groups of palisades or individually in "L" or "V" formations (Figure 2–1e).

Table 4.2–5. Differential Characteristics within the Genus *Corynebacterium*[a]

Tests	*C. diphtheriae*	*C. ulcerans*	*C. pseudodiphtheriticum*	*C. equi*[b]	*C. xerosis*	*C. pseudotuberculosis*	*C. haemolyticum*	*C. pyogenes*	Group JK
Catalase	+	+	+	+	+	+	−	−	+
Motility	−	−	−	−	−	−	−	−	−
Hemolysis	d	d	d	d	−	d	+	+	−
Nitrate reduction	+	−	+	d	+	d	−	−	−
Urease	−	+	+	d	−	+	−	−	−
Gelatin liquefaction	−	−	−	−	−	−	−	+	−
Acid production from									
Glucose	+	+	−	−	+	+	+ or (+)	+ or (+)	+ or (+)
Maltose	+	+ or (+)	−	−	+ or (+)	+ or (+)	+ or (+)	+ or (+)	d
Sucrose	−	d	−	−	+ or (+)	d	d	d	−

[a] Adapted from Hollis et al.[81]

[b] *C. equi* is a glucose oxidizer.

+, ≥90% positive reaction within 2 days; −, ≥90% negative reaction; (+), positive reaction within 3–7 days; d, different reactions.

c. Diagnostic Features

Cystine tellurite medium[63] is selective for the cultivation of *C. diphtheriae.* Tellurite is able to diffuse through the bacterial cell wall, where it is reduced to tellurium, which is precipitated inside the cell, giving colonies of *C. diphtheriae* a grayish-black appearance after 48 hr of incubation.[5] On tellurite agar, gravis-type strains produce large dark gray colonies that have irregular edges and radial striations and are flat and dry in appearance. The mitis-type colonies are small black, convex, moist with entire edge; the intermedius-type colonies are quite small and flat with a raised black center. Occasional strains of *Staphylococcus* will also grow on tellurite medium so that a Gram-stained smear of colonies growing on the medium should always be performed. Since occasional strains of *C. diphtheriae* will also be inhibited by tellurite, specimens should be cultivated on blood agar as well. Loeffler agar is also included as a primary isolation medium to enhance the development of the characteristic microscopic morphology of *C. diphtheriae* on this medium. Colonies of *C. diphtheriae* are creamy and circular with a slightly raised center after overnight incubation. Since Loeffler medium is nutritionally deficient for *C. diphtheriae,* unbalanced cell wall synthesis results and the bacilli exhibit extreme pleomorphism microscopically.[5] In a methylene blue stained smear, the bacteria are very pleomorphic, varying from 0.5 to 1 μm in width and 2 to 6 μm in length, and some are distinctly curved. The cells stain pale blue and are punctuated with volutin granules, the Babes-Ernst bodies, some of which are located at the ends of the bacilli, giving the cells a club-shaped appearance. The granules consist of polymetaphosphate. Since they have a strong affinity for the basic methylene blue dye, the granules usually stain metachromatically and appear pink-red (Figure 2–4).

d. Preliminary Test

Carbohydrate Fermentation

Purpose:
To differentiate various species within the genus *Corynebacterium* by acid production from fermentation of glucose, maltose, and sucrose.

Principle:
Corynebacterium diphtheriae can be differentiated from other *Corynebacterium* species by forming acid from the fermentation of glucose and maltose and no acidity from sucrose. Urease and nitrate reduction tests should be incorporated in the screening set to differentiate *C. ulcerans*

and *C. pseudotuberculosis* (Table 4.2–5). Carbohydrate fermentation broth with Andrade's indicator is the basal medium to which carbohydrate is added to give a final concentration of 1%. Horse serum can be incorporated in the medium to enhance the growth of *C. diphtheriae.*

Medium:
Carbohydrate fermentation broth, Andrade's.

Specimen:
Colonies growing on agar.

Procedure:

1. Inoculate each tube with a portion of an isolated colony and incubate at 35°C.
2. Examine the tubes for acid production daily. Reincubate negative tests for a total of 4 days.

Interpretation:

Positive test: Acidity is indicated by a color change of acid fuchsin, from light straw to red.
Negative test: No color change of indicator.

Controls:
The same organisms that are used for quality control of carbohydrate fermentation tests used in the identification of Enterobacteriaceae.

---

e. Definitive Test

Since diphtheria is caused only by toxigenic (*tox*$^+$) strains, it is necessary to determine the presence of this property. Because of the complexity of the in vitro and in vivo toxigenicity tests, it may be advisable to refer strains that are morphologically and biochemically typical of *C. diphtheriae* to a reference laboratory for determination of toxigenicity.

Toxigenicity Test: In Vitro

Purpose:
To demonstrate the toxin production in vitro by *Corynebacterium diphtheriae.*

Principle:
Toxin production by *C. diphtheriae* can be demonstrated by a precipitin reaction between toxin and antitoxin. This test is less expensive and easier

to perform than the in vivo toxigenicity test (p. 171). The test is performed by streaking the organism in a line across the surface of tellurite agar at right angles to a strip of antitoxin-impregnated filter paper that has been incorporated in the medium, and observing for the appearance of a precipitin line radiating at approximately 45° angles from the intercept of the line of inoculation of the culture and the filter paper strip.

The reliability of this test depends upon many factors,[10,43,74,98] including the type of peptone used in the medium, the purity and concentration of the antitoxin, the pH of the medium (optimally between 7.6 and 8.2), the concentration of NaCl in the medium, and the source of or presence of hemolysis in serum. Hermann et al[74] have shown that a serum substitute (KL Virulence Enrichment, Difco Laboratories) prepared from glycerin, Tween-80, and Bacto-casamino acids is a suitable substitute for serum.

Specimen:
Colonies on agar.

Media:

KL Virulence Agar (Difco Laboratories).
KL Virulence Strip (Difco Laboratories).
KL Virulence Enrichment (Difco Laboratories).

Reagent:
1% potassium tellurite aqueous solution, filter sterilized.

Procedure:

1. Melt two tubes containing 15 ml of KL agar each and maintain at 50°C in a water bath.
2. Aseptically place a KL antitoxin strip in the bottom of each of two sterile petri dishes.
3. Add 3 ml of KL Virulence Enrichment and 0.75 ml of potassium tellurite solution to each tube of molten agar. Mix thoroughly and pour the contents of each tube into each petri dish with the antitoxin strip.
4. Leave the petri dish lid ajar and allow the medium to solidify for at least 1 hr.
5. Inoculate an isolated colony onto each plate in a single thin line perpendicular to the antitoxin strip. Also inoculate a toxigenic and a nontoxigenic strain of *C. diphtheriae* approximately 1 cm on either side of the test isolate as positive and negative controls.
6. Incubate plates at 35°C for 72 hr. Examine plates daily for the appearance of precipitin lines.

Interpretation:
Toxigenic strains of *C. diphtheriae* will show a toxin-antitoxin (primary) precipitin line that forms at 45° angles with the line of inoculum. This

line will form an arc of identity with the precipitin line produced by the adjacent positive control strain. Nontoxigenic strains will show no precipitin line or only produce very weak secondary nonspecific lines near the antitoxin strips. These lines do not form arcs of identity with those formed by the known toxigenic strain. A negative in vitro toxigenicity test should be confirmed by the guinea pig inoculation test.

Controls:

Positive: *C. diphtheriae*, toxigenic strain
Negative: *C. diphtheriae*, nontoxigenic strain

Toxigenicity Test: In Vivo[73]

Purpose:
To demonstrate the in vivo toxigenicity of *C. diphtheriae* by guinea pig inoculation.

Principle:
A guinea pig unprotected by diphtheria antitoxin will succumb to the toxin produced by an inoculation of *C. diphtheriae* within 1 to 4 days; however, a protected control will show no toxic effect.

Specimen:
A 72 hr tryptic soy broth culture or the growth on a Loeffler agar slant should be suspended in 12 ml of broth to match the turbidity of a McFarland barium sulfate standard No. 3.

Procedure:

1. Select two white or light-colored guinea pigs.
2. Shave an area 3 cm in diameter on the abdomen of each animal. Clean the shaven area with alcohol pads.
3. Inject 1000 units of diphtheria antitoxin intraperitoneally into one guinea pig (control). One to 2 hr later inject this animal subcutaneously with 5 ml of inoculum.
4. Inject the other unprotected guinea pig subcutaneously with the same amount of inoculum.

Interpretation:
If the culture is toxigenic, the unprotected guinea pig will die usually within 1 to 4 days or will show an area of necrosis and inflammation at the sit of injection. The control animal will show no effects from the injection. If the culture is nontoxigenic, neither guinea pig will be affected within 10 days.

Guinea pigs that die within 10 days should be autopsied to rule out extraneous causes of death, such as pneumonia, trauma, or enteritis. Subcutaneous gelatinous exudate, enlarged or hemorrhagic adrenals, or excess abdominal or pleural fluid, or any combination thereof is a characteristic of toxigenic *C. diphtheriae.* These features are best observed in animals dying within 48 hr and autopsied promptly. Heart blood and adrenals can be cultured for *C. diphtheriae.*

C. Listeria

1. General Considerations

Listeria monocytogenes is the only clinically significant species within the genus *Listeria.* It was originally isolated from rabbits afflicted with a disease characterized by marked monocytosis. *Listeria* is found in a wide variety of mammalian hosts and birds and in environmental sources, such as silage, sewage, stream water, mud, and decaying vegetation.

In humans, listeriosis is manifested principally as meningoencephalitis, neonatal sepsis, and in opportunistic infections in immunosuppressed hosts. Diabetes and alcoholism also predispose to listeriosis. Listeriosis is not frequently reported, due in part perhaps to its close morphological resemblance to *Corynebacterium.*

2. Identification

a. Growth Characteristics

Since the initial isolation of *L. monocytogenes* may pose a problem, some authorities recommended cold enrichment, by which tissue suspension and body fluids should be recultured after several days, or even weeks, of storage at 5°C. *L. monocytogenes* is a facultatively anaerobic organism that grows well on infusion agar, or tryptose agar, with or without added glucose or blood. After overnight incubation on blood agar, colonies are smooth, translucent, grey, about 0.5 to 1 mm in diameter, and are surrounded by a very narrow rim of β-hemolysis, which might be evident only after 48 hr of incubation.

Listeria is catalase positive and displays a tumbling type of motility that is best demonstrated by examining a hanging drop from a 6-hr broth culture grown at 25°C. When inoculated into semisolid agar, motility is exhibited as diffused growth from the line of stab and as an area of umbrella-like growth several millimeters from the agar surface. The semisolid agar should be incubated at 22 to 25°C, rather than at 35°C, because motility is optimal at the lower temperatures. Its identification can be confirmed by the fluorescent antibody technique (p. 253).

Table 4.2–6. Differential Characteristics of *Listeria monocytogenes* and *Erysipelothrix rhusiopathiae*[a]

Test	*L. monocytogenes*	*E. rhusiopathiae*
Catalase	+	−
Oxidase	−	−
Motility	+	−
Methyl red	+	−
Voges-Proskauer	+	−
H_2S in TSI	−	+

[a] Adapted from Hollis and Weaver.[81]

b. Microscopic Morphology

Listeria is a short non–spore-forming gram-positive bacillus, usually 0.5 by 1–2 μm in size. Unlike *Corynebacterium* species, cells of *Listeria* stain very evenly; however, they do have a tendency to palisade (Figure 2–1d). Occasionally they may be seen in pairs and might be mistaken for streptococci, in which case a catalase test (p. 134) is a useful differential tool. Since the organism may also be confused morphologically with *Erysipelothrix rhusiopathiae*, motility is an important differential test (Table 4.2–6).

c. Preliminary Test

Two comparable methods for determining whether organisms exhibit motility are given below. Demonstration of the characteristic motility of certain genera, however, requires examination of a hanging drop preparation, while demonstration of flagellar arrangement requires a specific stain (p. 86).

Hanging Drop Test for Motility

Purpose:
To demonstrate bacterial motility.

Principle:
A wet mount of a drop of broth culture can be prepared and examined microscopically for motility. Broth cultures are usually examined after incubation at 35°C; however, *L. monocytogenes* demonstrates optimal motility at 22° to 25°C.

Specimen:
Isolated colony on blood agar.

Medium:
Nutrient broth.

Procedure:

1. Inoculate one colony into 5 ml of nutrient broth.
2. Incubate tube at 22° to 25°C for 6 hr.
3. Transfer a loopful of broth culture onto a thin coverslip.
4. Place a very small amount of petrolatum on the four corners of the coverslip and carefully invert it over the concave area of a glass well-slide. Press down gently to obtain a secure seal.
5. Examine the slide microscopically with oil immersion and reduced illumination.

Interpretation:
Cells of *L. monocytogenes* gyrate rapidly with a tumbling or end-over-end motion. Nonmotile bacteria show only Brownian movement, with slight vacillation along the same axis of movement, and they do not move out of the field of vision.

Motility Test in Semisolid Agar

Purpose:
To demonstrate motility in semisolid agar.

Principle:
The growth of motile organisms in a semisolid medium will be diffused and away from the stab line of inoculation; but that of a nonmotile isolate will be confined to the line of stab. Two agar tubes are used and incubated at 22° to 25°C and 35°C, respectively, to demonstrate the greater motility of *Listeria* at the lower temperature.

Specimen:
Isolated colony on agar.

Medium:
Motility agar.

Procedure:

1. Inoculate two motility agar tubes by stabbing halfway down the center of the agar, keeping the inoculating needle as vertical as possible.

2. Leave one tube at room temperature, and incubate the other at 35°C overnight.

Interpretation:
Listeria grows away from the line of stab, and an umbrella-like pattern of growth can be seen several millimeters below the agar surface, giving the medium a very cloudy appearance. Greater motility of *Listeria* is observed with agar incubated at room temperature than at 35°C. Nonmotile bacteria grow only along the line of stab, while the rest of the medium remains clear.

Controls:

Positive: *Edwardsiella tarda*
Negative: *Klebsiella pneumoniae*

Animal Inoculation Test

Instillation of a drop of a 24-hr broth culture of *Listeria* into the conjunctival sac of a young rabbit or guinea pig will cause severe purulent conjunctivitis, and intraperitoneal injection of 0.2 ml of a 24-hr broth culture into mice usually will cause death within 5 days.

D. Erysipelothrix

1. General Considerations

Although *Erysipelothrix rhusiopathiae* is capable of causing disease in a variety of animals and fowl, it is most important as the causative agent of swine erysipelas. In humans, *Erysipelothrix* infection is ordinarily manifested as an acute, localized, usually self-limited, cutaneous lesion known as erysipeloid. Occasionally, the infection becomes generalized and has caused arthritis and endocarditis. Erysipeloid is an occupational disease, the actual incidence of which is probably greater than that reported.[160]

2. Identification

a. Growth Characteristics

E. rhusiopathiae is a non–spore-forming gram-positive, facultatively anaerobic bacillus that grows poorly on ordinary bacteriological media, e.g., heart infusion or tryptic soy agar. Specimens should be inoculated directly

into an infusion broth containing 1% glucose incubated in air or in CO_2 at 35°C before subculturing to blood agar at 24-hr intervals. Conventional blood culture techniques (p. 24) are satisfactory in suspected cases of septicemia or endocarditis. After 18 to 24 hr on heart infusion agar containing 5% rabbit blood, colonies of *E. rhusiopathiae* are convex, circular, smooth, and transparent, about 0.5 to 1.0 mm in diameter. Rough colonies are larger and have a matte surface and fimbriated edge. Colonies tend to impart a green discoloration to blood agar.

b. Microscopic Appearance

Microscopically, organisms from smooth colonies are 0.2 to 0.4 μm by 0.5 to 2.5 μm, while those from rough colonies may show long and filamentous forms. The bacteria can decolorize easily and become gram-negative. *E. rhusiopathiae* is catalase and oxidase negative and nonmotile. A helpful characteristic and one rarely displayed by other morphologically similar gram-positive bacilli is the ability of *Erysipelothrix* to produce H_2S in the butt of triple-sugar iron agar (TSIA). Some of the differentiating biochemical characteristics between *E. rhusiopathiae* and *Listeria monocytogenes* are shown in Table 4.2–6, whereas some useful differential features among other genera of gram-positive bacilli are shown in Table 4.2–4.

E. Bacillus

1. General Considerations

Members of this genus are aerobic spore-forming, large, rod-shaped bacilli that are usually gram-positive and may or may not grow on erosin–methylene blue (EMB) agar. Because of their variability in Gram stain and oxidase reactions, they may resemble gram-positive bacilli when spores are not evident. All but one of the numerous recognized species are usually saprophytic and generally of no clinical significance, and therefore do not ordinarily require speciation. The isolation of saprophytic species from normally sterile body sites cannot, however, be completely disregarded since there have been isolated case reports of *Bacillus* infections, including pneumonia and septicemia.[149] Moreover, *B. cereus* is capable of causing two forms of food poisoning.[145,150] One, the emetic form, is associated with ingestion of cooked but unrefrigerated rice, has an incubation period of 1 to 6 hr, and is characterized principally by upper gastrointestinal symptoms, rather like staphylococcal food poisoning. The second, or predominantly diarrheal, form of infection, which has an incubation period of 6 to 24 hr, is related to a heat-labile enterotoxin. The diagnosis of *B. cereus* food poisoning can be confirmed by the isolation of 10^5 or more organisms per gram of incriminated food.

2. Bacillus anthracis

a. General Considerations

B. anthracis, the major pathogen in the genus, is the causative agent of anthrax. Human infection is most frequently manifested as a cutaneous lesion that develops initially as a small papule, usually on exposed parts of the body, and then progresses to a vesicle that becomes blue-black and ruptures to form an ulcer crater, the center of which develops into a black eschar ("malignant pustule"). Other forms of the disease are pulmonary (resulting from inhalation of spores) and gastrointestinal (resulting from ingestion of contaminated food). Meningitis may complicate all forms of anthrax. Anthrax is primarily an occupational disease among veterinarians, farm workers, and those handling animals or animal products. Because of its high infectivity, laboratory personnel should exercise extreme caution to prevent accidental aerosolization or cutaneous self-inoculation with infectious material. Cultures should be examined in a biological safety cabinet, and laboratory workbenches should be carefully decontaminated with 5% phenol. Instruments used on infected material should be autoclaved. Animals inoculated with infected material should be kept in separate cages. Carcasses and excreta should be autoclaved prior to incineration and cages autoclaved before cleaning.

b. Identification

(1) Growth Characteristics

After 18 to 24 hr of incubation at 35°C in air, colonies of *B. anthracis* are nonhemolytic, off-white, and about 4 to 5 mm in diameter with a ground-glass appearance. They are usually rough and flat with many comma-shaped outgrowths ("Medusa head"), which can be seen under the dissecting microscope as consisting of long filamentous chains of bacilli. When lifted with an inoculating needle, the edge of the colony stands up like egg white, a characteristic described as "tenacity."[58] Virulent strains produce smooth and mucoid colonies when cultivated on a sodium bicarbonate medium under CO_2 incubation. These smooth colonies will revert to rough colonies with an irregular margin when the bicarbonate plates are reincubated in air. Avirulent forms are usually not encapsulated.

(2) Microscopic Appearance

In a Gram-stained smear, *B. anthracis* are large gram-positive bacilli, about 1 to 1.3 by 3 to 10 μm, and the individual cells have square ends (Figure 2–1i). Oval, subterminal spores, which do not cause any significant swelling of the cells, may be present. Individual spores separated from the parent cells can also be observed. Sporulation can frequently be stimu-

Table 4.2–7. Differentiation of *Bacillus anthracis* and *Bacillus cereus*[a]

Test	*B. anthracis*	*B. cereus*
Blood agar	Rough, flat colonies, many comma-shaped outgrowths	Rough, flat colonies no or few comma-shaped outgrowths
Hemolysis	−	+
Tenacity	+	−
Bicarbonate medium (CO_2)	Mucoid, round	Flat, dry
Capsulation	+	−
Motility	−	+
Gelatin liquefaction	(+)	+
Litmus milk	d	+
Acid produced from salicin	−	+
Immunofluorescence antibody test	+	−
Animal pathogenicity	+	−

[a] Adapted from Feeley et al.[58]
(+), positive in 3–7 days; d, different reactions.

lated by cultivation on an artificial medium like esculin agar. Gram-stained smears of infected tissue or fluid or of growth on enriched media incubated in CO_2 frequently show bacterial cells surrounded by capsules. Characteristics helpful in differentiating *B. anthracis* from *B. cereus,* the organism most easily to be confused with the anthrax bacillus, are shown in Table 4.2–7.

F. Lactobacillus

1. General Considerations

Most species within the genus *Lactobacillus* are microaerophilic, but a few are strictly anaerobic. They are indigenous in humans in the oral cavity, intestinal tract, and vagina. Dispite their ubiquity on body surfaces, they rarely cause disease. Among the few infections reported are bacteremia and endocarditis.[7]

2. Identification

Lactobacilli are non–acid-fast non–spore-forming gram-positive bacilli. They are long and slender, and can sometimes be pleomorphic (Figure 2–11). They occur as single cells or in chains with a tendency to palisade. They are catalase negative and nonmotile, which will readily differentiate

them from *Corynebacterium* and *Listeria.* Unlike most *Bacillus* species, they are oxidase negative. They grow slowly on blood agar as tiny, gray-white, often rough, and α-hemolytic colonies and will not grow on eosin–methylene blue (EMB) or MacConkey agar. They grow best on a glucose-containing medium or other medium, such as Rogosa's selective agar, having an acidic pH (Table 4.2–4). Since occasional strains of *Bacillus* species can also grow on Rogosa medium, colonies should be Gram stained for their microscopic morphology.

IV. Gram-Negative Bacilli

A. General Considerations

Gram-negative bacilli are frequent causes of both community- and hospital-acquired infections. They are a very heterogeneous group of organisms comprising numerous families, genera, and species, the classification and identification of which are complex and are based on the interpretation of a variety of characteristics and biochemical tests.

Gram-negative bacilli can be broadly classified initially on the basis of their *growth characteristics* into those which grow rapidly on simple media (e.g., Enterobacteriaceae, *Pseudomonas*) and those which are slower growing, fastidious, and/or have special nutritional or incubation requirements (e.g., *Haemophilus, Gardnerella, Actinobacillus, Cardiobacterium, Eikenella, Brucella, Bordetella, Francisella, Legionella*). The organism's *colonial morphology* on agar may either suggest its identity or serve to distinguish it from other organisms in mixed cultures. On *microscopic examination* in a Gram-stained smear, the presence of small, pleomorphic, bacillary-to-coccobacillary rods may suggest the presence of *Haemophilus,* while that of curved rods should suggest the presence of *Vibrio* or *Campylobacter. Selective media* may be useful for the isolation of gram-negative bacilli by providing the essential nutritional requirements of a specific group of organisms, by containing substances that are inhibitory to organisms other than those being sought, or by having components that impart distinctive characteristics to specific groups of organisms (Table 3–6).

The identification of *Brucella, Bordetella pertussis,* or *Francisella tularensis* is usually made on the basis of the organism's growth characteristics on special isolation media and immunoserological tests.

The next step in the identification of gram-negative bacilli is to determine oxidase reactivity (p. 160, Figure 4.1–2). The isolation of *Haemophilus* is confirmed by the presence on chocolate agar of colonies of oxidase positive, small, gram-negative bacilli or coccobacilli which are V (and often X) dependent. In most other instances, however, determining whether, how, and which carbohydrates are utilized is necessary for

classifying gram-negative bacilli (Figure 4.1–2). Specific approaches to and techniques for making such determinations vary but are, in most instances, designed for the identification of members of the family Enterobacteriaceae, which are oxidase negative and ferment carbohydrates anaerobically (Figure 4.1–2) via the Embden-Meyerhof-Parnas pathway to yield mixed-acid products (e.g., *Escherichia coli*) or butylene glycol (e.g., *Klebsiella, Enterobacter*). Some other bacteria utilize glucose by alternative pathways that are variations of the hexose monophosphate shunt with pentose phosphate intermediates. The ultimate fate of pyruvic acid varies according to genus. Many end products of fermentation are organic acids so that the fermentation of a specific carbohydrate is readily detected by adding an acid-base indicator to the medium. On the other hand, some bacteria can aerobically oxidise carbohydrates via the citric acid (Krebs) cycle or pentose phosphate pathway to form CO_2 and water.

The practical significance of the fermentative and oxidative processes in the clinical laboratory is that the former yields greater amounts of acid than the latter so that the formation of acid oxidatively may be neutralized by alkaline breakdown products of peptones that are normally present in fermentation media, and the acid can only be detected reliably in a medium (O-F basal medium) containing a relatively high concentration of carbohydrate and a low concentration of peptone.[85] Hence, the importance of separating carbohydrate fermenters from nonfermenters.

B. Enterobacteriaceae

1. General Considerations

Organisms belonging to the family Enterobacteriaceae are facultatively anaerobic gram-negative bacilli that grow readily on conventional artificial media. They ferment glucose, are oxidase negative, and reduce nitrate to nitrite. Members of some genera are motile, and flagella, when present, are peritrichous. Nonmotile variants of motile species also occur. Many genera occur in abundance in the gastrointestinal tract of both humans and animals and in soil and plants. Some genera are opportunistic and may cause infections in debilitated patients or are associated with nosocomial infections, while others are responsible for enteric diseases, septicemia, urinary tract infection, pneumonia, neonatal meningitis, and wound infections (Table 4.2–8).

Traditionally, the classification of the Enterobacteriaceae has been based on the biochemical characteristics and serological relatedness among the species; however, antimicrobial susceptibility patterns, phage typing, computerized identification programs, and DNA hybridization studies have added new dimension to their taxonomy. Consequently,

Table 4.2–8. Diseases Frequently Associated with Enterobacteriaceae

Genera	Diseases
Cedecea	Clinical significance unknown
Citrobacter	Opportunistic and nosocomial infections, bacteriuria, wound infection, neonatal meningitis, brain abscess
Edwardsiella	Diarrhea, enteric fever, wound infection, septicemia, meningitis
Enterobacter	Opportunistic and nosocomial infections, bacteriuria, wound infection
Erwinia-Pectobacterium	Wound infection through contaminated soil or vegetation
Escherichia	Bacteriuria, septicemia, neonatal sepsis and meningitis, wound infection, diarrhea
Ewingella	Clinical significance unknown
Hafnia	Opportunistic and nosocomial infections, bacteriuria, septicemia, wound infection
Klebsiella	Bacteriuria, pneumonia, septicemia
Kluyvera	Clinical significance unknown
Moellerella	Clinical significance unknown
Morganella	Opportunistic and nosocomial infections, bacteriuria, wound infection
Proteus	Bacteriuria, septicemia
Providencia	Opportunistic and nosocomial infections, bacteriuria, wound infection
Salmonella	Septicemia, enteric fever, diarrhea
Serratia	Opportunistic and nosocomial infections, bacteriuria, wound infection, pneumonia, septicemia
Shigella	Diarrhea, dysentery
Tatumella	Clinical significance unknown
Yersinia	Plague (*Y. pestis*), mesenteric enteritis, diarrhea

the nomenclature used by Edwards and Ewing[40] has undergone substantial modification. The tribe concept was recently deleted, and the present classification of the Enterobacteriaceae consists of at least 22 genera and 118 species and "enteric groups."[54] The latter are members of the Enterobacteriaceae that do not conform biochemically to any of the designated species. Many of the "new" organisms are isolated very infrequently from clinical specimens and, when present, are of unknown significance. Genera and species that are associated with human infections, and the "new" species that have been isolated from clinical specimens can be found in Tables 4.2–11 to 4.2–16.

Because of their DNA relatedness, the genera *Escherichia* and *Shigella* are grouped together; *Arizona* is reclassified within the genus *Salmonella*,

Table 4.2–9. Classification within the genus *Salmonella*[a]

Genus	*Salmonella*	
Species	*S. choleraesuis*	Subgroup
Subspecies	*S. choleraesuis* subsp. *choleraesuis*	1
	S. choleraesuis subsp. *salamae*	2
	S. choleraesuis subsp. *arizonae*	3a
	S. choleraesuis subsp. *diarizonae*	3b
	S. choleraesuis subsp. *houtenae*	4
	S. choleraesuis subsp. *bongori*	5

[a] Adapted from Le Minor et al.[103]

which consists of only one species, *Salmonella choleraesuis. S. choleraesuis* can be further divided into six subgroups or subspecies by DNA hybridization and biochemical tests. Over 99% of strains isolated from clinical specimens belong to subgroup 1, or *S. choleraesuis* subsp. *choleraesuis,* which includes serotypes such as *typhi, paratyphi* A, *choleraesuis, typhimurium, enteritidis,* and others that are established etiologic agents of human diarrheal disease. What was known as *Arizona hinshawii* now belongs to subgroups 3a, or *S. choleraesuis* subsp. *arizonae.* Classification within the genus of *Salmonella* is shown in Table 4.2–9.[55,103] Differential characteristics of *Salmonella* serotypes with typical (*Salmonella* serotype *typhimurium*) and atypical reactions (*Salmonella* serotypes *choleraesuis* and *typhi*) can be found in Table 4.2–12.

2. Identification

The extent to which the Enterobacteriaceae are identified will depend on the availability of qualified personnel and the type of facility involved. The clinical laboratory, however, should be able to speciate clinically significant isolates for educational, therapeutic, and epidemiological reasons and to define serologically the major groups of *Salmonella* and *Shigella.*

Because of the large number of biochemical tests required, small laboratories were formerly limited in their ability to speciate the Enterobacteriaceae because of the need to prepare many different media. This problem has been resolved, however, by the increasing availability of commercially prepared devices, the advantages of which include prolonged shelf life, limited storage-space requirements, simplicity in use, and ease of interpretation. These advantages may, however, be limited in a larger laboratory that has adequate media preparation facilities and

skilled personnel and for which the devices may be more expensive to use than conventional tests, provide too many tests for routine use, or impose too much inflexibility in the selection of tests for a particular purpose. In addition, replica agar[23,151] or broth plating techniques offer convenience and economy to the larger laboratory, especially when these techniques are also used for antimicrobial susceptibility tests. Semiautomated systems, which combine antimicrobial susceptibility testing with bacterial identification, are equipped with computerized data management components. Some of these systems also have the capability of identifying gram-positive cocci, glucose nonfermenters, and yeasts. Although the relatively high purchase prices of such systems are generally spread out over a period of three to four years, the major expense associated with their use is, in fact, the costs of the expendable supplies and materials.

Regardless of what device or system is selected for use in any particular laboratory, care must still be taken to ensure the purity of the isolate being tested, to follow the manufacturer's directions, and to correlate the final result with the isolate's colonial morphology. Regardless of the device or system used, a small percentage of gram-negative bacilli will remain unidentified. Such isolates may be reported as unidentified gram-negative bacilli, identified and reported on the basis of conventional biochemical tests, or submitted to a reference laboratory for identification.

The configurations and components of commercially prepared devices vary considerably, however, since the tests that have been selected for use in the devices are for the most part counterparts of conventional tests described by Edwards and Ewing,[40] an understanding of the basic mechanisms of key reactions in the conventional tests should enable the laboratory worker to use any of the commercially prepared devices without too much difficulty, provided it is clearly understood that reactions in the devices must ordinarily be read within the same day or by 18 to 24 hr of incubation, while those in conventional tests may be observed for longer periods of time. For these reasons, certain key tests performed in conventional media will be described in detail.

A multisystem approach to the identification of Enterobacteriaceae and other gram-negative bacilli is used in our laboratory. Effective utilization of such an approach requires correlation of colonial morphology with results of biochemical tests. The different aspects of this approach are as follows:

The rapid spot indole (p. 199) and β-glucuronidase (p. 200) tests for identification of *Escherichia coli.*

A 4-hr kit (Micro-ID, General Diagnostics) for identification of blood culture isolates either with colonies on agar or with resuspended sediment of growth from blood culture bottles.

A basic 3-tube screening set for enteric pathogens.

A Computer-Assisted-Replica Plating (CARP) system for identification of Enterobacteriaceae not identified by the above steps, *Pseudomonas aeruginosa*, and *Acinetobacter*.[151]

A full array of conventional biochemical tests for confirmation of enteric pathogens, isolates not identified by the CARP system, glucose nonfermenters other than *Pseudomonas aeruginosa* and *Acinetobacter*, and other fastidious and infrequently encountered gram-negative bacilli.

a. Tests in Conventional Media

(1) Basic 3-Tube Screening Set for Enteric Pathogens

When examining cultures of feces on differential and selective media (Table 3–6) for the presence of colonies suspected of representing *Salmonella*, *Shigella*, or *Yersinia*, it is recommended that the initial screening tests be limited to triple sugar iron agar, lysine iron agar, and Christensen's urea agar (Table 4.2–10).

Table 4.2–10. Tentative Differentiation of Gram-Negative Bacilli in Stool Cultures[a]

Organism suspected	Triple sugar iron agar (TSIA)	Lysine iron agar (LIA)	Christensen's urea agar
Salmonella[b]	K/A, g, H_2S	K/K or K/N, H_2S	–
S. arizonae	A/A, g, H_2S	K/K, H_2S	–
Shigella[b]	K/A	K/A	–
Yersinia enterocolitica	A/A (K/A)	K/A	+ or –

[a] K, alkaline; N, neutral; A, acidic; K/A, alkaline slant with acidic butt; A/A, acidic slant with acidic butt; g, small amount of gas produced. Colonies yielding biochemical reactions other than those listed may be considered to represent normal fecal flora.

[b] Identify serologically.

(2) Definitive Tests

Approximately 10% of the clinically significant isolates in our laboratory from sources other than feces remain unidentified by the rapid methods or the CARP system. Speciation of these isolates and confirmation of possible fecal pathogens require additional tests. The number and choice of these tests depend on the initial biochemical patterns exhibited by these organisms. The biochemical reactions of the different genera are shown in Tables 4.2–11 through 4.2–16. Organisms resembling *Salmonella* and *Shigella* also require confirmation by serologic agglutination tests.

Table 4.2–11. Differential Characteristics of *Escherichia, Citrobacter, Kluyvera,* and *Ewingella*[a]

Test	*SHIGELLA*	*ESCHERICHIA*			*CITROBACTER*			*KLUYVERA*		*EWINGELLA*
		E. coli	*E. hermanni*	*E. vulneris*	*C. freundii*	*C. diversus*	*C. amalonaticus*	*K. ascorbata*	*K. cryocrescens*	*E. americana*
Indole	d	+	+	−	−	+	+	+ or −	+ or −	−
Methyl red	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−	−	−	−	+
Citrate, Simmons'	−	−	−	−	+	+	+ or −	+	+ or −	+
H_2S (TSI)	−	−	−	−	+	−	−	−	−	−
Urease	−	−	−	−	d	+ or −	+ or −	−	−	−
Phenylalanine deaminase	−	−	−	−	−	−	−	−	−	−
Lysine decarboxylase	−	+ or −	d	+	−	−	−	+	d	−
Arginine dihydrolase	−	− or +	−	d	d	d	+ or −	−	−	−
Ornithine decarboxylase	d	d	+	−	− or +	+	+	+	+	−
Motility	−	+ or −	+	+	+	+	+	+	+ or −	+
Malonate	−	−	−	+ or −	d	+	−	+	+ or −	−
Acid produced from										
Lactose	−	+	d	d	+ or (+)	d	d	+	+	+ or −
Adonitol	−	−	−	−	−	+	−	−	−	−
Sorbitol	d	+	−	−	+	+	+	d	d	−
Yellow pigment	−	−	+	d	−	−	−	−	−	−

[a] Adapted from Brenner,[15] Brenner et al.,[17,20] and Grimont et al.[67]
+, ≥90% positive reactions within 2 days; −, ≥90% negative reactions; (+), positive reactions in 3 to 7 days; + or −, reactions of most strains positive; − or +, reactions of most strains negative; w+, weakly positive reaction; d, different reactions [+, (+), or −].

Table 4.2–12. Differential Characteristics of *Edwardsiella,* and *Salmonella*[a]

Test	*EDWARDSIELLA*		*SALMONELLA serotype*			
	E. tarda	*E. hoshinae*	*choleraesuis*	*typhi*	*typhimurium*	*arizonae*
Indole	+	+	−	−	−	−
Methyl red	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−
Citrate, Simmons'	−	−	(+)	−	+ or (+)	+
H_2S (TSI)	+	+	d	+[b]	+	+
Urease	−	−	−	−	−	−
Phenylalanine deaminase	−	−	−	−	−	−
Lysine decarboxylase	+	+	+	+	+	+
Arginine dihydrolase	−	−	(+)	− or (+)	+ or (+)	(+) or +
Ornithine decarboxylase	+	+	+	−	+	+
Motility	+	+	+	+	+	+
Malonate	−	+	−	−	−	+
Acid produced from						
Lactose	−	−	−	−	−	d
Sucrose	−	+	−	−	−	−
Mannitol	−	+	+	+	+	+
Dulcitol	−	−	d	d[b]	+	−
Arabinose	−	−	−	−	+	+
Rhamnose	−	−	+	−	+	+
Trehalose	−	+	−	+	+	+

[a] Adapted from Brenner,[15] Farmer et al.,[55] and Grimont et al.[69]
[b] *Salmonella* serotype *paratyphi* A usually does not produce H_2S; ferments dulcitol, but not xylose.
For key to symbols see Table 4.2–11.

Table 4.2–13. Differential characteristics of *Klebsiella, Enterobacter, Tatumella,* and *Hafnia*[a]

	KLEBSIELLA					*ENTEROBACTER*						
Test	*K. pneumoniae*	*K. oxytoca*	*K. ozaenae*	*K. rhinoscleromatis*	*K. trevisanii*[b]	*E. cloacae*	*E. aerogenes*	*E. gergoviae*	*E. sakazakii*	*E. agglomerans*	*Tatumella ptyseos*	*Hafnia alvei*
Indole	−	+	−	−	− or +	−	−	−	− or +	− or +	−	−
Methyl red	− or +	d	+	+	+	−	−	d	− or +	d	−	d
Voges-Proskauer	+	+	−	−	+	+	+	+	+	d	+	d
Citrate, Simmons'	+	+	d	−	+	+	+	+	+	d	+ or (+)(25°C) − (36°C)	d
H_2S (TSI)	−	−	−	−	−	−	−	−	−	−	−	−
Urease	+	+	d	−	+	d	−	+	−	− or +	−	−
Phenylalanine deaminase	−	−	−	−	−	−	−	−	− or w+	− or +	+	−
Lysine decarboxylase	+	+	d	−	+	−	+	+ or (+)	−	−	−	+
Arginine dihydrolase	−	−	−	−	−	+	−	−	+	−	−	−
Ornithine decarboxylase	−	−	−	−	−	+	+	+	+	−	−	+
Motility	−	−	−	−	−	+	+	+	+	+ or −	d (25°C) − (36°C)	+
Malonate	+	+	−	+	+	+	+	+	+	+	−	d
Acid produced from												
Lactose	+	+	d	−	+	d	+	d	+	d	−	−
Sucrose	+	+	− or +	+ or −	+	+	+	+	+	+ or −	+	−
Mannitol	+	+	+	+	+	+	+	+	+	+	−	+
Adonitol	+	+	+	+	+	− or +	+	−	−	−	−	−
Sorbitol	+	+	+ or −	+	+	+	+	−	−	d	−	−
Yellow pigment	−	−	−	−	−	−	−	−	+	+ or −	−	−
DNase	−	−	−	−	−	−	−	−	(+)	−	−	−

[a] Adapted from Brenner,[15] Brenner et al.,[18] Ferragut et al.[60] Hollis et al.[80]
[b] Growth at 4°C, + or (+).
For key to symbols see Table 4.2–11.

Table 4.2–14. Differential Characteristics of *Cedecea,* and *Serratia*[a]

Test	*CEDECEA*			*SERRATIA*						
	C. davisae	*C. lapagei*	*C. neteri*	*S. marcescens*	*S. liquefaciens*	*S. rubidaea*	*S. ficaria*	*S. plymuthica*	*S. odorifera*	*S. fonticola*
Indole	–	–	–	–	–	–	–	–	+	–
Methyl red	+	d	+	– or +	+ or –	– or +	–	– or +	+ or –	+
Voges-Proskauer	+ or –	+	+	+	+ or –	+	+ or –	d	+ or –	–
Citrate, Simmons'	+	+ or –	+	+	+	+	+	d	+	+
H_2S (TSI)	–	–	–	–	–	–	–	–	–	–
Urease	–	–	–	– or +	–	–	–	–	–	– or +
Phenylalanine deaminase	–	–	–	–	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	+	+	d	–	–	+	+
Arginine dihydrolase	d	+ or –	+	–	–	–	–	–	–	–
Ornithine decarboxylase	+	–	–	+	+	–	–	–	d	+
Motility	+	+ or –	+	+	+	+ or –	+	+	+	+
Malonate	+ or –	+	+	–	–	+ or –	–	–	–	+
Acid produced from										
Lactose	– or +	d	+ or (+)	–	–	+	–	+ or –	+	+
Sucrose	+	–	+	+	+	+	+	+	d	– or +
Dulcitol	–	–	–	–	–	–	–	–	–	+
Adonitol	–	–	–	d	–	+	+	–	d	+
Sorbitol	–	–	+	+	+	–	+	d	+	+
Arabinose	–	–	–	–	+	+	+	+	+	+
Raffinose	–	–	–	–	+	+	+	+	d	+
Rhamnose	–	–	–	–	d	–	+	–	+	d
Xylose	+	–	+	–	+	+	+	+	+	+ or –
DNase	–	–	–	+	+ or –	+	+	+	+	–

[a] Adapted from Brenner,[15] Farmer et al.,[56] and Grimont et al.[68]
For key to symbols see Table 4.2–11.

Table 4.2–15. Differential Characteristics of *Proteus, Morganella, Providencia,* and *Moellerella*[a]

	PROTEUS			*MORGANELLA*	*PROVIDENCIA*				*MOELLERELLA*
Test	*P. vulgaris*	*P. mirabilis*	*P. penneri*	*M. morganii*	*P. rettgeri*	*P. alcalifaciens*	*P. rustigianii*	*P. stuartii*	*M. wisconsensis*
Indole	+	–	–	+	+	+	+	+	–
Methyl red	+	+	+	+	+	+	+ or –	+	+
Voges-Proskauer	–	– or +	–	–	–	–	–	–	–
Citrate, Simmons'	d	d	– or (+)	–	+	+	–	+	+
H_2S (TSI)	+	+	–	–	–	–	–	–	–
Urease	+	+	+	+	+	–	–	d	–
Phenylalanine deaminase	+	+	+	+	+	+	+	+	–
Lysine decarboxylase	–	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	–	+	–	+	–	–	–	–	–
Motility	+	+	+ or –	+ or –	+	+	–	+ or –	–
Malonate	–	–	–	–	–	–	–	–	–
Acid produced from									
Lactose	–	–	–	–	–	–	–	–	+
Galactose				+	+	–	+	+	+
Mannitol	–	–	–	–	+	–	–	d	+ or (+)
Adonitol	–	–	–	–	+	+	–	–[b]	+
Inositol	–	–	–	–	+	–	–	+[b]	–
Maltose	+	–	+	–	–	–	–	–	– or (+)
Xylose	+ or –	+	+	–	d	–	–	–	–
Trehalose	d	+	(+)	d	–	–	–	+	–
Raffinose	–	–	–	–	–	–	–	–	+

[a] Adapted from Brenner,[18] Hickman et al.,[76] and Hickman-Brenner et al.[77,78]
[b] Biotype 6 strains are adonitol-positive; biotype 4 strains are inositol-negative.
For key to symbols see Table 4.2–11.

Table 4.2–16. Differentiation within the Genus *Yersinia*[a]

Test	*YERSINIA*						
	Y. pestis	*Y. pseudo-tuberculosis*	*Y. entero-colitica*	*Y. inter-media*	*Y. kris-tenseni*	*Y. freder-icksenі*	*Y. ruckeri*
Indole	–	–	d	+	d	+	–
Methyl red	+	+	+	+	+	+	+
Voges-Proskauer	– (25°C) – (37°C)	– (25°C) – (37°C)	+ (25°C) – (37°C)	+ (25°C) – (37°C)	– (25°C) – (37°C)	+ (25°C) – (37°C)	– or (+)[w+](25°C) – (37°C)
Citrate, Simmons'	–	– (25°C)	– (25°C)	d (25°C)	– (25°C)	d (25°C)	(+)
H_2S (TSI)	–	–	–	–	–	–	–
Urease	–	+	+	+	+	+	–
Phenylalanine deaminase	–	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	–	–	–	+ or (+)
Arginine dihydrolase	–	–	–	–	–	–	–
Ornithine decarboxylase	–	–	+[b]	+	+	+	+
Motility	– (25°C) – (37°C)	+ (25°C) – (37°C)	+ (25°C) – (37°C)	+ (25°C) – (37°C)	+ (25°C) – (37°C)	+ (25°C) – (37°C)	+ or – (25°C)
Malonate	–	–	–	–	–	–	–
Acid produced from							
Lactose	–	–	d	–	d	d	– or (+)
Sucrose	–	–	+[b]	+	–	+	–
Rhamnose	–	+	–	+	–	+	–
Xylose	+	+	d	+	+	+	–
Sorbital	–	–	+[b]	+	+	+	–
Raffinose	–	d	–	+	–	–	–

[a] Adapted from Bercovier et al.,[9] Brenner et al.,[18] Highsmith et al.,[79] and Niléhn.[119]
[b] Most biotype 5 strains are negative.
For key to symbols see Table 4.2–11.

Triple Sugar Iron Agar (TSIA)

Purpose:
To determine whether a gram-negative bacillus utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulfide (H_2S).

Principle:
Demonstration of the organism's fermentative properties depend on the proportions of each of the carbohydrates (10 parts lactose:10 parts sucrose:1 part glucose), as well as their concentrations relative to that of peptone in the medium. Phenol red and ferrous sulfate serve as indicators of acidification and H_2S formation, respectively, TSIA is slanted when tubed and is inoculated with a straight wire by stabbing the butt and streaking the slant of the medium. When glucose is utilized by a fermentative organism, the entire medium becomes acidic (yellow) in 8 to 12 hr. The butt remains acidic after the recommended 18 to 24 hr incubation period due to the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the medium in the tube. The slant, however, reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions on the slant with the formation of CO_2 and H_2O and because of the oxidation of peptones in the medium to alkaline amines (Figure 4.2–3a). When, in addition to glucose, lactose and/or sucrose are fermented, the large amounts of fermentation products formed on the slant will more than neutralize the alkaline amines and render it acidic (yellow), provided the reaction is read in 18 to 24 hr (Figure 4.2–3b). Reactions in TSIA should *not* be read beyond 24 hr of incubation since aerobic oxidation of the fermentation products from lactose and/or sucrose does proceed and the slant will eventually revert to the alkaline state. The formation of CO_2 and H_2 is indicated by the presence of bubbles or cracks in the agar or by separation of the agar from the sides or bottom of the tube (Figure 4.2–3b). The production of H_2S requires an acidic environment and is manifested by blackening of the butt of the medium in the tube (Figure 4.2–3c).

Specimen:
Isolated colony on agar.

Medium:
TSIA (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. With a straight inoculating needle, touch the top of a well-isolated colony.

2. Inoculate TSIA by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of agar slant. The same inoculum can be used to inoculate the rest of the media in the set.
3. Leave cap on *loosely* (i.e., *do not tighten the cap on the tube*) and incubate the tube at 35°C for 18 to 24 hr.

Interpretation:

1. Alkaline slant/alkaline butt (K/K) = nonfermenter.
2. Alkaline slant/acid butt (K/A) = glucose fermentation only (Figure 4.2–3a).
3. Acid slant/acid butt (A/A) = glucose, sucrose, and/or lactose fermenter (Figure 4.2–3b).

Patterns 2 and 3 can be accompanied by black precipitate of ferrous sulfide and gas production (Figure 4.2–3c).

Controls:

Citrobacter freundii = A/A with gas and H_2S
Providencia rettgeri = K/A
Pseudomonas cepacia = K/K

Lysine Iron Agar (LIA)[41]

Purpose:
To determine whether a gram-negative bacillus decarboxylates or deaminates lysine and forms hydrogen sulfide (H_2S).

Principle:
In an acidic environment and in the presence of a coenzyme (usually pyridoxal phosphate) and a specific amino acid substrate, bacteria possessing a specific decarboxylase can decarboxylate the amino acid (lysine, arginine, ornithine, tyrosine, histidine, or glutamine) to its corresponding amine, LIA contains, in addition to lysine, peptones, a small amount of glucose, ferric ammonium citrate, and sodium thiosulfate. Like TSIA, LIA is slanted when tubed and is inoculated by stabbing the butt and streaking the slant. When glucose is fermented, the butt of the medium becomes acidic (yellow). When the organism possesses lysine decarboxylase, cadaverine is formed, according to the following equation:

$$\text{Lysine} \xrightarrow[\text{pyridoxal phosphate}]{\text{lysine decarboxylase}} \text{cadaverine} + CO_2 \uparrow$$

Cadaverine neutralizes the organic acids formed by glucose fermentation, and the butt of the medium reverts to the alkaline state (purple). If the decarboxylase is not present, the butt remains acidic. Unless deamination of lysine occurs, the LIA slant remains purple in a manner analogous to the TSIA slant when only glucose is fermented. Oxidative deamination of lysine by *Proteus* and *Providencia* sp. yields a compound that, in the presence of ferric ammonium citrate and a coenzyme, flavin mononucleotide, forms a burgundy red color.

Specimen:
Isolated colony on agar.

Medium:
LIA (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. With a straight inoculating needle, inoculate LIA by stabbing through the center of the medium to the bottom of the tube and then streaking the slant.
2. Leave cap on *loosely* and incubate the tube at 35°C for 18 to 24 hr.

Interpretation:

1. Alkaline slant/acid butt (K/A) = glucose fermentation.
2. Alkaline slant/alkaline butt (K/K) = lysine decarboxylation or no fermentation.
3. Red slant/acid butt (R/A) = lysine deamination and glucose fermentation.

Patterns 1 and 2 can be accompanied by a black precipitate of ferrous sulfide.

Controls:

Citrobacter freundii = K/A with H_2S
Edwardsiella tarda = K/K with H_2S
Providencia rettgeri = R/A

Citrate Utilization Test

Purpose:
To determine an organism's ability to use citrate as the sole source of carbon. The test is useful for differentiating *Escherichia-Shigella* from most of the other genera of the Enterobacteriaceae.

Principle:
Organisms possessing a transport system or permease, which permits citrate to enter the cell, and citrate lyase, which cleaves citrate, produce an alkaline reaction by an as yet poorly understood mechanism. The alkaline reaction in Simmons' citrate agar is sufficient to change the indicator, bromthymol blue, from green to blue (Figure 4.2–4, see color insert in the center of the book). The medium should be lightly inoculated, and a positive reaction should be limited to organisms producing the change to an alkaline pH. Growth on the medium without the pH change to alkalinity usually indicates growth by a species not belonging to the family Enterobacteriaceae.

Specimen:
Isolated colony on agar or growth on TSIA slant.

Medium:
Simmons' citrate medium (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. Streak a portion of an isolated colony on the surface of the agar slant. Too dense an inoculum may cause a false positive result.
2. Leave cap on *loosely* and incubate the tube at 35°C for 18 to 24 hr. Negative tests can be reincubated for up to 4 days.

Interpretation:

Positive test: Growth with a deep Prussian blue color of the agar slant (Figure 4.2–4, left).
Negative test: Absence of growth or color change of the medium (Figure 4.2–4, right).

Controls:

Positive: *Citrobacter freundii*
Negative: *Edwardsiella tarda*

Urease Test[29,139]

Purpose:
To demonstrate the hydrolysis of urea. Urease is produced in large quantity by *Proteus, Morganella,* and some *Providencia* sp., and in smaller amounts by members of other genera (e.g., *Klebsiella, Enterobacter, Serratia, Citrobacter,* and *Yersinia*). The test is also useful for screening colonies of yeasts for possible cryptococci.

Principle:
The hydrolysis of urea by urease produces ammonia and carbon dioxide according to the equation:

$$O{=}C\begin{matrix} \diagup NH_2 \\ \diagdown NH_2 \end{matrix} + 2\ H_2O \xrightarrow{\text{urease}} 2\ NH_3 + CO_2 + H_2O$$

The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta at pH 8.1. Stuart's urea broth is highly buffered and is better suited to detect reactions caused by strong urease producers; therefore, it is more specific for the *Proteus* sp. Christensen's urea agar contains a smaller amount of buffer and is more sensitive in the detection of weak urease reactions. The added peptone and glucose in the latter medium also allows more luxuriant growth of bacteria. The agar should be slanted with an adequate butt portion to allow gradation of positive reactions (Figure 4.2–5, see color insert in the center of the book).

Specimen:
Isolated colony on agar.

Medium:
Christensen's urea agar (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. Streak the surface of urea agar slant with portion of a well-isolated colony.
2. Leave cap on *loosely* and incubate tube for 8 to 24 hr at 35°C. Negative test can be reincubated up to 4 days.

Interpretation:

Positive test: Urea agar slant will change from light orange to magenta color. Color change also occurs in the butt of the medium as ammonia diffuses into the medium; the degree of this change depends on the amount of urease that is elaborated by the bacteria (Figure 4.2–5). The *Proteus* sp. will give a positive test within 1 to 6 hr of incubation.
Negative test: Agar slant and butt remain light orange.

Controls:

Positive: *Providencia rettgeri,* 4+
Negative: *Edwardsiella tarda*

Ornithine Motility Test[111]

Purpose:
To detect the activity of the enzyme ornithine decarboxylase and the motility exhibited by certain genera of the Enterobacteriaceae. It can also be used for speciation of the pseudomonads.

Principle:
Like lysine decarboxylation, ornithine decarboxylation requires a specific decarboxylase enzyme, a coenzyme, and an acidic environment. Decarboxylation of ornithine to putrescine proceeds according to the following equation:

$$\text{Ornithine} \xrightarrow[\text{pyridoxal phosphate}]{\text{ornithine decarboxylase}} \text{putrescine} + CO_2\uparrow$$

By incorporating a small amount of agar (0.3%) in Moeller's decarboxylase broth to render it semisolid, it is possible to determine both decarboxylase activity and motility (Figure 4.2–6, see color insert in the center of the book). When the broth decarboxylase medium is used, it must be overlaid with mineral oil to ensure that fermentation (vs. oxidation) of glucose takes place and to prevent oxidative degradation of the peptone, resulting in alkalinization, from occurring at the surface of the medium and eventually diffusing throughout the medium. When the semisolid agar formulation is used, no oil overlay is required unless it is to be incubated for more than 24 hr. The medium will initially change from purple to yellow due to acid production from glucose fermentation. In the event of decarboxylation, the alkalinity produced by the amine will cause the yellow color of the medium to revert to purple. Motility is determined after overnight incubation of the semisolid agar medium by the presence of turbidity resulting from growth extending out from the stab line of inoculation.

Decarboxylation of other amino acids can be similarly determined by incorporating the one to be tested into the decarboxylase basal medium (broth or semisolid agar) (Figure 4.2–6). The decarboxylation of lysine results in cadaverine, while that of ornithine results in putrescine. Arginine is actually hydrolyzed in a two-step reaction to ornithine which, in turn, is decarboxylated to putrescine, a process generally requiring 2 days. Hence, the term arginine dihydrolase is used for this test. When performing decarboxylase tests, it is important to inoculate as a control a tube containing the decarboxylase medium with no added amino acid. Alkalinization of this medium during the period of observation of decarboxylase tests will invalidate their results. Decarboxylase activity is also exhibited by some nonfermenters; however, the activity is not optimal since the medium is not initially acidified by glucose fermentation. Nevertheless, the formation of alkaline amines will render the medium more

alkaline and, therefore, a deeper purple than that in the control medium. With a slight modification in the composition of the medium, it is also possible to determine indole production by adding a few drops of Kovacs' reagent (p. 198) to the surface of the medium[38]; however, the test is not as sensitive as the conventional indole tests in broth (p. 199).

Specimen:
Isolated colony on agar or growth on TSIA slant.

Medium:
Moeller decarboxylase broth base (BBL Microbiology Systems or Difco Laboratories) with 1% of L-ornithine hydrochloride (Grand Island Biological Company).

Procedure:

1. With a straight inoculating needle, introduce inoculum by stabbing through the center of the medium to the bottom of the tube.
2. If the ornithine test is to be incubated longer than 24 hr or if it is used for speciation of pseudomonads, also inoculate a control medium (i.e., without ornithine) and overlay both media with sterile mineral oil to a height of 1.5 cm.
3. Incubate tubes at 35°C for 18 to 24 hr.

Interpretation:

Decarboxylation: Purple color throughout the medium (Figure 4.2–6). With certain *Pseudomonas* sp., the medium acquires a deeper purple color than the control medium. Growth must be present for test results to be valid.
No decarboxylation: The medium is yellow except for a very small rim of purple at the agar surface (Figure 4.2–6).
Motility: Turbidity representing bacterial growth extending out from stab line (Figure 4.2–6).
No motility: Bacterial growth is confined along the stab line. The rest of the medium remains clear.

Controls:
Ornithine and motility positive: *Edwardsiella tarda*
Ornithine and motility negative: *Klebsiella pneumoniae*

Indole Test

Purpose:
To detect the production of indole from tryptophan. The test is especially useful in the identification of *Escherichia coli* and the differentiation of *Proteus mirabilis* from *Proteus vulgaris*.

Principle:
Bacteria that produce the enzyme tryptophanase will deaminate tryptophan to indole, pyruvic acid, and ammonia in the presence of a coenzyme, pyridoxal phosphate. The reaction can be represented as follows:

$$\text{Tryptophan} \xrightarrow[\text{pyridoxal phosphate}]{\text{tryptophanase}} \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

When Kovacs' reagent, which is an acidic alcoholic solution of *p*-dimethylaminobenzaldehyde, is added to the medium, indole combines with the aldehyde to form a red-colored complex. If Ehrlich's reagent is used, extraction of indole from the aqueous medium with xylene, xylol, or chloroform is required before adding the aldehyde. Erhlich's method is more sensitive, especially when testing bacteria (e.g., anaerobes), which produce small amounts of indole. The peptone broth used as growth medium should have a high content of tryptophan. By performing the test in 2 ml of medium, accurate results can be obtained after 18 to 24 hr of incubation. Well-isolated, nonmucoid colonies that have a green metallic sheen on EMB agar and resemble *Escherichia coli* and swarming colonies of *Proteus* can be presumptively identified with a rapid indole spot test (p. 199). The identification of indole-negative, swarming colonies of *Proteus* from sources other than urine should be confirmed with the ornithine decarboxylase test since some colonies of *P. vulgaris* yield a falsely negative spot test result. Colonies for the spot test should be taken from a blood agar plate and not from a medium containing inhibitors or dyes, e.g., EMB agar. The carryover of dye will produce a color change on reagent-impregnated filter paper.

Specimen:
Isolated colony on agar.

Medium:
Indole test broth.

Reagent:

Kovacs' Reagent

Amyl or isoamyl alcohol (Mallinckrodt)	150 ml
p-Dimethylaminobenzaldehyde (Eastman Organic Chemicals)	10 g
Concentrated hydrochloric acid	50 ml

Dissolve aldehyde slowly in the alcohol and then add the acid. The solution should have a light yellow color. If a brown color results, the reagent should not be used. Kovacs' reagent is stable at 4°C for 1 year.

Ehrlich's Reagent

Ethyl alcohol, 95%	95 ml
p-Dimethylaminobenzaldehyde	1 g
Concentrated hydrochloric acid	20 ml

Prepare and store like Kovacs' reagent.

Reagent for Indole Spot Test

p-Dimethylaminocinnamaldehyde	1 g
Hydrochloric acid, 10% aqueous solution	100 ml

Solution is stable for 1 year at 4°C.

Procedure:

A. Broth Test

1. Inoculate portion of a colony into 2 ml of indole broth.
2. Mix on mechanical mixer (Vortex).
3. Incubate broth at 35°C for 18 to 24 hr.
4. Add 0.5 ml of Kovacs' reagent and mix by shaking.

Interpretation:

Positive test: Red color in the upper organic layer (Figure 4.2–7, left, see color insert in the center of the book).
Negative test: Light yellow color in the upper organic layer (Figure 4.2–7, right, see color insert in the center of the book).

Controls:

Positive: *Providencia rettgeri*
Negative: *Enterobacter aerogenes*

B. Spot Test[105]

1. Place a small piece of Whatman No. 1 filter paper in a petri dish.
2. Moisten paper with indole reagent.
3. With an inoculating loop, gently rub a portion of an isolated colony onto the filter paper.

Interpretation:

Positive test: Development of a turquoise color within 20 sec at the site of bacterial deposit.
Negative test: Salmon-pink color at the site of bacterial deposit.

Controls:

Positive: *Escherichia coli*
Negative: *Proteus mirabilis*

β-Glucuronidase (PGUA) Test[95]

Purpose:
The PGUA test detects the presence of β-glucuronidase, an enzyme found exclusively among the Enterobacteriaceae in *E. coli* and some *Shigella.* The test is used in conjunction with the spot indole test for the identification of *E. coli* from sources other than urine. Colonies tested should be lactose-fermenting, oxidase negative, and spot indole test positive.

Principle:
A number of chromogenic substrates have been used for rapid detection of inducible bacterial glycosidases produced from a heavy suspension of viable but not multiplying organisms. In the PGUA test, *p*-nitrophenyl-β-D-glucuronide is hydrolysed by β-glucuronidase to yield a yellow product, *o*-nitrophenol.

Specimen:
Isolated colonies on blood agar.

Reagent:

1. 0.05 M Tris buffer

Tris-(hydroxymethyl)-aminomethane (Sigma Chemical Company)	0.61 g
Distilled water	80 ml

Titrate with 1 M HCl to pH 8.5 ± 0.2. Adjust volume to 100 ml with distilled water.

2. *p*-Nitrophenyl-β-D-glucuronide

(Sigma Chemical Company)	100 mg
0.05 M Tris buffer	100 ml

Dispense 0.2 ml in 12 × 75 mm polystyrene tube. Store at 4°C, and use within 6 months.

Procedure:

1. Inoculate PGUA with 5 to 10 colonies.
2. Incubate for 1 hr at 35°C.

Interpretation:

Positive test: Yellow color production
Negative test: No color change

Controls:

Positive: *Escherichia coli*
Negative: *Proteus mirabilis*

Methyl Red Test[6]

Purpose:
To demonstrate an acidic pH change resulting from mixed acid fermentation of glucose in an indicator medium. The test is of particular value in differentiating *E. coli* from *Enterobacter* sp.

Principle:
The Enterobacteriaceae convert glucose to pyruvic acid by the Embden-Meyerhof-Parnas pathway; however, the metabolism of pyruvic acid by this group of bacteria yields either mixed acids (lactic, acetic, formic, and succinic acids) or small amounts of mixed acids and acetoin and large amounts of 2,3-butylene glycol. Mixed acid fermentation results in a pH in the medium of approximately 4.4, which is little affected by oxidative metabolism. In contrast, the combination of neutral products formed by butylene glycol and oxidative metabolism precludes a significant drop in the pH of the medium. Methyl red, the indicator in the medium, is red at pH <5.0 and yellow at pH >5.8 (Figure 4.2–8, see color insert in the center of the book). *Escherichia coli* have a mixed acid fermentation pathway and are, therefore, methyl red positive, while *Enterobacter* sp. have a butylene glycol fermentation pathway and are methyl red negative. The medium's content of glucose and peptone, as well as its volume and buffering capacity and duration of incubation are crucial factors in the accuracy of the MR test. Accurate results can be obtained in 18 to 24 hr with a heavy inoculum in 0.5 ml of the medium.

Specimen:
Growth on TSIA slant or agar.

Medium:
MR-VP broth (BBL Microbiology Systems or Difco Laboratories).

Reagent:

Methyl red indicator
Methyl red (Difco Laboratories) 0.1 g

Ethyl alcohol, 95%	300 ml
Distilled water	200 ml

Dissolve methyl red in ethyl alcohol and then dilute with water. Reagent is stable for 1 year at 4°C.

Procedure:

1. Inoculate 0.5 ml of MR-VP broth heavily with bacterial growth from TSIA slant or agar.
2. Incubate the broth at 35°C for 18 to 24 hr.
3. Add two drops of methyl red indicator.

Interpretation:

Positive test: Color of reagent remains red (Figure 4.2–8, left).
Negative test: Color of reagent turns yellow (Figure 4.2–8, right).

Controls:

Positive: *Citrobacter freundii*
Negative: *Enterobacter aerogenes*

Voges-Proskauer (V-P) Test[8]

Purpose:
To detect the production of acetoin, an intermediate in the formation of butylene glycol, by organisms that ferment glucose by way of the butylene glycol fermentation pathway. The test is of particular value in differentiating *Klebsiella, Enterobacter* and *Serratia* from *E. coli.*

Principle:
Organisms, such as *Klebsiella, Enterobacter* and *Serratia* sp. that utilize the butylene glycol fermentation pathway yield a large quantity of neutral products, such as butylene glycol and ethanol, and small amounts of acetoin and organic acids. In the presence of air and potassium hydroxide (KOH), acetoin, the precursor of butylene glycol, is oxidized to diacetyl, which yields a red-colored complex (Figure 4.2–9, see color insert in the center of the book). The test is made more sensitive by adding α-naphthol before the KOH. The mechanism of action of α-naphthol is not understood. Accurate results can be obtained in 18 to 24 hr with a heavy inoculum in 0.5 ml of the medium.

Specimen:
Growth on TSIA slant or agar.

Medium:
MR-VP broth (BBL Microbiology Systems or Difco Laboratories).

Reagent:

1. α-Naphthol (Fisher Scientific Company)	5 g
Ethyl alcohol, 95%	100 ml
2. Potassium hydroxide (J.T. Baker Chemical Co.)	40 g
Distilled water	100 ml

Both reagents are stable for 1 year at 22°C.

Procedure:

1. Inoculate 0.5 ml of MR-VP broth heavily with bacterial growth from TSIA slant or agar.
2. Incubate the broth at 35°C for 18 to 24 hr.
3. Add six drops of α-naphthol, followed by three drops of KOH to the broth culture.
4. Agitate the tube for maximal exposure of the culture to atmospheric oxygen.
5. Let broth stand for 10 to 15 min.

Interpretation:

Positive test: A red color develops within 15 min (Figure 4.2–9, left) indicating the presence of diacetyl.
Negative test: No color development (Figure 4.2–9, right).

Controls:

Positive: *Enterobacter aerogenes*
Negative: *Citrobacter freundii*

Phenylalanine Deaminase Test[46]

Purpose:
To detect the enzymatic oxidative conversion of phenylalanine to an α-keto acid. Within the family Enterobacteriaceae, such activity is limited to *Proteus, Providencia, Tatumella,* and some strains of *Enterobacter agglomerans.*

Principle:
Some bacteria are able to deaminate phenylalanine to phenylpyruvic acid by an amino acid oxidase enzyme according to the equation:

$$\text{Phenylalanine} \xrightarrow{\text{amino acid oxidase}} \text{phenylpyruvic acid} + \text{ammonia} \uparrow$$

Subsequent addition of 10% ferric chloride ($FeCl_3$) produces a dark green color by a reaction the mechanism of which is not known (Figure 4.2–10, see color insert in the center of the book).

Specimen:
Growth on TSIA slant or agar.

Medium:
Phenylalanine agar (BBL Microbiology Systems or Difco Laboratories).

Reagent:

Ferric chloride, 10%

Ferric chloride, anhydrous (Eastman Organic Chemicals)	10 g
Distilled water	100 ml

Solution is stable for 1 year at 22°C.

Procedure:

1. Streak the surface of the phenylalanine agar slant with growth on TSIA slant or agar.
2. Incubate the tube at 35°C for 18 to 24 hr.
3. Add five drops of 10% $FeCl_3$ to the agar slant.

Interpretation:

Positive test: A dark green color develops on the agar slant and in the syneresis fluid within a few seconds (Figure 4.2–10, left).
Negative test: No green color develops (Figure 4.2–10, right).

Controls:

Positive: *Providencia rettgeri*
Negative: *Enterobacter aerogenes*

Deoxyribonuclease (DNase) Test[132]

Purpose:
To detect an organism's ability to break down deoxyribonucleic acid (DNA) to smaller units composed of nucleotides. The test is useful for differentiating *Serratia* from *Enterobacter, Staphylococcus aureus* from coagulase-negative staphylococci, and *Branhamella catarrhalis* from *Neisseria* sp.

Principle:
DNase is an extracellular enzyme that breaks down DNA to smaller units composed of nucleotides. The length of these subunits depends on the type of DNase involved in the hydrolytic process. For the differentiation of *Serratia* from *Enterobacter,* the optimal method is one in which toluidine blue O is incorporated in the DNA medium.[132] The true staining of toluidine blue O is blue; however, the nucleotides-dye complex has an absorption spectrum that differs from that of the DNA-dye complex, and metachromatic staining results when DNA has been hydrolyzed. When this occurs, there develops an area of pink around the bacterial growth on the DNA agar. Since toluidine blue O is inhibitory to some strains of gram-positive cocci, the medium of Jeffries et al[88] should be used for speciation of staphylococci.

Specimen:
Growth on TSIA slant or agar.

Medium:
DNase test agar (BBL Microbiology Systems or
Difco Laboratories).
Toluidine blue O (Fisher Scientific Company) 100 mg/l

Procedure:

1. Inoculate half of DNase agar plate in a thin streak with growth on TSIA slant or agar.
2. Incubate plate at 35°C for 18 to 24 hr. Negative test can be reincubated for 24 hr.

Interpretation:

Positive test: A red zone around the streak of bacterial growth indicates hydrolysis of DNA.
Negative test: No red zone around bacterial growth.

Controls:

Positive: *Serratia marcescens*
Negative: *Enterobacter aerogenes*

Acetate Utilization Test[148]

Purpose:
To determine an organism's ability to use acetate as the sole source of carbon. The test is useful for differentiating *Escherichia coli,* especially the nonmotile, anaerogenic biotype, from *Shigella.*

Principle:
Most strains of *E. coli* are able to utilize acetate as the sole carbon source. Their growth on acetate agar, which has the same composition as citrate agar except that citrate is replaced with acetate, produces alkaline product(s) that will cause a change in color of the medium from green to a deep Prussian blue. This test is usually performed only when other biochemical reactions fail to differentiate *E. coli* from *Shigella.*

Specimen:
Growth on TSIA slant or agar.

Medium:
Acetate Differential Agar (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. Inoculate acetate agar slant lightly with bacterial growth on TSIA slant or agar. Too dense an inoculum may cause a false positive result.
2. Incubate tube at 35°C for 18 to 24 hr. Negative test can be reincubated up to 48 hr.

Interpretation:

Positive test: Growth with a deep Prussian blue color.
Negative test: Absence of growth or color change of the medium.

Controls:

Positive: *Escherichia coli*
Negative: *Edwardsiella tarda*

Malonate Utilization Test[46]

Purpose:
To determine an organism's ability to utilize malonate as a carbon source. The test is especially useful in differentiating *Salmonella choleraesuis* subsp. *arizonae* (malonate positive) from subsp. *choleraesuis* (malonate negative). *Klebsiella* and most species of *Enterobacter* also utilize malonate.

Principle:
Malonate medium contains yeast extract and a trace amount of glucose to enhance bacterial growth and especially that of *Salmonella.* Thus, both glucose and malonate serve as carbon sources. Since all Enterobac-

teriaceae ferment glucose, those that cannot utilize malonate acidify the medium, changing the bromthymol blue indicator to yellow. Organisms that utilize malonate produce alkaline products and change the bromthymol blue indicator from green to a deep Prussian blue. As with the citrate and acetate utilization tests, the nature of the alkaline products produced in this test is not exactly known.

Specimen:
Bacterial growth on TSIA slant or agar.

Medium:
Malonate broth (BBL Microbiology Systems or Difco Laboratories).

Procedure:

1. Inoculate malonate broth with bacterial growth on TSIA slant or agar.
2. Incubate tube at 35°C for 18 to 24 hr. Reincubate negative tubes for up to 4 days.

Interpretation:

Positive test: Growth with a deep Prussian blue color. A turquoise color indicates a weakly positive reaction.
Negative test: Growth with the color of the medium remaining green or becoming yellow.

Controls:

Positive: *Enterobacter aerogenes*
Negative: *Edwardsiella tarda*

Carbohydrate Fermentation Test

Purpose:
To determine an organism's ability to ferment a specific carbohydrate with the production of acid with or without the production of gas.

Principle:
An organism's ability to ferment a particular carbohydrate depends on the presence of specific enzyme that can degrade the substrate. The differential fermentation pattern exhibited by various members of the Enterobacteriaceae forms a useful framework for their identification. In the fermentation test, a filter-sterilized solution of a specific carbohydrate is added to a sterile basal medium to give a final carbohydrate concentration of 0.5 to 1.0%. Filtration will prevent possible degradation of those

carbohydrates that are susceptible to autoclaving. Acid production is manifested by the color change of an indicator incorporated in the medium. While gas production can be conveniently detected by the entrapment of air bubbles in an inverted Durham tube. Although the choice of a particular basal medium may be influenced by personal preference, the clarity and ease with which color change of the indicator can be visualized should be the primary consideration.

Specimen:
Bacterial growth on TSIA or agar.

Medium:
Carbohydrate fermentation broth with Andrade's indicator.

Procedure:

1. Inoculate carbohydrate broth with bacterial growth on TSIA slant or agar.
2. Incubate tube at 35°C for 18 to 24 hr. Negative test can be reincubated up to 4 days.

Interpretation:

Positive test: Acidification is indicated by a change in the fermentation broth from light straw color to red. Gas production is seen as air in the inverted Durham tube.
Negative test: Fermentation broth retains light straw color.

Controls:
Any members of the Enterobacteriaceae that give the appropriate reactions can be used as positive and negative controls. The idea is to use the least number of strains to achieve the purpose.

---

b. Serological Examination

The extent to which most clinical laboratories pursue the serological identification of shigellae and salmonellae varies according to expertise, budgetary considerations, and space limitations. It is expected that, by using commercially available antisera, clinical laboratories should be able to identify serologically the common somatic O antigen groups of *Salmonella* and *Shigella*. Complete serological testing, including typing (based on flagellar or capsular antigens), may be obtained by forwarding a pure subculture of the isolate to a reference laboratory after the isolate has been identified biochemically.

Agglutination Tests

Purpose:
To determine the somatic (O) antigenic group of *Salmonella* and *Shigella*. Group-specific agglutination provides a rapid method for presumptive identification of these genera. Because of serological cross-reactivity among some genera of the Enterobacteriaceae, serological reactions require confirmation of identification with biochemical tests.

Principle:
The somatic, or O, antigens are heat-stable, phospholipid-lipopolysaccharide complexes that form granular aggregates in agglutination reactions with homologous antisera.

Specimen:
Fresh bacterial growth on TSIA slant or nutrient agar slant.

Reagent:

Salmonella polyvalent O antiserum; O antisera for groups A, B, C_1 C_2, and D; and Vi antiserum.
Shigella O antisera for groups A, B, C, and D.

These antisera are obtainable from Lederle Diagnostics, Inc. Each batch should be tested for agglutination with suspensions of bacteria belonging to the appropriate serological group.

Procedure:

1. Salmonella

a. With a wax pencil marker, divide the interior surface of a 100-mm petri dish bottom into several rectangular areas.
b. Prepare a dense bacterial suspension in 2 ml of physiological saline with bacterial growth on the TSIA slant or, preferably, a nutrient agar slant.
c. In one rectangle, mix one drop of saline suspension with one drop of phenolized buffered saline to check for roughness of the antigen to prevent a false-positive result.
d. Place a drop of saline suspension in one end of a second rectangle and one drop of polyvalent antiserum in the other end of the rectangle.
e. With a wooden applicator stick, mix the bacterial suspension with the antiserum along an oval track.
f. Tilt the petri dish back and forth for further mixing of antigen and antiserum, up to one minute.

Interpretation:

Positive test: Prompt and complete agglutination as the petri dish is tilted back and forth.
Negative test: No or weak agglutination after one minute of mixing.

If agglutination occurs in polyvalent O serum, test the bacterial suspension with group specific antisera: A, B, C_1, C_2, and D. Though additional group specific antisera are also available, these groups represent those most commonly isolated *Salmonella.*

2. *Salmonella* serotype *typhi*
If biochemical reactions in TSIA (K/A, trace H_2S), LIA (K/K), and urea agar (—) suggest the isolation of *S.* serotype *typhi,* proceed with agglutination as for other *Salmonella,* except use *Salmonella* polyvalent, group D, and Vi antisera. The presence of the capsule-like, heat-labile Vi antigen may mask the somatic O antigen, so that agglutination will occur in Vi antiserum only or in Vi antiserum with a weak reaction in the polyvalent or group D antiserum. If this is the case, proceed as follows:

a. Prepare a heavy suspension of bacteria in 0.5 ml of physiological saline.
b. Heat the suspension in a boiling water bath for 15 min.
c. Cool and repeat the agglutination test in the polyvalent, group D, and Vi antisera.

Interpretation:
Agglutination in polyvalent O and group D antisera but not in Vi antiserum provides a presumptive diagnosis of *S.* serotype *typhi.*

3. *Shigella*

a–c. are the same as for *Salmonella* (*1.*a–c).
d. In four separate rectangular areas test the bacterial suspension in antisera for *Shigella* groups A, B, C, and D.

Interpretation:

Positive test: The most rapid and strongest agglutination in one group antiserum constitutes a positive test.
Negative test: No agglutination or weak cross-agglutination in other groups of antisera.

If no agglutination occurs, the test should be repeated with a heat-killed suspension of bacteria prepared similarly to that of *S.* serotype *typhi.* *Shigella* groups B and D usually do not require heating for agglutination, whereas groups A and C frequently do because of interference by the heat-labile K envelope antigen.

Table 4.2–17. Differential Characteristics of *Vibrio, Aeromonas,* and *Plesiomonas*[a]

Test	*V. cholerae*	*Vibrio,* halophilic	*A. hydrophila*	*P. shigelloides*
Lysine decarboxylase[b]	+	d	−	+
Arginine dihydrolase[b]	−	d	d	+
Ornithine decarboxylase[b]	+	d	−	+
Inositol	−	−	−	+
Mannitol	+	d	+	−
DNase	−	−	+	−
Motility	monotrichous	monotrichous	monotrichous	lophotrichous
Growth in nutrient broth without NaCl	+	−	+	+

[a] Adapted from Brenner et al.,[19] Davis et al.,[33] and Ewing et al.[47]
[b] NaCl added to medium to a final concentration of 1%.
For key to symbols see Table 4.2–11.

C. Vibrionaceae

1. General Considerations

The Vibrionaceae comprise fermentative, facultatively anaerobic gram-negative bacilli that, in contrast to the Enterobacteriaceae, are oxidase positive and, with the exception of *Plesiomonas shigelloides,* are motile by means of a monotrichous polar flagellum. Differential characteristics of the genera *Vibrio, Aeromonas,* and *Plesiomonas* are summarized in Table 4.2–17.

2. Vibrio

a. General Considerations

The genus *Vibrio* includes a number of species, some of which are halophilic and occur in the marine environment and of which the best known is *V. cholerae,* which proliferates in the small intestine and elaborates a heat-labile enterotoxin to produce a severe, watery diarrhea. The mode of acquisition of many species is by consumption of seafood or exposure to sea water.

Long epidemic in portions of South and Southeast Asia, Africa, the

Near East, and Eastern Europe, cholera has occurred sporadically in other countries, including recently in the United States.[28] With the frequency, ease, and speed of travel today, the diagnosis of cholera should be considered in anyone who gives a history of travel to an endemic area and who abruptly develops painless, watery diarrhea. Similar symptomatology occurs with *V. parahaemolyticus* infections related to the ingestion of contaminated seafood.[4] In contrast to *V. cholerae*, extraintestinal infections, principally of the blood stream and soft tissue, have been associated with *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*.[4,12,82] *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, and *V. mimicus* have been recovered in feces or from people with acute gastroenteritis.[12,19,135] *V. damsella*, *V. hollisae* and *V. mimicus* have been recovered rarely from blood and wounds.[112,135]

Noncholera or nonagglutinable vibrios are biochemically indistinguishable from *V. cholerae* but do not agglutinate in *Vibrio* O group 1 antiserum. These have been isolated from feces, the gastrointestinal and biliary tracts, and a variety of other sites, including wounds, blood, and cerebrospinal fluid.[86]

b. Identification

Vibrios are asporogenous, short, straight or curved, comma-shaped, facultatively anaerobic, gram-negative bacilli. They are oxidase and catalase positive and ferment glucose usually without the production of gas. Unlike other vibrios, *V. metschnikovii* is oxidase and nitrate negative; *V. hollisae* and *V. furnissii* are aerogenic. All *Vibrio* sp. are motile with one polar flagellum. Some species require a high concentration of salt for growth. Their differential halophilic characteristics can be readily demonstrated in nutrient broth containing various concentrations of sodium chloride. Tests useful for differentiating various vibrios are summarized in Table 4.2–18.

V. cholerae does not grow well on some gram-negative differential and selective media, such as EMB and SS, but will grow on MacConkey agar, producing colorless colonies after 18 to 24 hr of incubation. For optimal recovery of *V. cholerae*, a selective medium, TCBS agar, should be heavily inoculated with fecal material. The differential characteristics of colonies on TCBS agar depend on the ability of some *Vibrio* species to ferment sucrose. Colonies of *V. cholerae* and its biotype El Tor grow well after 18 to 24 hr as yellow colonies. Colonies of *V. alginolyticus* are also yellow, but tend to be larger, while those of *V. vulnificus* and *V. parahaemolyticus* are blue-green. Enterococci and most enteric bacilli are suppressed on this medium or grow as small, translucent colonies. However, sucrose-fermenting *Proteus* species produce yellow colonies

Table 4.2–18. Differential Characteristics of *Vibrio* species[a]

Test	*V. cholerae*	*V. mimicus*	*V. damsela*	*V. parahaemolyticus*	*V. alginolyticus*	*V. vulnificus*	*V. fluvialis*	*V. metschnikovii*	*V. hollisae*	*V. furnissii*
Indole[b]	+ or −	+ or −	−	+ or −	d	+	−	d	+	− or +
Voges-Proskauer[b]	− or +	−	+	−	+	−	−	+	−	−
Lysine decarboxylase[b]	+	+	d	+	+	+	−	d	−	−
Ornithine decarboxylase[b]	+	+	−	+ or −	d	d	−	−	−	−
Arginine dihydrolase[b]	−	−	+	−	−	−	+	d	−	+
Glucose (gas)	−	−	−	−	−	−	−	−	+	+
Acid produced from										
Lactose	(+) or −	(+) or −	−	−	−	+	−	d	−	−
Sucrose	+	−	−	−	+	− or +	+	+	−	+
Mannitol	+	+	−	+	+	d	+	+	−	+
Maltose	+	+	+	+	+	+	+	+	−	+
Arabinose	−	−	−	+ or −	−	−	+	−	+	+
Nitrate reduction	+	+	+	+	+	+	+	−	+	+
Oxidase	+	+	+	+	+	+	+	−	+	+
Growth in nutrient broth plus NaCl (%)										
0	+	+	−	−	−	−	−	−	−	−
1	+	+	+	+	+	+	+	+	+	+
6	− or (+)	− or (+)	+	+	+	+	+	+	(+)	+
8	−	−	−	+	+	−	+ or −	d	−	+ or −
10	−	−	−	−	+	−	d	d	−	− or (+)
12	−	−	−	−	−	−	−	−	−	−

[a] Adapted from Brenner et al.,[19] Davis et al.[33]
[b] NaCl was added to medium to a final concentration of 1%.
For key to symbols see Table 4.2–11.

that might resemble those of *Vibrio. Pseudomonas* colonies, when present, are blue-green.

V. cholerae produces A/A and K/K reactions without H_2S or gas in TSIA and LIA, respectively. A presumtpive identification can be made if a saline suspension of growth from the TSIA slant agglutinates with *V. cholerae* polyvalent antiserum.[57] Confirmatory biochemical tests are as listed in Table 4.2–18.

V. parahaemolyticus produces K/A and K/K reactions without gas or H_2S in TSIA and LIA, respectively. Confirmatory biochemical reactions are shown in Table 4.2–18.

3. Aeromonas

a. General Considerations

Classification within the genus *Aeromonas* remains unsettled. Members of the genus are found in water, food, sewage, and feces of lower animals. They have been associated with a variety of focal and systemic infections,[156] including bacteremia in patients with leukemia, sarcoma, aplastic anemia, and underlying liver disease; bacteriuria; and infected traumatically acquired wounds. *Aeromonas* has also been associated with diarrhea; however, the mechanism by which it causes intestinal disease remains unclear.

b. Identification

A. hydrophila is an oxidase and catalase positive, gram-negative, asporogenous bacillus that measures 1.0 to 3.5 μm by 0.4 to 1.0 μm, is motile with a single polar flagellum, and ferments glucose, with or without the production of gas. *Aeromonas* grows readily on most conventional media after overnight incubation. On sheep blood agar colonies are usually 1 to 2 mm in diameter, gray, slightly rough, and may be surrounded by a wide zone of complete hemolysis. To the inexperienced microbiologist, their colonial morphology might resemble that of *Pseudomonas aeruginosa.* On EMB agar, colonies are clear and tend to be smaller. Colonies of lactose and sucrose fermenting strains will be pink. *Aeromonas* can be recovered also from CIN agar (Table 3–6), used for the isolation of *Yersinia.* Although *Aeromonas* may give reactions resembling those of *Escherichia coli, Enterobacter,* or *Providencia* in the biochemical tests used for the identification of Enterobacteriaceae, a positive oxidase reaction will readily make the distinction. Additional tests listed in Table 4.2–17 can be performed to confirm the identity of *Aeromonas.*

4. Plesiomonas

a. General Considerations

Formerly known as *Aeromonas shigelloides*, *Plesiomonas shigelloides* has been isolated from feces of humans and lower animals, as well as from blood and cerebrospinal fluid. It has been associated with gastrointestinal illness, but its mechanism of pathogenesis remains unclear.

b. Identification

P. shigelloides grows readily on conventional media. Unlike *A. hydrophila*, colonies do not hemolyze sheep blood. Colonies may be clear or pink on EMB agar depending on whether the strain ferments lactose readily. Unlike other members of the Vibrionaceae, motile strains of *P. shigelloides* possess one to five long polar flagella. Colonies are oxidase and catalase positive. They can be differentiated from *Aeromonas* and *Vibrio* species with tests listed in Table 4.2–17.

D. Campylobacter

1. General Considerations

The genus *Campylobacter* consists of oxidase and catalase positive, gram-negative, microaerophilic, curved, motile bacilli that do not ferment or oxidize carbohydrates, but will reduce nitrate to nitrite. *C. jejuni* and *C. fetus* subsp. *fetus* (formerly *C. fetus* subsp. *intestinalis*) cause disease in humans.

The organism is widely distributed in animals, including poultry and wild birds, and is associated with abortion and sterility in sheep and cattle and with enteritis in other animals. Human campylobacteriosis is associated with direct contact with infected animals or with the consumption of contaminated water and food. Infection can be manifested systemically as bacteremia, meningitis, abortion, endocarditis, pericarditis, and arthritis[13] or as gastroenteritis.[27,138] Gastroenteritis is usually due to *C. jejuni*, while systemic disease is usually due to *C. fetus* subsp. *fetus*. The latter is infrequently isolated in the laboratory.

2. Identification

a. Growth Characteristics

Campylobacter is a fastidious organism that fails to grow on simple nutrient media but will grow on Brucella agar supplemented with hemin,

vitamin K_1, and 5% defibrinated sheep blood. *C jejuni* thrives better at 42°C than at 35°C. Compounds such as ferrous sulfate, sodium metabisulfite, and sodium pyruvate, which enhance the aerotolerance of the organism, and antimicrobials may be added to provide a suitable selective medium for isolating the organism from fecal material. Plates are incubated at 42°C in a microaerophilic atmosphere, which can be achieved either by evacuation and replacement of an anaerobe jar with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 or with a Campy-Pak H_2 and CO_2 generator envelope (BBL Microbiology Systems) in a GasPak jar without the catalyst. Duplicate sets of plates should be inoculated with specimens other than feces for incubation at 35°C and 42°C since *C. fetus* subsp. *fetus* does not grow at the higher temperature.

b. Colonial Morphology

After 24 to 48 hr of incubation, colonies of *C. jejuni* are about 1 mm in diameter, gray, flat, nonhemolytic, and very watery. On further incubation, colonies appear rough and frosted. Colonies of *C. fetus* subsp. *fetus* are usually smaller, raised, opaque, and sometimes yellowish.

c. Microscopic Morphology

Campylobacter cells are small, slender, curved, gram-negative bacilli, interspersed with occasional larger, filamentous, spiral forms, which are more numerous and prominent as the culture ages or after repeated subcultures. Flagellar stain will show a single flagellum at one or both poles.

d. Presumptive Identification

The isolate can be identified presumptively as *Campylobacter* if the colonies are oxidase and catalase positive and an aqueous suspension of the colony shows rod-shaped organisms with a characteristic corkscrew, darting motility.

e. Definitive Tests

Biochemical tests for confirmation and differentiation of *Campylobacter* are shown in Table 4.2–19. Bacterial growth on selective medium is subcultured onto chocolate blood agar prior to biochemical testing. A heavy suspension is used for the hippurate hydrolysis test (p. 145). A light suspension is streaked with a cotton swab over the surface of chocolate blood agar, and a nalidixic acid (30 μg) disk is applied. The plate is incubated at 42°C for 1–2 days. Any zone of inhibition is considered as demonstrating susceptibility to the drug.

Table 4.2-19. Biochemical Characteristics of *Campylobacter*[a]

Test	*C. jejuni*	*C. fetus* subsp. *fetus*
Catalase	+	+
Oxidase	+	+
Growth		
35°C	+	+
42°C	+	−
Hippurate hydrolysis[b]	+	−
Nalidixic acid (30 μg)	resistant	susceptible

[a] *Campylobacter coli,* which resembles *C. jejuni* in biochemical characteristics, does not hydrolyze sodium hippurate.

E. Pseudomonas

1. General Considerations

The genus *Pseudomonas* is composed of oxidase positive, aerobic, gram-negative bacilli that are usually motile by means of polar flagella. Some species reduce nitrate to nitrite or to nitrogen.

Other than *Pseudomonas pseudomallei,* which causes melioidosis, a glanders-like disease endemic in Southeast Asia, clinically significant pseudomonads are largely limited to the species, *Pseudomonas aeruginosa.* This organism, which is widely distributed in soil and water, is a frequent cause of nosocomially acquired bacteremia, bacteriuria, pneumonia, and wound infection, especially in the debilitated, chronically ill, or immunosuppressed host. *P. aeruginosa* is ordinarily susceptible to carboxy- and ureidopenicillins, and to aminoglycosides. Resistance to the aminoglycosides, however, may occur due to the presence of aminoglycoside-modifying enzymes or, more frequently, to reduced permeability of bacterial cell wall to these antimicrobials.

P. cepacia is being isolated with increasing frequency from the sputum of patients with cystic fibrosis and may cause pneumonia and bacteremia in this group. *P. cepacia* has also been associated with a variety of hospital-acquired infections, including bacteremias secondary to contaminated devices or products. Other *Pseudomonas* species are frequently encountered in the hospital environment but seldom cause disease.[154] They may occasionally contaminate equipment or supplies and thereby cause infections. The finding, therefore, of a number of isolates of pseudomonads from similar kinds of specimens should alert one to the possibility of a common-source outbreak of infection.

Table 4.2–20. Differential Characteristics of *Pseudomonas*[a]

Species	O-F			Oxidase	Nitrate reduction	Urease	Motility polar flagella	Pigment			L-Lysine decar-boxylase	L-Arginine dihy-drolase	Comments
	Glucose	Maltose	Xylose					Pyocyanin	Fluorescein	Other			
P. aeruginosa	+	−	+ or −	+	+ or gas	+ or −	+, 1–2	+ or −	+	brown or red	−	+	Growth at 42°C on BHIA[b]
P. fluorescens	+	− or +	+	+	− or +	− or +	+, >2	−	+	− to tan	−	+	No growth at 42°C on BHIA
P. putida	+	− or +	+	+	−	+ or −	+, >2	−	+ or −	−	−	+	No growth at 42°C on BHIA
P. pseudo-mallei	+	+	+ or −	+	+, gas	− or +	+, 1–5	−	−	white to yellow	−	+	Some wrinkled colonies, A/K on TSIA
P. cepacia	+	+	+	d	− or +	− or +	+, 1–6	−	−	− to yellow	+	−	Catalase w+, ONPG +
P. stutzeri	+	+	+	+	+, gas	− or +	+, 1–2	−	−	yellow to −	−	−	Rough, dry, wrinkled colonies
P. maltophilia	+	+	+ or −	−	− or +	−	+, 1–8	−	−	brown	+	−	
P. alcaligenes	−	−	−	+	− or +	− or +	+, 1–2	−	−	−	−	−	Susceptible to polymyxin
P. putrefaciens	+ or (+)	d	−	+	+	−	+, 1–2	−	−	brown to apricot	−	−	H_2S in butt of TSIA
P. paucimobilis	+ or (+)	+ or (+)	+ or (+)	+ or w+	−	−	+, 1	−	−	yellow to −	−	−	
P. diminuta	−	−	−	+	−	−	+, 1	−	−	tan	−	−	+ or − susceptible to polymyxin

[a] Adapted from Hugh and Gilardi,[84] and King.[96]
[b] BHIA = Brain heart infusion agar.
For key to symbols see Table 4.2–11.

2. Identification

Pseudomonas species grow readily at 35°C on conventional media, including gram-negative differential media such as EMB and MacConkey. Carbohydrate utilization patterns, flagellar arrangements, and other characteristics listed in Table 4.2–20 are helpful in the differentiation of *Pseudomonas* sp.

Speciation of the nonfermentative gram-negative bacilli in general is time consuming, expensive, and not always clinically important, although it may have considerable epidemiological significance in certain instances. The laboratory should be able to identify *P. aeruginosa,* colonies of which on blood agar are grayish, dull, flat with an irregular edge, often strongly hemolytic, and usually associated with a pungent odor and a blue-green pigment. The TSIA reaction is K/K, and growth on the slant usually displays a dark pigment with a silvery sheen. The blue-green pigment pyocyanin is best demonstrated on *Pseudomonas* agar P. However, in practice it is rarely necessary to inoculate this medium since typical colonial morphology on blood agar, positive oxidase reaction, and K/K reaction in TSIA provide sufficient criteria for the identification of *P. aeruginosa.* With the increasing availability of kits and devices, it should become possible for more laboratories to speciate reliably and conveniently the commonly isolated nonfermentative bacilli.

a. Definitive Tests

Definitive identification requires performance of a number of tests, including ones for oxidase reactivity (p. 160) and motility (Table 4.2–20). Motility can usually be demonstrated in a semisolid agar medium without ornithine but is often more reliably demonstrated in a hanging drop (p. 173). It is frequently helpful to perform a stain to determine the arrangement and number of flagella (p. 86). Other tests that have already been described include lysine decarboxylase and arginine dihydrolase (p. 196).

Oxidation-Fermentation (O-F) Test[85]

Purpose:
To demonstrate whether bacteria utilize carbohydrates fermentatively or oxidatively.

Principle:
Glucose can be degraded either by a fermentative (anaerobic) or an oxidative (aerobic) process with the formation of pyruvic acid as the key intermediate in both metabolic pathways. In fermentation, pyruvic acid ulti-

mately transfers its electrons to organic compounds with the formation of a large amount of mixed acids, whereas in oxidation, pyruvic acid further enters the Krebs cycle where it ultimately transfers its electrons to oxygen to form water. Citric acid produced in the Krebs cycle is a weak acid compared with the mixed acids produced by fermentation. O-F medium is specifically formulated to detect weak acidity because it contains a lower amount of peptone, the metabolism of which yields less alkaline amine to neutralize any acid that has been produced, and a high carbohydrate content. Its structure as a semisolid medium can better localize any acid formed. Glucose or another carbohydrate is added to a final concentration of 1%, and bromthymol blue is incorporated as an indicator. Two tubes of O-F glucose are inoculated, one of which is overlaid with sterile mineral oil or melted paraffin to create an anaerobic environment and the other of which is exposed to atmospheric oxygen.

Specimen:
Colonies on agar.

Medium:
O-F basal medium (Difco Laboratories) with the appropriate carbohydrate in a final concentration of 1%.

Procedure:

1. Inoculate colonies on agar into two tubes of O-F glucose medium.
2. Overlay one tube ("closed") to a height of 1.5 cm with sterile mineral oil. Leave the other tube open.
3. Incubate at 35°C for 18 to 24 hr.

Interpretation:
The medium remains blue if no acid is formed and becomes yellow when acid is formed. There are three possible reactions:

1. Acidity in closed and open tubes = glucose fermenter.
2. Acidity in open tube only = glucose oxidizer.
3. No acidity in either tube = inactive (glucose not used fermentatively or oxidatively).

Other carbohydrates may be incorporated into O-F medium to facilitate speciation of glucose oxidizers.

Controls:

Glucose oxidizer: *Pseudomonas cepacia*
Glucose nonoxidizer: *Acinetobacter calcoaceticus* var. *lwoffi*

Nitrate Reduction Test

Purpose:
To determine the ability of an organism to reduce nitrate to nitrite, which can be further reduced to nitrogen if the bacterium is a true denitrifier.

Principle:
Nitrate is reduced by a class of enzymes known as nitrate reductases. Members of the Enterobacteriaceae reduce nitrate to nitrite. Other bacteria (e.g., some *Pseudomonas* species) are true denitrifiers and further reduce nitrite to nitrogen. True denitrifiers are obligate aerobes, but are able to thrive in anaerobic environment by using the nitrate ion instead of oxygen as the terminal electron receptor in their energy metabolism. In the nitrate reduction test, bacteria are inoculated into nutrient broth containing 0.1% of potassium nitrate. After overnight incubation, sulfanilic acid and *N,N*-dimethyl-1-naphthylamine are added to the broth culture. If nitrate has been reduced to nitrite, the latter reacts with sulfanilic acid to form a diazonium salt which, in turn, couples with *N,N*-dimethyl-1-naphthylamine to produce a red diazo dye. However, since the reagents detect the presence of nitrite and not nitrogen, a small amount of zinc dust should be added to the nitrate broth when no red color develops after addition of the reagents to check if nitrate is still present in the medium. The development of a red color after the addition of zinc dust indicates nitrate reduction has not occurred, while the absence of a red color denotes complete reduction of nitrate beyond the nitrite stage.

Specimen:
Colonies on agar.

Medium:
Nitrate broth.

Reagent:

Reagent A

Sulfanilic acid (J. T. Baker Chemical Company)	1.6 g
Acetic acid, 5 *M* (J. T. Baker Chemical Company)	200 ml

Reagent B

N,N-dimethyl-1-naphthylamine (Eastman Organic Chemicals)	1 g
Acetic acid, 5 *M*	200 ml

5 *M* acetic acid is prepared by adding 1 part of glacial acetic acid to 2.5 parts of distilled water. Store reagent A and reagent B at 22°C and use within 1 year.

Zinc dust (Eastman Organic Chemicals).

Procedure:

1. Inoculate nitrate broth with colonies on agar.
2. Incubate broth at 35°C for 18 to 24 hr.
3. Add three drops each of reagent A and reagent B.
4. Add a small amount of zinc dust if no red color develops after step 3.

Interpretation:
There are three possible reactions:

1. Red color after addition of reagents A and B = reduction of nitrate to nitrite.
2. No red color after addition of reagents A and B and zinc dust = reduction of nitrate to nitrogen.
3. Red color after addition of reagent A and B and zinc dust = nitrate has not been reduced.

Since some bacteria reduce nitrate slowly, one can use half the broth culture to perform the test after 24 hr of incubation, and reincubate the rest of the broth for another day before adding reagents.

Controls:

Positive: *Pseudomonas cepacia*
Negative: *Acinetobacter calcoaceticus*

Pigment Production Test

Purpose:
To determine the ability of a pseudomonad to produce pyocyanin and/or fluorescein. Most stains of *Pseudomonas aeruginosa* produce both pigments, while *P. fluorescens* and *P. putida* produce only fluorescein.

Principle:
P. aeruginosa can synthesize a variety of water-soluble pigments: pyocyanin, pyoverdin (fluorescein), pyomelanin, and pyorubin. Since the incidence of strains producing the brown-black pyomelanin or red pyorubin

is low, these two pigments have not been used in the identification of *P. aeruginosa;* however, their presence might mask the production of pyocyanin. Pigment production depends on the composition of the medium on which the organism is cultivated and can usually be enhanced by leaving the culture at 22°C after incubation overnight at 35°C. Pyocyanin is a water- and chloroform-soluble, blue-green, nonfluorescent pigment, synthesis of which is enhanced by cultivating the organism on Pseudomonas agar P or on Tech agar. Fluorescein is a yellow, water-soluble, chloroform-insoluble, fluorescent pigment, synthesis of which is enhanced on Pseudomonas agar F or Flo agar, which also suppresses the production of pyocyanin.

Specimen:
Colonies on agar.

Media:
Pseudomonas agar P (Difco Laboratories).
Flo agar (BBL Microbiology Systems).

Procedure:

1. Inoculate P and F agar slants with colonies on agar.
2. Incubate both media at 35°C for 18 to 24 hr; then leave them at 22°C for one or more days if no pigment has developed.

Interpretation:

Pseudomonas agar P: A blue-green pigment diffused into the agar along the slant indicates production of pyocyanin.

Flo agar: A bright yellow pigment diffused into the agar along the slant indicates fluorescein production. In equivocal cases, the agar can be viewed under a Wood's lamp. Pyoverdin fluoresces yellow under long UV wavelength illumination.

Controls:

Positive pyocyanin and fluorescein: *Pseudomonas aeruginosa*
Negative pyocyanin and fluorescein: *Pseudomonas cepacia*

If a pseudomonad cannot be speciated or definitively identified with the tests listed in Table 4.2–20, additional tests can be performed. These include O-F medium with lactose, mannitol, and sucrose; esculin hydrolysis; indole production; and the ability of the organism to grow on citrate, SS, and cetrimide agars. Complete biochemical reactions can be found in Hugh and Gilardi[84] and King.[96]

F. Miscellaneous Gram-Negative Bacilli

1. General Considerations

There are many other gram-negative bacilli that are weakly or nonfermentative, which may be encountered in the clinical laboratory with varying degrees of frequency, and some of which can be clinically and epidemiologically important. *Acinetobacter calcoaceticus,* which is the second most frequently isolated nonfermenter after *Pseudomonas aeruginosa,* has been associated with nosocomially acquired pneumonia, bacteriuria, and wound infection.[65] *Achromobacter xylosoxidans* has been associated with otitis externa and a variety of other infections.[83,123] *Flavobacterium meningosepticum,* considered to be questionably or weakly fermentative, is a cause of neonatal meningitis.[128]

Notable for their role in causing subacute bacterial endocarditis are *Haemophilus aphrophilus* (p. 226), *Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens,* and *Kingella kingae* (formerly group M1). These organisms are small, slow-growing rods that usually require longer than a week's incubation in cultures of blood for their detection. *H. aphrophilus* and *E. corrodens* are also commonly involved in infections in and about the oropharynx.[11,21]

2. Identification

This group of gram-negative bacilli can be broadly classified according to their manner of carbohydrate utilization, oxidase reactivity, and ability to grow on MacConkey agar.[144] Some groups remain unnamed and are designated by letters and Roman numerals. The differential characteristics of some organisms are listed in Tables 4.2–21 and 4.2–22. The commonly isolated *A. calcoaceticus* can be rapidly identified by inoculating an oxidase negative nonfermentative colony from EMB to a TSIA and a 10% lactose agar slant. An alkaline slant and butt in TSIA and acidity on lactose agar slant is a definitive identification of the organism.

G. Gram-Negative Bacilli with Special Growth Requirements

1. General Considerations

Other clinically important gram-negative bacilli include such genera as *Haemophilus, Gardnerella, Brucella, Bordetella, Pasteurella,* and *Francisella,* the taxonomic status of many of which remains uncertain.

Organisms belonging to these genera can be coccoid or extremely

Table 4.2–21. Differential Characteristics of Miscellaneous Nonfermentative Gram-Negative Bacilli[a]

Organism	O-F			Oxidase	Nitrate reduction	Urease	Motility flagella	L-Lysine decarboxylase	L-Arginine dihydrolase	Growth on MacConkey	Comments
	Glucose	Maltose	Xylose								
Acinetobacter calcoaceticus var. *anitratus*	+	d	+	−	−	+ or −	−	−	−	+	∓ fruity odor; 10% lactose +
A. calcoaceticus var. *lwoffi*	−	−	−	−	−	− or +	−	−	−	+	10% lactose −
Alcaligenes sp.	−	−	−	+	+ or −	− or +	+ peritrichous	−	−	+ or −	∓ fruity odor
Achromobacter xylosoxidans	+ or (+)	−	+	+	+ or gas	−	+ peritrichous	−	−	+	
Bordetella bronchiseptica	−	−	−	+	− or +	rapidly +	+ peritrichous	−	−	+	
Flavobacterium meningosepticum[b]	+	+	−	+	−	−	−	−	−	d	∓ slightly yellow; indole, DNase, and ONPG +
Flavobacterium sp.[b]	+ or (+)	+ or (+)	d	+	− or +	− or +	−	−	−	− or (+)	very yellow; ∓ fruity odor; indole +, DNase, and ONPG −
Flavobacterium odoratum	−	−	−	+	−	+	−	−	−	+	yellow; indole, DNase and ONPG −
Moraxella sp.	−	−	−	+	d	− or +	−	−	−	d	

[a] Adapted from Gilardi,[64] Tatum et al.[144]
[b] Weakly fermentative.
For key to symbols see Table 4.2–11.

Table 4.2–22. Differentiation of *Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens,* and *Kingella kingae*[a]

Organism	Oxi-dase	Cata-lase	δ-ALA[b] utiliza-tion	V Factor require-ment	In-dole	Urease	Nitrate reduc-tion	Motil-ity	Fermentation[c]						Mac-Conkey
									Glu-cose	Lac-tose	Su-crose	Mal-tose	Man-nitol	Xy-lose	
H. aphrophilus	−	−	+	−	−	−	+	−	+	+	+	+	−	−	−
H. paraphrophilus	+	−	+	+	−	−	+	−	+	+	+	+	−	−	−
A. actinomyce-temcomitans	d	+	+	−	−	−	+	−	+ or (+)	−	−	+ or (+)	d	d	−
C. hominis	+	−	+	−	+	−	−	−	+ or (+)	−	+ or (+)	+ or (+)	+ or (+)	−	−
E. corrodens	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−
K. kingae	+	−	+	−	−	−	−	−	(+) or +	−	−	+ or (+)	−	−	d

[a] Based on Kilian,[93] King,[96] Weaver et al.[158]
[b] δ-ALA, δ-aminolevulinic acid.
[c] Fermentation broth with Andrade's indicator, supplemented with several drops of horse serum.
For key to symbols see Table 4.2–11.

pleomorphic on microscopic examination. Many species require increased carbon dioxide and moisture, as well as special factors, for growth. They do not grow on gram-negative differential media, such as EMB and MacConkey. Most species are zoonotic, and some are highly pathogenic in humans.

2. Haemophilus

a. General Considerations

The genus *Haemophilus* consists of facultatively anaerobic gram-negative bacilli that ordinarily require either a heat-stable factor, X, or a heat-labile factor, V, or both for growth. Factor X or hemin is derived from lysed erythrocytes, while factor V is one of the two codehydrogenases, NAD or NADP. Growth of some species is enhanced by 5 to 10% CO_2. *Haemophilus* species are obligate parasites that inhabit the mucous membranes of both humans and animals. Based on detailed taxonomic studies, Kilian[93] has indicated that *H. aegyptius, H. parahaemolyticus,* and *H. paraphrohaemolyticus* are biotypes of *H. influenzae* and *H. parainfluen-*

Table 4.2–23. Differential Characteristics of *Haemophilus* Species[a]

		Tests					
Species	Biotype	δ-ALA[b]	Indole	Ornithine decarboxylase	Urease	Oxidase	Catalase
H. influenzae	I	−	+	+	+	+	+
	II	−	+	−	+	+	+
	III	−	−	−	+	+	+
	IV	−	−	+	+	±	±
	V	−	+	+	−	+	+
	VI	−	+	−	−	+	+
H. parainfluenzae	I	+	−	+	−	+	±
	II	+	−	+	+	+	±
	III	+	−	−	+	+	+
H. aphrophilus	−	+	−	−	−	−	−
H. paraphrophilus	−	+	−	−	−	+	−
H. ducreyi	−	−	−	−	−	−	−

[a] Based on Kilian.[94]
[b] δ-ALA = δ-aminolevulinic acid.

zae and should not be assigned species status. Some of the differential characteristics of *Haemophilus* species of human origin are listed in Table 4.2–23. *H. influenzae* is a frequent cause of epiglottitis, otitis media, sinusitis, and conjunctivitis. *H. influenzae* type b, most frequently of biotype I, is the leading cause of acute bacterial meningitis in children between 3 months and 6 years of age. The same sero- and biotype is also the major etiologic agent in acute epiglottitis, bacteremia, osteomyelitis, and pericarditis. Nontypable stains are frequently associated with conjunctivitis, otitis media, and chronic bronchitis. Due to the production of a plasmid-mediated β-lactamase, 5 to 25% of *H. influenzae* are resistant to ampicillin. A permeability defect or alteration in the penicillin-binding proteins is thought to be responsible for ampicillin resistance in those strains that do not have detectable β-lactamase. *H. parainfluenzae* represents normal flora in the throat and oral cavity but may rarely cause endocarditis or meningitis. *H. aphrophilus* is an important cause of subacute bacterial endocarditis and is often associated with infections of the head and neck. *H. ducreyi* causes chancroid, an ulcerative sexually transmitted disease.

b. Identification

(1) Growth Characteristics

Haemophilus species are small, nonmotile, coccoid to coccobacillary, gram-negative bacilli which are usually oxidase and catalase positive, sometimes encapsulated, and require enriched media for growth. The growth of some species is enhanced in 5 to 10% CO_2.

H. influenzae and *H. parainfluenzae* grow readily in 18 to 24 hr on chocolate blood agar in the presence of 5 to 10% CO_2. Colonies of the former are smooth, slightly convex, grayish, and translucent. Colonies of encapsulated strains of *H. influenzae* are more mucoid (1 to 2 mm, diameter) and larger than those of nonencapsulated strains (0.5 to 1 mm, diameter). Colonies of *H. parainfluenzae* are convex, smooth, grayish-white or yellowish in color, and opaque. Colonies of some strains are flat, dry, rough, or wrinkled, while those of others (biotype III) are smooth, butyrous, gray, and translucent.

Organisms in the aphrophilus group are CO_2 dependent but X factor independent. The colonies are small (0.5 to 1 mm), very convex, granular, opaque, and yellowish on chocolate blood agar.

H. ducreyi is difficult to isolate because of its slow rate of growth and overgrowth by contaminants. It may be grown after one or more weeks of incubation in 10% CO_2 on chocolate blood agar containing 5 to 10% fetal calf serum and 3 μg of vancomycin per ml.[71] Colonies are small (0.5 mm, diameter), smooth, convex, translucent, and grayish.

(2) Microscopic Morphology
Organisms are usually small, coccoid to coccobacillary, pleomorphic with occasional to many filamentous forms (Figure 2–1a). Cells of *H. ducreyi* are often arranged in pairs, chains, or parallel rows ("school of fish" arrangement).

(3) Diagnositc Features
The differential characteristics of *Haemophilus* species are listed in Table 4.2–23.

Porphyrin Test[92]

Purpose:
To differentiate *Haemophilus influenzae* and *H. parainfluenzae.*

Principle:
Specific enzymes [porphobilinogen (PBG) synthase, uroporphyrinogen I synthase, and uroporphyrinogen decarboxylase] are required for *Haemophilus* to convert δ-aminolevulinic acid (ALA) to PBG and porphyrin in the biosynthesis of hemin. *H. influenzae* lacks these enzymes and, therefore, requires that hemin be present in media for growth to occur. Hemin is used in the synthesis of iron-containing respiratory enzymes such as cytochrome, cytochrome oxidase, catalase, and peroxidase. In the porphyrin test, a bacterial suspension is incubated with the substrate ALA. Production of porphyrin is indicated by the appearance of orange-red fluorescence under a Wood's lamp illumination (wavelength of 360 nm). By adding Kovacs' reagent to the bacterial-substrate mixture, any PBG that has been produced is extracted into the lower aqueous phase. PBG complexes with *p*-dimethylaminobenzaldehyde to give a red color.

The differentiation of *H. influenzae* and *H. parainfluenzae* is commonly made by subculturing the organism onto a blood-free nutrient agar; applying disks or strips containing V factor alone and X and V factors together onto the agar surface; and determining the organism's growth requirements based on the presence of growth around the XV disk or strip only (*H. influenzae*) or both the XV and V disks or strips (*H. parainfluenzae*). This method is, however, subject to error due to carryover of trace amounts of hemin (X factor) from the primary isolation medium (chocolate blood agar) to the blood-free medium. When this occurs, sufficient amounts of hemin are present to enable an X factor– and V factor–dependent organism (*H. influenzae*) to grow in the vicinity of the V factor disk or strip and mistakenly be identified as a V-dependent species (*H. parainfluenzae*). The porphyrin test obviates this problem and is, therefore, the recommended method for differentiating these species.

Specimen:
Bacterial growth on chocolate blood agar.

Reagents:

1. Phosphate buffer, M/15, pH 6.9

Na_2HPO_4, M/15 (Mallinckrodt, MW = 141.98)	550 ml
KH_2PO_4, M/15 (Mallinckrodt, MW = 136.09)	450 ml

Na_2HPO_4, M/15, and KH_2PO_4, M/15, are prepared by making a 1:15 dilution of the respective 1 *M* solutions in distilled water.

2. $MgSO_4$, 0.8 mM in M/15 phosphate buffer

$MgSO_4$ (Mallinckrodt, MW = 120.39)	96.31 mg
Phosphate buffer, M/15	1 liter

3. δ-Aminolevulinic acid, 2mM in $MgSO_4$ buffer solution

δ-Aminolevulinic acid hydrochloride (Sigma, MW = 167.8)	16.78 mg
$MgSO_4$ buffer solution	50 ml

Filter sterilize ALA solution and distribute 0.5 ml amounts in sterile polystyrene tubes. Store at −10°C, and use within 6 months.

Procedure:

1. Inoculate ALA heavily with strain to be tested and incubate at 35°C in 5 to 10% CO_2 for 18 to 24 hr.
2. Examine the tube under Wood's lamp with long wavelength illumination.
3. Add one drop of Kovacs' reagent, agitate to mix completely.

Interpretation:

Positive test: An orange-red fluorescence under Wood's lamp indicates conversion of ALA to porphyrin; development of a red color in the lower aqueous phase of bacterial substrate mixture after addition of Kovacs' reagent indicates production of porphobilinogen. *Haemophilus* giving a positive test does not require hemin for growth.

Negative test: No red fluorescence and no red color after addition of Kovacs' reagent.

Alternatively, the ALA test can be adapted as a spot test by transferring one colony onto a piece of filter paper fitted in a petri dish and overlaying it with 2 to 3 drops of ALA reagent. After incubating the covered petri dish at 35°C in 5 to 10% CO_2 for 2 hr, the bacterial deposit is examined

under a Wood's lamp. An orange-red fluorescence indicates the conversion of ALA to hemin. Negative tests are reincubated for another 2 hr.

Controls:

Positive: *Haemophilus parainfluenzae*
Negative: *Haemophilus influenzae*

---

3. Gardnerella

a. General Considerations

The genus *Gardnerella* consists of one species, *G. vaginalis,* which has undergone several taxonomic changes. It was previously classified as a member of the genus *Haemophilus* or the genus *Corynebacterium.* It is frequently isolated from the human genitourinary tract and appears to have a worldwide distribution. Its role in nonspecific vaginosis remains controversial[109,122] (p. 59). It is rarely a cause of bacteriuria[1] and bacteremia.[152]

b. Identification

(1) Growth Characteristics

G. vaginalis is a fastidious facultatively anaerobic, non-spore-forming, non-motile gram-negative to gram-variable bacillus. It does not require X or V factor for growth; however, growth is enhanced on media containing proteose or similar peptones such as those in Casman or Columbia colistin–nalidixic acid (CNA) agar. The HBT medium, which is a bilayer of CNA with amphotericin B, Tween 80, proteose peptone, and human blood, provides a satisfactory selective and differential medium for the isolation of *G. vaginalis.*[147] Tween 80 and the bilayer composition enhance the β-hemolysis produced by *G. vaginalis,* and thus allows easier detection of the organism amidst growth of other vaginal flora. After 48 hr of incubation in 5 to 10% CO_2, colonies of *G. vaginalis* on HBT medium are β-hemolytic, approximately 0.5 mm in diameter, round, grayish-white, smooth and transparent. They increase in size with further incubation, but their viability decreases rapidly. Colonies do not produce hemolysis in medium containing sheep blood.

(2) Microscopic Morphology

Microscopically, *G. vaginalis* are small, pleomorphic bacilli or coccobacilli, approximately 0.5 μm by 1.5–2.5 μm, that are gram-negative or gram-variable. Frequently, they appear in a diphtheroidal arrangement.

(3) Diagnostic Features

G. vaginalis can be identified presumptively on the basis of the growth of small β-hemolytic, catalase- and oxidase-negative colonies on HBT medium that exhibit compatible microscopic morphology. Confirmatory tests include hippurate hydrolysis (p. 145) and α- and β-glucosidase tests.

α- and β-Glucosidase Tests[124]

Purpose:

To identify *Gardnerella vaginalis* that possesses α- but not β-glucosidase.

Principle:

In the α- or β-glucosidase test, a chromogenic substrate is hydrolyzed by the corresponding bacterial enzyme to yield a yellow product, nitrophenol.

Specimen:

Colonies subcultured onto CNA agar from HBT medium.

Reagents:

1. *Phosphate buffer M/15, pH 8.0*

Na_2HPO_4, M/15	950 ml
KH_2PO_4, M/15	50 ml

2. 0.1% 4-Nitrophenyl-α-D-glucopyranoside and 0.1% 4-nitrophenyl-β-D-glucopyranoside (Sigma Chemical Company, respectively), in phosphate buffer, M/15

Filter sterilize substrates and distribute 0.5 ml amount in 12 × 75 mm sterile polystyrene tubes. Store at 4°C, and use within 6 months.

Procedure:

1. Inoculate substrates with bacteria on CNA agar to produce a heavy suspension.
2. Incubate at 35°C in 5 to 10% CO_2 for 18 hr.

Interpretation:

Positive test: Yellow color production.
Negative test: No color change.

Controls:

α-Glucosidase positive; β-glucosidase negative: *Gardnerella vaginalis*
α-Glucosidase negative; β-glucosidase positive: *Enterobacter cloacae*

---

4. Brucella

a. General Considerations

Brucella is a parasite of animals, such as sheep, cattle, goats, swine, and dogs. Infection in humans is primarily an occupational disease, though brucellosis can be caused by ingestion of unpasteurized dairy products and, rarely, exposure to infected pet dogs. Incidence of the disease has greatly decreased in the last two decades as the result of eradication programs. Clinically significant species are *B. abortus, B. suis, B. melitensis,* and *B. canis.*

b. Identification

(1) Growth Characteristics

Brucella organisms are fastidious, aerobic, small, non-spore-forming, nonmotile, gram-negative bacilli that are oxidase and catalase positive, reduce nitrate to nitrite, and may produce H_2S or urease. *B. suis* characteristically hydrolyzes urease very rapidly. They do not thrive under strictly anaerobic conditions, but will grow better with incubation in CO_2. Increased CO_2 is required for primary isolation of some biotypes of *B. abortus.* Primary isolation of *Brucella* from clinical specimens may require prolonged incubation; hence blood cultures for *Brucella* should be held for 3 weeks before being discarded as negative. After 3 to 4 days of incubation with CO_2, *Brucella* colonies on sheep blood agar are pinpoint, nonhemolytic, smooth, convex, gray, and translucent. They will increase to 1 to 1.5 mm in diameter with further incubation.

(2) Microscopic Morphology

The organisms are pale staining, coccoid, gram-negative bacilli about 0.4 by 1.5 μm, usually occurring singly or in pairs. The gram-negative characteristic can be enhanced by prolonged counterstaining with safranin for 1 to 3 min. Direct staining of tissue will show the intracellular location of the organisms.

(3) Diagnostic Features

Differential inhibition by thionine and basic fuchsin as a means of speciating *Brucella* is not always clear-cut. Biotypes within species can demonstrate various growth patterns in the presence of different concentrations of the dyes. Moreover, the actual concentrations of the dyes that will adequately differentiate between the species depend on the composition of the basal medium, the bacteriostatic action, and the purity of each dye lot. Definitive species identification can be done by agglutination in monospecific sera. The presence of *Brucella* in clinical material or in cultures may be detected by fluorescent antibody staining (p. 267).

5. Bordetella

a. General Considerations

The genus *Bordetella* consists of aerobic, coccoid, gram-negative bacilli. *Bordetella pertussis,* strictly a human parasite, is the causative agent of pertussis, a contagious childhood disease that is characterized by an inspiratory whoop following a long paroxysm of coughing. *B. parapertussis,* which is also a human parasite, can produce a mild form of the disease.[120] *B. bronchiseptica* causes enzootic infections in various wild and domestic animal species and may be isolated from the upper respiratory tract of humans who have been in close contact with animals; however, it is not clearly established that this species causes a pertussis-like syndrome.

b. Identification

(1) Growth Characteristics

B. pertussis, which is the most fastidious of the *Bordetella* species was first isolated in 1906 by Bordet and Gengou. Its isolation requires the presence of blood and the incorporation of charcoal or ion-exchange resin in the medium for the removal of inhibitory substances, such as fatty acids, peroxides, sulfur, and manganese. Since penicillin inhibits some strains of *B. pertussis,* cephalexin is incorporated in the primary isolation medium to inhibit organisms that are indigenous to the nasopharynx.[140]

After 3 to 4 days of incubation at 35°C with added CO_2, *B. pertussis* appears on charcoal agar as small ($\leq$1 mm diameter), smooth, and raised colonies with a pearl-like luster. It will grow on subculture on blood agar after 4 to 5 days of incubation as small (1 mm, diameter) gray, slightly raised colonies. *B. parapertussis* appears on blood agar after 48 hr as larger colonies which may impart a slight brownish coloration to the medium. The production of this brown soluble pigment is more prominent in a blood-free peptone agar. Colonies of *B. bronchiseptica* resemble those of *B. parapertussis,* but usually become larger with prolonged incubation. Typically, *B. bronchiseptica* grows readily on EMB or MacConkey agar. All three species of *Bordetella* are hemolytic; however, the degree of hemolysis varies according to the type and concentration of erythrocytes used and the length of incubation.

(2) Microscopic Morphology

Bordetella species are non-spore-forming, encapsulated, bipolar, pale-staining, small gram-negative bacilli which can occur singly, in pairs, or in clumps. Staining characteristics can be enhanced by prolonged counterstaining with safranin. In young cultures, they are coccoid and usually 1.0 by 0.3 to 0.5 μm; however, in older cultures, they can be long and filamentous.

Table 4.2–24. Differential Characterisitcs within the Genus *Bordetella*[a]

Test	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
Growth			
Sheep blood agar	+, 4–5 d on subculture	+, 2 d	+, 1 d
EMB agar	−	+	+
Peptone agar	−	+	+
Hemolysin	+	+	+
Pigment production, peptone agar	−	+, brown	−
Urease	−	+	+, 4 h
Oxidase	+	−	+
Catalase	+, 70%	+	+
Nitrate reduction	−	−	+
Motility	−	−	+, peritrichous
Citrate utilization	−	+	+

[a] Adapted from Pittman.[125]

(3) Diagnostic Features

B. bronchiseptica is the only motile species and is also a strong urease producer, usually giving a positive reaction on Christensen's urea agar within 4 hr of incubation. Otherwise, *Bordetella* is rather inert biochemically. Some of the differential characteristics within the genus are shown in Table 4.2–24. *B. pertussis* can be identified in nasopharyngeal specimens and in cultures by fluorescent antibody staining (p. 267).

6. Pasteurella

a. General Considerations

The genus *Pasteurella* consists of facultatively anaerobic gram-negative bacilli of uncertain taxonomic status. Although it has been associated with bacteremia, bacteriuria, spontaneous peritonitis, chronic bronchitis, osteomyelitis, and septic arthritis, *P. multocida* is usually isolated from infected wounds resulting from dog bites and cat bites or scratches. *Pasteurella* sp., new species 1, also produces infection in humans; the mode of acquisition is again through contact with animals, mainly by dog or cat bites. *P. pneumotropica, P. haemolytica, P. ureae,* and *P. aerogenes* may be found in healthy or diseased animals but rarely in humans.

Table 4.2–25. Differentiation within the Genus *Pasteurella*[a]

Test	*P. multocida*	*P.* sp., new species 1	*P. pneumotropica*	*P. haemolytica*	*P. ureae*	*P. aerogenes*
Hemolysis on blood agar	−	−	−	+ or −, β	−	−
Growth on MacConkey	−	−	d	d	−	+
Oxidase	+	+	+	+	+	+
Indole	+	+	+	−	−	−
Motility	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+
Urea, Christensen's	−	d	+	−	+	+
TSIA	A/A	A/A	A/A	A/A	A/A	A/A
Acid produced from						
Xylose	+ or −	−	+ or (+)	d	−	+ or −
Mannitol	+ or −	−	−	d	+	−
Lactose	−	−	d	d	−	d
Maltose	−	+	+	+ or (+)	+	+
Sucrose	+	+	+	+	+	+

[a] Adapted from Weaver et al.[158]
For key to symbols see Table 4.2–11.

b. Identification

Pasteurella species grow readily on enriched media, such as sheep blood, but with the exception of *P. haemolytica,* not on EMB or MacConkey agar. Growth is enhanced by increased CO_2. Organisms are nonmotile, non-spore-forming, and oxidase and catalase positive. They reduce nitrate to nitrite and are fermentative in their carbohydrate metabolism, giving an A/A reaction in TSIA. Differential characteristics within the genus are shown in Table 4.2–25.

After 18 to 24 hr of incubation, colonies of virulent strains of *P. multocida* are smooth or mucoid, gray-white, translucent, nonhemolytic, about 1.0 to 2 mm in diameter, with a characteristic musty odor. Rough or avirulent strains produce colonies that are difficult to suspend in saline. With prolonged incubation, colonies may impart a brown coloration to blood agar. Microscopically, *Pasteurella* species are small, coccoid, gram-negative bacilli with characteristic bipolar staining (Figure 2–1o). The bacterial cells may be encapsulated.

7. Francisella

a. General Considerations

Francisella tularensis is the causative agent of tularemia, a disease which is primarily transmitted to humans by blood-sucking arthropods, by direct or indirect inoculation of the skin as the result of handling diseased animals, by the ingestion of infected meat or water contaminated with excreta of infected animals, or by contact with or inhalation of infected aerosols. Thus, the disease can manifest itself as ulceroglandular, with the primary lesion in the skin accompanied by regional lymphadenopathy; pneumonic; or occuloglandular, exhibited by conjunctivitis.

b. Identification

F. tularensis is a fastidious, obligately aerobic, gram-negative bacillus, the isolation of which requires an enriched medium with added cystine or cysteine. Glucose-cysteine agar with thiamine or cystine heart agar with defibrinated rabbit blood or packed human red cells are both suitable isolation media. Penicillin, polymyxin B, and cycloheximide can be incorporated in the medium for culturing contaminated material. The surface of the agar plate should be free from moisture which is detrimental to the recovery of the organism.[42] Buffered charcoal-yeast extract agar (the *Legionella* medium) with 2.5% glucose has been found to support the growth of *F. tularensis* from infected guinea pig tissue better than glucose-cysteine-blood agar.[107]

On glucose-cysteine-blood agar, pinpoint colonies may appear after 18 to 24 hr of incubation at 35°C. They will become discernible as smooth, gray, droplike colonies by 48 hr and will increase to 3 to 4 mm in diameter with further incubation. A greening will appear in the medium around individual colonies or around an area of confluent growth. Depending on the concentration of organisms in the clinical specimen, up to 3 weeks may be necessary for colonies to develop on agar media.

Microscopically, *F. tularensis* is a bipolar, pale-staining, coccoid, gram-negative bacillus, approximately 0.3 to 0.5 by 0.2 μm in size. Organisms in colonies older than 24 hr may become very pleomorphic. Biochemical tests are usually not used for identification, since diagnosis of tularemia can be readily made by fluorescent antibody staining (Section 4.3).

8. Legionella

a. General Considerations

Legionella is the only genus in the family of Legionellaceae. It consists of aerobic, fastidious, motile, gram-negative rod-shaped or filamentous bacilli that require cysteine and some form of iron compound for growth. Since the first description of *L. pneumophila* in 1979, numerous other species have been described.[16] Ecologic studies indicate that *Legionella* are aquatic bacteria that can maintain themselves over a wide range of temperatures. They are widely distributed in lakes, ponds, streams, rivers, and often in thermally polluted water. Potable water has been associated with nosocomial outbreaks of infection. Epidemiological studies indicate that legionellosis is acquired from environmental sources, with the mode of dissemination of infection very likely through an infectious aerosol.[22]

The most frequently isolated species, *Legionella pneumophila,* is frequently associated with pneumonia accompanied by malaise, myalgia, mild headache, high unremitting fever, relative bradycardia, nonproductive cough, hypophosphatemia, and elevated liver enzymes.[99] It has also been associated with self-limited illness characterized by fever, myalgia, malaise, and headache with few or no respiratory manifestations and no pneumonia. The latter syndrome, originally designated as "Pontiac fever," is a milder form of the disease and has not been associated with any fatality.[26,66] The signs, symptoms, and radiographic findings of Legionnaires' disease are sufficiently nonspecific that the disease is often confused with pneumonias caused by viruses, mycoplasmas, rickettsiae, and other bacteria. The disease is being recognized with increasing frequency both worldwide and in the United States. Although sporadic cases have been reported, most of the cases occur in clusters. Immunosuppressed patients, such as renal transplant recipients, are more prone to legionellosis than

are other healthy individuals. *L. micdadei*, sometimes referred to as the Pittsburgh pneumonia agent, is associated with a severe pneumonia that has a high mortality rate among patients with nosocomially acquired disease.[129] *L. micdadei* pneumonia is clinically indistinguishable from that caused by other *Legionella* species. An outbreak of Pontiac fever was associated with the newly described species, *L. feeleii*.[75] A small number of cases of pneumonia have also been associated with other species. Extrapulmonary infections causes by *Legionella* are rare and usually occur as complications of pulmonary infections.

b. Identification

(1) Growth Characteristics

L. pneumophila is a fastidious, motile, gram-negative bacillus that is genetically unrelated to other known pathogens. It grows optimally at 35°C, with lighter growth at 30°C, but will not grow at 25°C or at 42°C. It grows best aerobically in an atmosphere containing 2.5% CO_2, as is generated in a candle extinction jar. No growth has been observed anaerobically. It has a narrow pH range for growth, the optimal pH being 6.9. L-Cysteine hydrochloride and soluble ferric pyrophosphate or other iron compounds are essential growth factors. The nonselective charcoal yeast extract (CYE) agar or buffered CYE agar with α-ketoglutarate (BCYEα) is suitable for cultivation of *Legionella* from normally sterile material, e.g., pleural fluid or lung tissue.[59,121] BCYEα medium provides greater sensitivity than the original Feeley-Goram (F-G) agar as a primary isolation medium. *Legionella* also grows better on BCYEα medium that is incubated at 35°C in air than in air plus 2.5% CO_2. Acid treatment of specimens (p. 107), followed by cultivation on BCYEα medium containing polymyxin B, anisomycin, and either cefamandole or vancomycin facilitates the recovery of *Legionella* from contaminated sources such as sputum, bronchial washings, or postmortem lung tissue.[24,37] Cefamandole provides greater selectivity than vancomycin; however, it can be inhibitory to some of the *Legionella* species, especially *L. micdadei*.

On BCYEα agar, growth of *L. pneumophila* is apparent in confluent areas after about 3 to 5 days of incubation at 35°C. By 5 to 7 days, well-isolated colonies may reach 3 to 4 mm in diameter. The colonies are gray, glistening, convex, and circular with an entire edge. In the area of confluent growth, the colonies appear slightly moist. When examined under a dissecting microscope, young colonies appear greenish, and have a characteristic cut-glass texture which is rapidly lost with additional incubation.[157] When viewed under long wavelength ultraviolet light (366 nm), colonies on charcoal-free BYEα medium display dull yellow fluorescence. Other species exhibit a dull yellow or blue-white fluorescence. Most species produce a diffusible melanin-like brown pigment on tyrosine-containing agar or F-G agar.

Table 4.2–26. Differential Characteristics within the Genus *Legionella*[a]

Test	*L. pneumophila*	*L. bozemanii*	*L. micdadei*	*L. dumoffii*	*L. gormanii*	*L. longbeachae*	*L. jordanis*	*L. oakridgensis*	*L. wadsworthii*	*L. feeleii*
Growth										
Blood agar	–	–	–	–	–	–	–	–	–	–
CYE or BCYEα	+	+	+	+	+	+	+	+	+	+
Browning of YE agar with tyrosine[b]	+	+	–	+	+	+	+	+	–	w+
Autofluorescence	dull yellow	blue white	dull yellow	blue white	blue white	dull yellow	dull yellow	dull yellow	dull yellow	–
Catalase	+	+	+/–	+	+	+	+	+	+	+
Oxidase	+ or +/–	+/–	+	–	–	+	+	–	–	–
Motility	+	+	+	+	+	+	+	–	+	+
Hippurate hydrolysis[c]	+	–	–	–	–	–	–	–	–	+/–
β-lactamase[d]	+	+/–	–	+	+	+/–	+	w+	+	–

[a] Adapted from Brenner;[16] +/–, weakly or not always positive; w+, weakly positive.
[b] YE agar = BCYE agar without charcoal; tyrosine is added to a final concentration of 0.045%.
[c] Incubate for 18–24 hr.
[d] With chromogenic cephalosporin test.

(2) Microscopic Appearance

In Gram-stained smears, *Legionella* is a pale-staining, pleomorphic, gram-negative bacillus about 0.5 to 0.7 μm by 2 to 3 μm. However, extremely long, filamenous, and frequently curved forms greater than 20 μm can be seen. Flagella are curved or straight with a polar or lateral arrangement. The staining characteristics can be enhanced by prolonging the application of safranin for 3 to 5 min. On primary isolation and in early passages on artificial media, many cells are punctuated with vacuoles that can cause slight swelling of the cells.[157] The vacuoles can be demonstrated as blue–black or blue-gray inclusions by the Sudan black B stain, and are thought to contain poly-β-hydroxybutyrate.

(3) Diagnostic Features

Legionellae are catalase and gelatinase positive, and most species produce oxidase and β-lactamase. Otherwise, they are rather inert biochemically. They are urease negative, do not reduce nitrate, and neither ferment nor oxidize carbohydrates.[157] Some of the differential characteristics within the genus are shown in Table 4.2–26. It will not be possible to identify most of the described species solely on the basis of these tests.[16] However, testing a very small number of each species, except for *L. pneumophila* and *L. micdadei,* Vickers and colleagues demonstrated that it might be possible to differentiate members of the Legionellaceae on the basis of pigment production and fluorescence on media supplemented with various aromatic substrates.[153] Presently, the identification of *Legionella* is confirmed with an immunofluorescent antibody stain (p. 262). However, with the emergence of new species and new serogroups within each species, it becomes increasingly difficult to maintain sufficient reagents for fluorescent-antibody testing.

References

1. Abercrombie, G. F., Allen, J., and Maskell, R. *Corynebacterium vaginale* urinary tract infection in a man. *Lancet* 1:766, 1978.
2. Auckenthaler, R., Hermans, P. E., and Washington, J. A., II. Group G streptococcal bacteremia: Clinical study and review of the literature. *Rev. Infect. Dis.* 5:196, 1983.
3. Austrian, R. *Streptococcus pneumoniae* (pneumococcus). *In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology.* Washington, D.C., American Society for Microbiology, 1974, p. 109.
4. Barker, W. H., Weaver, R. E., Morris, G. K., and Martin, W. T. Epidemiology of *Vibrio parahaemolyticus* infection in humans. *In* Schlessinger, D. (ed.), *Microbiology—1974.* Washington, D.C., American Society for Microbiology, 1975, p. 257.
5. Barksdale, L. *Corynebacterium diphtheriae* and its relatives. *Bacteriol. Rev.* 34:378, 1970.

6. Barry, A. L., Bernsohn, K. L., Adams, A. P., and Thrupp, L. D. Improved 18-hour methyl red test. *Appl. Microbiol.* **20**:866, 1970.
7. Bayer, A. S., Chow, A. W., Betts, D., and Guze, L. B. Lactobacillemia—Report of nine cases. Important clinical and therapeutic considerations. *Am. J. Med.* **64**:808, 1978.
8. Benjaminson, M. A., De Guzman, B. C., and Weil, A. J. Voges-Proskauer test: Expeditious techniques for routine use. *J. Bacteriol.* **87**:234, 1964.
9. Bercovier, H., and Mollaret, H. H. Genus XIV. *Yersinia. In* Krieg, N. R., and Holt, J. G. (eds.), *Bergey's Manual of Systematic Bacteriology,* Volume 1. Baltimore, Maryland, Williams and Wilkins, 1984, p. 498.
10. Bickham, S. T., and Jones, W. L. Problems in the use of in vitro toxigenicity test for *Corynebacterium diphtheriae. Am. J. Clin. Pathol.* **57**:244, 1971.
11. Bieger, R. C., Brewer, N. S., and Washington, J. A., II. *Haemophilus aphrophilus:* A microbiologic and clinical review and report of 42 cases. *Medicine* **57**:345, 1978.
12. Blake, P. A., Weaver, R. E., and Hollis, D. G. Diseases of humans (other than cholera) caused by vibrios. *Ann. Rev. Microbiol.* **34**:341, 1980.
13. Bokkenheuser, V. *Vibrio fetus* infection in man. I. Ten new cases and some epidemiologic observations. *Am. J. Epidemiol.* **91**:400, 1970.
14. Breese, B. B., and Hall, C. B. *Beta Hemolytic Streptococcal Diseases.* Boston, Houghton Mifflin, 1978.
15. Brenner, J. D. Family 1. *Enterobacteriaceae. In* Krieg, N. R., and Holt, J. G. (eds.), *Bergey's Manual of Systematic Bacteriology,* Volume 1. Baltimore, Maryland, Williams and Wilkins, 1984, p. 408.
16. Brenner, D. J. Classification of Legionellae. *In* Thornsberry, C., Balows, A., Feeley, J. C., and Jakubowski, W. (eds.), *Legionella,* Proceedings of the 2nd International Symposium. Washington, D.C., American Society for Microbiology, 1984, p. 55.
17. Brenner, D. J., Davis, B. R., Steigerwalt, A. G., Riddle, C. F., McWhorter, A. C., Allen, S. D., Farmer, J. J., III, Saitoh, Y., and Fanning, G. R. Atypical biogroups of *Escherichia coli* found in clinical specimens and description of *Escherichia hermanii* sp. nov. *J. Clin. Microbiol.* **15**:703, 1982.
18. Brenner, D. J., Farmer, J. J., III, Hickman, F. W., Asbury, M. A., and Steigerwalt, A. G. *Taxonomic and Nomenclature Changes in Enterobacteriaceae.* U.S. Department of Health, Education, and Welfare. Public Health Service. Center for Disease Control. Atlanta, Ga., 1977 (HEW Publication No. [CDC] 78-8356).
19. Brenner, D. J., Hickmann-Brenner, F. W., Lee, J. V., Steigerwalt, A. G., Fanning, G. R., Hollis, D. G., Farmer III, J. J., Weaver, R. E., Joseph, S. W., and Seidler, R. J. *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human feces and the environment. *J. Clin. Microbiol.* **18**:816, 1983.
20. Brenner, D. J., McWhorter, A. C., Leete Knutson, J. K., Steigerwalt, A. G. *Escherichia vulneris:* A new species of *Enterobacteriaceae* associated with human wounds. *J. Clin. Microbiol.* **15**:1133, 1982.
21. Brooks, G. F., O'Donoghue, J. M., Rissing, J. P., Soapes, K., and Smith, J. W. *Eikenella corrodens,* a recently recognized pathogen: Infections in medical-surgical patients and in association with methylphenidate abuse. *Medicine* **53**:325, 1974.

22. Broome, C. V. Current issues in epidemiology of Legionellosis, 1983. *In* Thornsberry, C., Balows, A., Feeley, J. C., and Jakubowski, W. (eds.), *Legionella,* Proceedings of the 2nd International Symposium. Washington, D.C., American Society for Microbiology, 1984, p. 205.
23. Brown, S. D., and Washington, J. A., II. Evaluation of the Repliscan system for identification of *Enterobacteriaceae. J. Clin. Microbiol.* **8**:695, 1978.
24. Bueseching, W. J., Brust, R. A., and Ayers, L. W. Enhanced primary isolation of *Legionella pneumophila* from clinical specimens by low-pH treatment. *J. Clin. Microbiol.* **17**:1153, 1983.
25. Carlson, J. R., and McCarthy, L. R. Modified coagglutination procedure for the serological grouping of streptococci. *J. Clin. Microbiol.* **9**:329, 1979.
26. Center for Disease Control. Epidemic of obscure illness—Pontiac, Michigan. *Morbid. Mortal. Weekly Rep.* **17**:315, 1968.
27. Center for Disease Control. Waterborne *Campylobacter* gastroenteritis—Vermont. *Morbid. Mortal. Weekly Rep.* **27**:207, 1978.
28. Center for Disease Control. Follow-up on *Vibrio cholerae* serotype Inaba infection—Louisiana. *Morbid. Mortal. Weekly Rep.* **27**:388, 1978.
29. Christensen, W. B. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* **52**:461, 1946.
30. Christie, R., Atkins, N. E., Munch-Petersen, E. A note on a lytic phenomenon shown by group B streptococci. *Aust. J. Exp. Biol.* **22**:197, 1944.
31. Cooksey, R. C., Thompson, F. S., and Facklam, R. R. Physiological characterization of nutritionally variant streptococci. *J. Clin. Microbiol.* **10**:326, 1979.
32. Darling, C. L. Standardization and evaluation of the CAMP reaction for the prompt presumptive identification of *Streptococcus agalactiae* (Lancefield group B) in clinical material. *J. Clin. Microbiol.* **1**:171, 1975.
33. Davis, B. R., Fanning, G. R., Madden, J. M., Steigerwalt, A. G., Bradford, H. B., Jr., Smith, H. L., Jr., and Brenner, D. J. Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species *Vibrio mimicus. J. Clin. Microbiol.* **14**:631, 1981.
34. Davis, J., Tirrell, J., and Martin, W. J. Comparison of three coagulase test methods with two commercial rapid tests for identification of *Staphylococcus aureus.* 83rd Annual Meeting of the American Society for Microbiology, 1983, Abstract C378, p. 374.
35. DiSalvo, J. W. Desoxyribonuclease and coagulase activity of micrococci. *Med. Tech. Bull.* **9**:191, 1958.
36. Duthie, E. S. Evidence for two forms of staphylococcal coagulase. *J. Gen. Microbiol.* **10**:427, 1954.
37. Edelstein, P. H. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* **14**:289, 1981.
38. Ederer, G. M., and Clark, M.: Motility-indole-ornithine medium. *Appl. Microbiol.* **20**:849, 1970.
39. Edwards, M. S., Baker, C. J., Wagner, C. L., Taber, L. H., Barrett, F. F. An etiologic shift in infantile osteomyelitis: the emergence of the group B *Streptococcus. J. Pediatr.* **93**:378, 1978.
40. Edwards, P. R., and Ewing, W. H.: *Identification of Enterobacteriaceae,* 3rd ed. Minneapolis, Burgess, 1972.

41. Edwards, P. R., and Fife, M. A. Lysine-iron agar in the detection of *Arizona* cultures. *Appl. Microbiol.* **9**:478, 1961.
42. Eigelsbach, H. T. *Francisella tularensis. In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 316.
43. Elek, S. D. The plate virulence test for diphtheria. *J. Clin. Pathol.* **2**:250, 1949.
44. Evans, J. B. Anaerobic fermentation of mannitol by staphylococci. *J. Bacteriol.* **54**:266, 1947.
45. Evans, J. B., and Kerbaugh, M. A. Recognition of *Aerococcus viridans* by the clinical microbiologist. *Hlth. Lab. Sci.* **7**:76, 1970.
46. Ewing, W. H., Davis, B. R., and Reavis, R. W. Phenylalanine and malonate media and their use in enteric bacteriology. *Publ. Hlth Lab.* **15**:153, 1957.
47. Ewing, W. H., and Hugh, R. *Aeromonas. In* Lennette, E. H., Spaulding, E. H., Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 230.
48. Facklam, R. R. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. *Appl. Microbiol.* **23**:1131, 1972.
49. Facklam, R. R. Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Appl. Microbiol.* **26**:138, 1973.
50. Facklam, R. R. A review of the microbiological techniques for the isolation and identification of streptococci. *Crit. Rev. Clin. Lab. Sci.* **6**:287, 1976.
51. Facklam, R. R. Physiological differentiation of viridans streptococci. *J. Clin. Microbiol.* **5**:184, 1977.
52. Facklam, R. R., and Moody, M. D. Presumptive identification of group D streptococci: The bile-esculin test. *Appl. Microbiol.* **20**:245, 1970.
53. Facklam, R. R., Padula, J. F., Thacker, L. G., Wortham, E. C., and Sconyers, B. J. Presumptive identification of group A, B, and D streptococci. *Appl. Microbiol.* **27**:107, 1974.
54. Farmer, J. J., III. *Enterobacteriaceae* and *Vibrionaceae:* Update. Centers for Disease Control, Atlanta, Georgia. Teleconference Series—1982, Number 7—Microbiology Update.
55. Farmer, J. J., III, McWhorter, A. C., Brenner, D. J., and Morris, G. K. The *Salmonella-Arizona* group of *Enterobacteriaceae:* nomenclature, classification, and reporting. *Clin. Microbiol. Newsletter.* **6**:63, 1984.
56. Farmer, J. J., III, Sheth, N. K., Hudzinski, J. A., Rose, H. D., and Asbury, M. F. Bacteremia due to *Cedecea neteri* sp. nov. *J. Clin. Microbiol.* **16**:775, 1982.
57. Feeley, J. C., and Balows, A. *Vibrio. In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 238.
58. Feeley, J. C., and Brachman, P. S. *Bacillus anthracis. In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 143.
59. Feeley, J. C., Gibson, R. J., Gorman, G. W., Langford, N. C., Rasheed, J. K., Mackel, D. C., and Baine, W. B. Charcoal-yeast extract agar: Primary isolation medium for *Legionella pneumophila. J. Clin. Microbiol.* **10**:437, 1979.
60. Ferragut, C., Izard, D., Gavani, F., Kersters, K., De Ley, J., and LeClerc,

H. *Klebsiella trevisanii:* A new species from water and soil. *Int. J. Syst. Bacteriol.* **33**:133, 1983.

61. Flandrois, J. P., and Carret, G. Study of the staphylococcal affinity to fibrinogen by passive hemagglutination: a tool for the *Staphylococcus aureus* identification. *Zbl. Bakt. Hyg. I. Abt. Orig.* A251, 171, 1981.
62. Flynn, J., and Waitkins, S. A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae. J. Clin. Pathol.* **25**:525, 1972.
63. Frobisher, M., Jr. Cystine-tellurite agar for *C. diphtheriae. J. Infect. Dis.* **60**:99, 1937.
64. Gilardi, G. L. Identification of miscellaneous glucose nonfermenting gram-negative bacilli. *In* Gilardi, G. L. (ed.), *Glucose Nonfermenting Gram-Negative Bacteria in Clinical Microbiology.* West Palm Beach, Florida, CRC Press, 1978, p. 45.
65. Glew, R. H., Moellering, R. C., and Kunz, L. J. Infections with *Acinetobacter calcoacetius* (*Herellea vaginicola*): Clinical and laboratory studies. *Medicine* **56**:79, 1977.
66. Glick, T. H., Gregg, M. B., Berman, B., Mallison, G., Rhodes, W. W., Jr., and Kassanoff, I. Pontiac fever—An epidemic of unknown etiology in a health department. I. Clinical and epidemiologic aspects. *Am. J. Epidemiol.* **107**:149, 1978.
67. Grimont, P. A. D., Farmer, J. J., III, Grimont, F., Asbury, M. A., Brenner, D. J., and Deval, C. *Ewingella americana* gen. nov. sp. nov., a new *Enterobacteriaceae* isolated from clinical specimens. *Ann. Microbiol.* (Inst. Pasteur) **134A**:39, 1983.
68. Grimont, P. A. D., and Grimont, F. Genus VII *Serratia. In* Krieg, N. R., and Holt, J. G. (eds.), *Bergey's Manual of Systematic Bacteriology,* Volume 1. Baltimore, Maryland, Williams and Wilkins, 1984, p. 477.
69. Grimont, P. A. D., Grimont, F., Richard, C., and Sakazaki, R. *Edwardsiella hoshinae,* a new species of *Enterobacteriaceae. Current Microbiol.* **4**:347, 1980.
70. Hable, K. A., Washington, J. A., II, and Herrmann, E. C., Jr. Bacterial and viral throat flora: Comparison of findings in children with acute upper respiratory tract disease and in healthy controls during winter. *Clin. Pediatr.* **10**:199, 1971.
71. Hammond, G. W., Lian, C. J., Wiet, J. C., and Ronald, A. R. Comparison of specimen collection and laboratory techniques for isolation of *Haemophilus ducreyi. J. Clin. Microbiol.* **7**:39, 1978.
72. Hawn, C. V. Z., and Beebe, E. Rapid method for demonstrating bile solubility of *Diplococcus pneumoniae. J. Bacteriol.* **90**:549, 1965.
73. Hermann, G. J. Diphtheria. *In* Bodily, H. L., Updyke, E. L., and Mason, J. O. (eds.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections,* 5th ed. New York, American Public Health Association, 1970, p. 97.
74. Hermann, G. J., Moore, M. S., and Parsons, E. I. A substitute for serum in the diphtheria in vitro toxigenicity test. *Am J. Clin. Pathol.* **29**:181, 1958.
75. Herwaldt, L. A., Gorman, G. W., McGrath, T., Toma, S., Brake, B., Hightower, A. W., Jones, J., Reingold, A. L., Boxer, P. A., Tang, P. W., Moss, C. W., Wilkinson, H., Brenner, D. J., Steigerwalt, A. G., and Broome, C. V. A new *Legionella* species, *Legionella feeleii* species nova, causes Pontiac fever in an automobile plant. *Ann. Intern. Med.* **100**:333, 1984.
76. Hickman, F. W., Steigerwalt, A. G., Farmer, J. J., III, and Brenner, D. J.

Identification of *Proteus penneri* sp. nov., formerly known as *Proteus vulgaris* indole negative or as *Proteus vulgaris* biogroup I. *J. Clin. Microbiol.* **15**:1097, 1982.

77. Hickman-Brenner, F. W., Farmer, J. J., III, Steigerwalt, A. G., and Brenner, D. J. *Providencia rustigianii:* A new species in the family *Enterobacteriaceae* formerly known as *Providencia alcalifaciens* biogroup 3. *J. Clin. Microbiol.* **17**:1057, 1983.
78. Hickman-Brenner, F. W., Huntley-Carter, G. P., Saitoh, Y., Steigerwalt, A. G., Farmer, J. J., III, and Brenner, D. J. *Moellerella wisconsensis,* a new genus and species of *Enterobacteriaceae* found in human stool specimens. *J. Clin. Microbiol.* **19**:460, 1984.
79. Highsmith, A. K., Feeley, J. C., and Morris, G. K. *Yersinia enterocolitica:* A review of the bacterium and recommended laboratory methodology. *Hlth. Lab. Sci.* **14**:253, 1977.
80. Hollis, D. G., Hickman, F. W., Fanning, G. R., Farmer, J. J., III, Weaver, R. E., and Brenner, D. J. *Tatumella ptyseos* gen. nov., sp. nov., a member of the family *Enterobacteriaceae* found in clinical specimens. *J. Clin. Microbiol.* **14**:79, 1981.
81. Hollis, D. G., and Weaver, R. E. *Gram-Positive Organisms: A Guide to Identification.* Centers for Disease Control, Atlanta, Georgia, 1980, p. 1.
82. Hollis, D. G., Weaver, R. E., Baker, C. N., and Thornsberry, C. Halophilic *Vibrio* species isolated from blood cultures. *J. Clin. Microbiol.* **3**:425, 1976.
83. Holmes, B., Snell, J. J. S., and Lapage, S. P. Strains of *Achromobacter xylosoxidans* from clinical material. *J. Clin. Pathol.* **30**:595, 1977.
84. Hugh, R., and Gilardi, G. L. *Pseudomonas. In* Lennette, E. H., Balows, A., Hausler, W. J., Jr., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 289.
85. Hugh, R., and Leifson, E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacilli. *J. Bacteriol.* **66**:24, 1953.
86. Hughes, J. M., Hollis, D. G., Gangarosa, E. J., and Weaver, R. E. Noncholera vibrio infections in the United States. Clinical, epidemiologic, and laboratory features. *Ann. Intern. Med.* **88**:602, 1978.
87. Hwang, M., and Ederer, G. M. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. *J. Clin. Microbiol.* **1**:114, 1975.
88. Jeffries, C. D., Holtman, D. F., and Guse, D. G. Rapid method for determining the activity of microorganisms on nucleic acids. *J. Bacteriol.* **73**:590, 1957.
89. Johnson, W. D., and Kaye, D. Serious infections caused by diphtheroids. *Ann. N.Y. Acad. Sci.* **174**:568, 1970.
90. Jordan, P. A., Iravani, A., Richard, G. A., and Baer, H. Urinary tract infection caused by *Staphylococcus saprophyticus. J. Infect. Dis.* **142**:510, 1980.
91. Kellogg, D. S., Jr., Holmes, K. K., and Hill, G. A. Laboratory diagnosis of gonorrhoea. *In* Marcus, S., and Sherris, J. C. (coordinating eds.), *Cumitech 4.* Washington, D. C., American Society for Microbiology, 1976.
92. Kilian, M. A rapid method for the differentiation of *Haemophilus* strains. The porphyrin test. *Acta Pathol. Microbiol. Scand.* **82(B)**:835, 1974.

93. Kilian, M. A taxonomic study of the genus *Haemophilus,* with the proposal of a new species. *J. Gen. Microbiol.* **93**:9, 1976.
94. Kilian, M. *Haemophilus. In* Lennette, E. H., Balows, A., Hausler, W. J., Jr., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 330.
95. Kilian, M., and Bülow, P. Rapid diagnosis of *Enterobacteriaceae. Acta. Pathol. Microbiol. Scand.* **84B**:245, 1976.
96. King, E. O. *The Identification of Unusual Pathogenic Gram-Negative Bacteria.* (Preliminary revision by R. E. Weaver, H. W. Tatum, and D. G. Hollis). Atlanta, National Communicable Disease Center. Department of Health, Education and Welfare, September 1972.
97. King, E. O., Frobisher, M., Jr., and Parsons, E. I. The in vitro test for virulence of *Corynebacterium diphtheriae. Am. J. Publ. Hlth.* **39**:1314, 1949.
98. King, E. O., Frobisher, M., Jr., and Parsons, E. I. Further studies on the in vitro test for virulence of *Corynebacterium diphtheriae. Am. J. Publ. Hlth.* **40**:704, 1950.
99. Kirby, B. D., Snyder, K. M., Meyer, R. D., and Finegold, S. M. Legionnaires' Disease: Clinical features of 24 cases. *Ann. Intern. Med.* **89**:297, 1978.
100. Kloos, W. E. Coagulase-negative staphylococci. *Clin. Microbiol. Newsletter* **4**:75, 1982.
101. Kloos, W. E., and K. H. Schleifer. Simplified scheme for routine identification of human *Staphylococcus* species. *J. Clin. Microbiol.* **1**:82, 1975.
102. Kloos, W. E., and Wolfshohl, J. F. Identification of *Staphylococcus* species with the API STAPH-IDENT System. *J. Clin. Microbiol.* **16**:509, 1982.
103. Le Minor, L., Veron, M., and Popoff, M. Proposition pour une nomenclature des Salmonella. *Ann. Microbiol.* **133B**:245, 1982.
104. Levchak, M. E., and Ellner, P. D. Identification of group D streptococci by SeroSTAT. *J. Clin. Microbiol.* **15**:58, 1982.
105. Lowrance, B. L., Reich, P., Traub, W. H. Evaluation of two spot-indole reagents. *Appl. Microbiol.* **17**:923, 1969.
106. Lund, E. Laboratory diagnosis of pneumococcus. *Bull. World Hlth. Organ.* **23**:5, 1960.
107. MacLeod, K. B., Patton, C. M., Klein, G. C., and Feeley, J. C. Growth of *Francisella tularensis* on media for the cultivation of *Legionella pneumophila. In* Thornsberry, C., Balows, A., Feeley, J. C., and Jakubowski, W., (eds.), *Legionella,* Proceedings of the 2nd International Symposium. Washington, D.C., American Society for Microbiology, 1984, p. 20.
108. Maxted, W. R. The use of bacitracin for identifying group A hemolytic streptococci. *J. Clin. Pathol.* **6**:224, 1953.
109. McCormack, W. M., Hayes, C. H., Rosner, B., Evrard, J. R., Crockett, V. A., Alpert, S., and Zinner, S. Vaginal colonization with *Corynebacterium vaginale* (*Haemophilus vaginalis*). *J. Infect. Dis.* **136**:740, 1977.
110. Meers, P. D. A case of classical diphteria, and other infections due to *Corynebacterium ulcerans. J. Infect.* **1**:139, 1979.
111. Moeller, V. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta Pathol. Microbiol. Scand.* **36**:158, 1955.
112. Morris, J. G., Miller, H. G., Wilson R., Tackett, C. O., Hollis, D. G., Hickman, F. W., Weaver, R. E., and Blake, P. A. Illness caused by *Vibrio damsela* and *Vibrio hollisae. Lancet* **i**:1294, 1982.

113. Mossel, D. A. A. Attempt in classification of catalase-positive staphylococci and micrococci. *J. Bacteriol.* **84**:1140, 1962.
114. Mulder, R. R., Yu, V. L., Zuravleff, J. J. Pneumonia due to Pittsburgh pneumonia agent: new clinical perspective with a review of the literature. *Medicine* **62**:120, 1983.
115. Murray, P. R., Wold, A. D., Hall, M. M., and Washington, J. A., II. Bacitracin inhibition for presumptive identification of group A β-hemolytic streptococci. Comparison of primary and purified plate testing. *J. Pediatr.* **89**:576, 1976.
116. Murray, P. R., Wold, A. D., and Washington, J. A., II. Recovery of group A and non-group A β-hemolytic streptococci from throat swab specimens. *Mayo Clin. Proc.* **52**:81, 1977.
117. Myerowitz, R. L., Pasculle, A. W., Dowling, J. N., Pazin, G. J., Puerzer, M., Rinaldo, C. R., Yu, R. B., and Hakala, T. R. Opportunistic lung infection due to Pittsburgh pneumonia agent. *N. Engl. J. Med.* **301**:953, 1979.
118. Nestle, M., and Roberts, W. K. An extracellular nuclease from *Serratia marcescens.* II. Specificity of the enzyme. *J. Biol. Chem.* **244**:5219, 1969.
119. Niléhn, B. Studies on *Yersinia enterocolitica:* With special reference to bacterial diagnosis and occurrence in human acute enteric disease. *Acta Pathol. Microbiol. Scand. Suppl.* **206**:5, 1969.
120. Olson, L. C. Pertussis. *Medicine* **54**:427, 1975.
121. Pasculle, A. W., Feeley, J. C., Gibson, R. J., Cordes, L. G., Myerowitz, R. L., Patton, C. M., Gorman, G. W., Carmack, C. L., Ezzell, J. W., and Dowling, J. N. Pittsburgh pneumonia agent: direct isolation from human lung tissue. *J. Infect. Dis.* **141**:727, 1980.
122. Pheifer, T. A., Forsyth, P. S., Durfee, M. A., Pollock, H. M., and Holmes, K. K. Nonspecific vaginitis. Role of *Haemophilus vaginalis* and treatment with metronidazole. *N. Engl. J. Med.* **298**:1429, 1978.
123. Pien, F. D., and Higa, H. Y. *Achromobacter xylosoxidans* isolates in Hawaii. *J. Clin. Microbiol.* **7**:239, 1978.
124. Piot, P., Van Dyck, E., Totten, P. A., and Holmes, K. K. Identification of *Gardnerella (Haemophilus) vaginalis. J. Clin. Microbiol.* **15**:19, 1982.
125. Pittman, M. Genus *Bordetella. In* Krieg, N. R., and Holt, J. G. (eds.), *Bergey's Manual of Systematic Bacteriology,* Volume 1. Baltimore, Maryland, Williams and Wilkins, 1974, p. 388.
126. Ragsdale, A. R., and Sanford, J. P. Interfering effect of incubation in carbon dioxide on the identification of pneumococci by optochin discs. *Appl. Microbiol.* **22**:854, 1971.
127. Riley, P. S., Hollis, D. G., Utter, G. B., Weaver, R. E., and Baker, C. N. Characterization and identification of 95 diphtheroid (group JK) cultures isolated from clinical specimens. *J. Clin. Microbiol.* **9**:418, 1979.
128. Rios, I., Klimek, J. J., Maderazo, E., and Quintiliani, R. *Flavobacterium meningosepticum* meningitis: Report of selected aspects. *Antimicrob. Agents Chemother.* **14**:444, 1978.
129. Rubin, J. E., Wing, E. J., and Yee, R. L. An ongoing outbreak of *Legionella micdadei. In* Thornsberry, C., Balows, A., Feeley, J. C., and Jakubowski, W. (eds.), *Legionella,* Proceedings of the 2nd International Symposium. Washington, D.C., American Society for Microbiology, 1984, p. 227.
130. Schiemann, D. A. *Yersinia enterocolitica:* observation on some growth char-

acteristics and response to selective agents. *Can. J. Microbiol.* **26**:1232, 1980.
131. Schliefer, K. H., and Kloos, W. E. A simple test system for the separation of staphylococci from micrococci. *J. Clin. Microbiol.* **1**:337, 1975.
132. Schreier, J. B. Modification of deoxyribonuclease test medium for rapid identification of *Serratia marcescens. Am. J. Clin. Pathol.* **51**:711, 1969.
133. Schulman, J. A., and Nahmias, A. J. Staphylococcal infections: clinical aspects. *In* Cohen, J. O. (ed.), *The Staphylococci.* New York, Wiley, 1972, p. 457.
134. Sewell, C. M., Clarridge, J. E., Young, E. J., and Guthrie, R. K. Clinical significance of coagulase-negative staphylococci. *J. Clin. Microbiol.* **16**:236, 1982.
135. Shandera, W. X., Johnston, J. M., Davis, B. R., and Blake, P. A. Disease from infection with *Vibrio mimicus,* a newly recognized *Vibrio* species. Clinical characteristics and epidemiology. *Ann. Int. Med.* **99**:169, 1983.
136. Shattock, P. M. F. Enterococci. *In* Ayres, J. C., Kraft, A. A., Snyder, H. E., and Walker, H. W. (eds.), *Chemical and Biological Hazards in Food.* Ames, Iowa, Iowa State University Press, 1962, p. 303.
137. Shtibel, R., and Toma, S. *Neisseria gonorrhoeae:* Evaluation of some methods used for carbohydrate utilization. *Can. J. Microbiol.* **24**:177, 1978.
138. Skirrow, M. B. *Campylobacter* enteritis: A "new" disease. *Brit. Med. J.* **2**:9, 1977.
139. Stuart, C. A., Van Stratum, E., and Rustigian, R. Further studies on urease production by *Proteus* and related organisms. *J. Bacteriol.* **49**:437, 1945.
140. Sutcliffe, E. M., and Abbott, J. D. Selective medium for the isolation of *Bordetella pertussis* and *parapertussis. J. Clin. Pathol.* **25**:732, 1972.
141. Swan, A. The use of bile-aesculin medium and of Maxted's technique of Lancefield grouping in the identification of enterococci (group D streptococci). *J. Clin. Pathol.* **7**:160, 1954.
142. Tager, M., and Drummond, M. C. Current views on the mechanisms of coagulase action in blood clotting. *In* Yotis, W. W. (ed.), *Recent Advances in Staphylococcal Research. Ann. N.Y. Acad. Sci.* **236**:277, 1974.
143. Taranta, A., and Moody, M. D. Diagnosis of streptococcal pharyngitis and rheumatic fever. *Pediat. Clin. N. Am.* **18**:125, 1971.
144. Tatum, H. W., Ewing, W. H., and Weaver, R. E. Miscellaneous gram-negative bacteria. *In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology. 1974, p. 271.
145. Terranova, W., and Blake, P. A. *Bacillus cereus* food poisoning. *N. Engl. J. Med.* **298**:143, 1978.
146. Tomasz, A. The mechanism of the irreversible antimicrobial effects of penicillins: How the beta-lactam antibiotics kill and lyse bacteria. *Annu. Rev. Microbiol.* **33**:113, 1979.
147. Totten, P. A., Amsel, R., Hale, J., Piot, P., and Holmes, K. K. Selective differential human blood bilayer media for isolation of *Gardnerella* (*Haemophilus*) *vaginalis. J. Clin. Microbiol.* **15**:141, 1982.
148. Trabulsi, L. R., and Ewing, W. H. *Sodium acetate medium for the differentiation of Shigella and Escherichia cultures.* Communicable Disease Center, Public Health Services, U.S. Department of Health, Education and Welfare, Atlanta, Georgia, 1962, p. 137.
149. Turnbull, P. C. B., Jorgensen, K., Kramer, J. M., Gilbert, R. J., and Parry,

J. M. Severe clinical conditions associated with *Bacillus cereus* and the apparent involvement of exotoxins. *J. Clin. Pathol.* 32:289, 1979.

150. Turnbull, P. C. B., Kramer, J. M., Jorgensen, K., Gilbert, R. J., and Melling, J. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. *Am. J. Clin. Nutr.* 32:219, 1979.
151. Uhl, J. R., Anhalt, J. P. Identification and susceptibility testing using an IBM-PC. 84th Annual Meeting of the American Society for Microbiology, 1984, Abstract C221, p. 273.
152. Venkataramani, T. K., and Rathbun, H. K. *Corynebacterium vaginale* (*Hemophilus vaginalis*) bacteremia: Clinical study of 29 cases. *Johns Hopkins Med. J.* 139:93, 1976.
153. Vickers, R. M., and Yu, V. L. Clinical laboratory differentiation of *Legionellaceae* family members with pigment production and fluorescence on media supplemented with aromatic substrates. *J. Clin. Microbiol.* 19:583, 1984.
154. Von Graevenitz, A., and Weinstein, J. Pathogenic significance of *Pseudomonas fluorescens* and *Pseudomonas putida*. *Yale J. Biol. Med.* 44:265, 1971.
155. Ward, J. I., Koornhof, H., Jacobs, M., and Applebaum, P. Clinical and epidemiological features of multiply resistant pneumococci, South Africa. *In* Schlessinger, D. (ed.), *Microbiology—1979*. Washington, D.C., American Society for Microbiology, 1979, p. 283.
156. Washington, J. A., II. *Aeromonas hydrophila* in clinical bacteriologic specimens. *Ann. Intern. Med.* 76:611, 1972.
157. Weaver, R. E., and Feeley, J. C. Cultural and staining characteristics. *In* Jones, G. L., and Hébert, G. A. (eds.), *"Legionnaires'": The Disease, the Bacterium and Methodology*, rev. ed. Center for Disease Control, Atlanta, Georgia, 1979, p. 18.
158. Weaver, R. E., and Hollis, D. Gram-negative fermentative bacteria and *Francisella tularensis*. *In* Lennette, E. H., Balows, A., Hausler, W. J., Jr., and Truant, J. P. (eds.), *Manual of Clinical Microbiology*, 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 242.
159. Wilkowske, C. J., Facklam, R. R., Washington, J. A., II, and Geraci, J. E. Antibiotic synergism: enhanced susceptibility of group D streptococci to certain antibiotic combinations. *Antimicrob. Agents Chemother.* 195, 1970–1971.
160. Wood, R. L. Erysipelothrix infection. *In* Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (eds.), *Diseases Transmitted from Animals to Man*, 6th ed. Springfield, Illinois, Charles C Thomas, 1975, p. 271.
161. Zarzour, J. Y., and Belle, E. A. Evaluation of three test procedures for identification of *Staphylococcus aureus* from clinical sources. *J. Clin. Microbiol.* 7:133, 1978.
162. Zinnemann, K., and Turner, G. C. The taxonomic position of *"Haemophilus vaginalis"* (*Corynebacterium vaginale*). *J. Pathol.* 85:213, 1963.

Section 4.3

Fluorescent Antibody Procedures and Counter-immunoelectrophoresis

John P. Anhalt, Ph.D., M.D.

I. Overview

The fluorescent antibody (FA) technique and counterimmunoelectrophoresis (CIE) are rapid methods for the accurate detection and identification of certain medically significant bacteria. These techniques are not only rapid when used to identify bacteria isolated in culture, but they also can be used for direct examination of clinical specimens or mixed cultures and are more specific and sensitive than the Gram stain. Moreover, because they can be used on nonviable organisms, they may allow a bacteriological diagnosis when cultures fail. They should not, however, be viewed as substitutes for cultures or for complete serological identification. Culture methods must be used sometimes to confirm the presumptive results obtained by the FA technique or CIE and to provide viable isolates for susceptibility testing or other studies.

In many instances, CIE and the FA technique can be used interchangeably. For example, *Haemophilus influenzae* can be detected rapidly in cerebrospinal fluid by either method. Although good parallel comparisons of both methods on clinical specimens are not available, comparisons between separate studies[4,30] show that the techniques have approximately equal sensitivity, that is, either method will detect approximately 90% of meningitis cases due to *Haemophilus influenzae.* In other instances, one technique is definitely preferred over the other. For example, the FA technique is preferred for detection of group A streptococci in clinical specimens, whereas CIE is preferred for detection of soluble bacterial antigens in urine from patients with meningitis. These examples illustrate the basic difference between CIE and the FA technique with regard to

specimen requirements. CIE requires that the antigen be in solution so that it can migrate through an agar gel by electrophoresis. In contrast, the FA technique requires that the antigen be fixed to a particle that will adhere to a microscope slide during the staining procedure.

Cherry and Moody[7] have written an excellent review of FA techniques, and a complete manual of laboratory procedures has been published by the Centers for Disease Control (*Fluorescent Antibody Techniques and Bacterial Applications*, cat. no. PB 297–961/PTX; NTIS, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, VA 22161. Price: $6.00[18]). The FA procedures described in this section have been adopted, for the most part, directly from this manual, from other publications of the Centers for Disease Control, or from technical publications by Difco Laboratories.[11] Clinical applications of CIE for detection of microbial antigens have been reviewed by Anhalt et al.[1] and Rytel.[26]

Particle agglutination tests can be used as alternatives to CIE for detection of soluble bacterial antigens or identification of bacterial isolates. The tests in common laboratory use for diagnosis of meningitis are based on staphylococcal coagglutination[8,12,27] or latex agglutination.[8,12,27,29] Comparisons of these tests with CIE are difficult to evaluate, because each study uses different methods and tests different patient populations. These differences can have significant effects on the apparent sensitivity of each method. Considering only detection of *Haemophilus influenzae* in unconcentrated CSF, Feigin et al.[15] found that CIE had a sensitivity of 89% in a study of 64 cases; whereas, Thirumoorthi and Dajani[27] found a sensitivity of only 75% with 65 cases. The reported sensitivity of latex agglutination ranges from 100%[29] to 77%.[12] In studies, staphylococcal coagglutination has been found to be more sensitive than latex agglutination[8] or much less sensitive than CIE.[12] In the latter study,[12] sensitivity of CIE was 92%, latex agglutination was 77%, and staphylococcal coagglutination was 57%. This is one of the few studies in which CIE was more sensitive than latex agglutination. In most studies, latex agglutination has been found to be as sensitive as and usually more sensitive than CIE. The relative sensitivity reported for staphylococcal coagglutination is more variable. The apparent effect of antiserum on sensitivity of CIE is markedly evident in Surveys of the College of American Pathologists. In one Survey (specimen ID-4, 1983), sensitivity for detection of *H. influenzae*, type b antigen at a concentration of 0.1 μg/ml varied from 64% to 100%, depending on antiserum source. This concentration of antigen is higher than that found in many cases of meningitis.[24] Specificity is even more difficult to evaluate for the different methods. Reported incidence of false positives by each method is very low. Early studies seemed to show a false-positive rate for latex agglutination of 1 to 2%,[9,30] but some more recent studies[29] do not show such a high incidence. Many of the false-positive reactions of agglutination tests can be avoided by heating the samples or by treatment with dithiothreitol.[29] Nevertheless, in Surveys

of the College of American Pathologists, the incidence of false-positive results has been higher among participants using agglutination tests (see Survey critiques for specimens ID-7, 1983 and ID-3, 1982). Other methods of antigen detection include radioimmunoassay (RIA)[9] and enzyme-linked immunosorbent assay (ELISA).[10] These methods have not attained widespread use.

A recent application of latex agglutination has been to detection of group A streptococci directly from throat swabs. Directigen™ (Hynson, Westcott and Dunning) and Culturette™ 10-minute Group A Strep ID (Marion Scientific) are available for this purpose. The proper place for the methods in laboratory diagnosis of group A streptococcal infection has yet to be established.

II. Fluorescent Antibody Techniques

A. General Considerations

1. Definitions

FA procedures can be categorized as *direct,* in which the antibody against the antigen of interest is labeled with a fluorescent molecule, or *indirect,* in which the antibody against the antigen of interest is not labeled. In the indirect method, fluorescent staining is achieved by using a second antibody that is labeled with a fluorescent molecule and has specificity directed against the first antibody, not the microbial antigen of interest. Direct FA procedures are simple and require only a single incubation step with the antibody; however, direct procedures require that each antibody of intended use be labeled. Indirect procedures require two incubation steps; however, in contrast to direct procedures, a single labeled antibody (for example, a labeled antibody produced in sheep against rabbit immunoglobulin) can be used for any number of tests. Indirect procedures are also useful for measuring the titer of antibody in a clinical specimen. Both categories of procedures use the same techniques for fluorescence microscopy.

2. Fluorescence Microscope

The laboratory manual from the Centers for Disease Control[18] describes in detail the principles and operation of a fluorescence microscope and the various filter combinations required for tests using fluorescein-labeled and rhodamine-labeled antibodies. All the following procedures use fluorescein-labeled antibodies, and the stained specimens are examined using

incident light. An Osram HBO-200 mercury arc lamp is used for illumination, and a Schott BG-38 filter (Fish-Schurman Corp.) is used to absorb the red portion of the spectrum from the source. A 3-mm Schott BG-12 filter is used as the primary filter, which provides light for excitation with wavelengths from 350 to 450 nm. A K530 filter (Leitz) is used as the barrier (secondary) filter, which blocks reflected excitation light and allows only light from fluorescence of the labeled antibody to pass through to the eyepiece.

3. Examination of Stained Specimens

Carefully clean the microscope objective with lens paper. Place the slide into the mechanical stage and place a drop of low fluorescence immersion oil on the cover glass. Use a low-viscosity, nondrying oil, such as Cargille type A (Fisher Scientific Co., cat. no. 12–368), which does not contain polychlorinated biphenyl (PCB) compounds. Use the high-power, oil immersion objective to focus and read the slide. After reading, remove the slide leaving the objective in place. Add oil to the next slide and position it under the objective. Very little manipulation of the fine adjustment should be required to focus on subsequent slides.

4. Interpretative Criteria

An FA stain must be interpreted only after the morphology of the organism, the type of staining, the intensity and consistency of fluorescence, and the appearance of the background are considered. The intensity and type of staining can be graded as described in Table 4.3–1. The distinctions are made easier by concurrent testing of known positive (4+) and negative (1+ or 2+) control organisms. Examples of FA stains are shown in Figure 4.3–1 (see color insert in the center of the book).

Table 4.3–1. Grading Criteria for FA Stains[18]

Grade	Definition
4+	Brilliant yellow-green fluorescence with clear-cut cell outline; sharply defined nonstaining cell center.
3+	Less brilliant fluorescence with clear-cut cell outline; sharply defined nonstaining cell center.
2+	Less brilliant but still definite fluorescence with less clear-cut cell outline; nonstaining cell center poorly defined with indistinct margins.
1+	Very subdued fluorescence of cell outline; cell center indistinguishable from cell outline.

5. Titration of FA Conjugates

Before a new lot of conjugate is used, it should be titrated and tested in parallel with a conjugate of known reactivity.

Conjugate Titration

Purpose:
To determine the optimal working dilution of an FA conjugate.

Principle:
Dilutions of a conjugate of unknown reactivity are prepared and tested concurrently with a conjugate of known activity against positive and negative control organisms or serum specimens. The dilution of the conjugate of unknown reactivity giving results comparable to those obtained with the conjugate of known reactivity is the optimal working dilution.

Reagents:
The appropriate reagents are used as described below for specific tests. Use sterile PBS, pH 7.5, for dilutions.

Procedure:

1. Prepare slides of the test organisms and fix according to specific procedures described below. Generally, control smears are made from a suspension of the appropriate organism in phosphate buffered saline (PBS), pH 7.5, or 0.5% (v/v) formalinized 0.85% (w/v) saline adjusted to the turbidity of a McFarland No. 1 standard and allowed to air dry.
2. Prepare dilutions of the unknown conjugate in PBS, pH 7.5, so as to exceed by several dilutions the titer stated by the manufacturer. For example, with a stated titer of 1:16, prepare twofold dilutions through 1:128.
3. Test each dilution, as well as undiluted conjugate, against a known positive control. The conjugate of known activity should be tested on the same day or concurrently at its working dilution against both a positive and negative control.
4. Select the dilution of the unknown conjugate that is one twofold dilution lower than the greatest dilution giving maximal fluorescence (4+) with the positive control. For example, if maximal fluorescence (4+) is observed at a dilution of 1:64 and a dilution of 1:128 produces less brilliant fluorescence (3+ or 2+), select a dilution of 1:32 for the working solution.
5. Test the working dilution of the new conjugate against several known positive and negative controls. For example, a conjugate for presump-

tive identification of group A streptococci should be tested against several isolates or strains of group A streptococci, as well as organisms of other species or serogroups that are likely to cross-react, such as streptococci of groups C and G and *Staphylococcus aureus.* The tests against the group A strains are to assess sensitivity and reproducibility because some homologous strains may require a lower dilution of conjugate for adequate staining. The tests against other species or serogroups are to assess specificity (Appendix C). Nonspecific reactions, when they occur, often can be avoided by using a higher dilution of conjugate, if sensitivity is not affected adversely, or by adding a nonfluorescent blocking antibody.[11] These adjustments in the working dilution to achieve optimal results often can be made using less than twofold dilutions. For example, a conjugate that shows slight nonspecific reactions at a 1:20 dilution may work well at a 1:25 or 1:30 dilution without decreasing sensitivity.

6. Technical precautions
   a. Antigen fixation

      Avoid overfixation of the antigen, particularly when heat is used. When alcohol or acetone is used, avoid using the same solution for too many slides. No more than 50 slides should be fixed per 200 ml of alcohol or acetone.

   b. Application of conjugate

      Use only a small drop of conjugate on each smear. Spread the drop carefully with a clean piece of applicator stick or a toothpick held almost horizontally, catching the meniscus of the fluid in a manner that avoids touching and thereby disturbing the smear.

   c. Incubation

      Drying of the conjugate on the slide during incubation could result in what might be interpreted as false-positive reactions. Therefore, incubate the slide for the prescribed time in a humid atmosphere. A satisfactory chamber consists of a piece of moist filter paper affixed to the lid of a petri dish. Place the slides on a cloth or paper towel and cover with this chamber during incubation.

   d. Removal of excess conjugate or serum

      Several smears are often prepared on a single slide and stained with different conjugates (direct procedures) or different dilutions of serum (indirect tests). Care must be taken not to allow the different conjugates or dilutions to run together. Remove excess conjugate by holding the slide with the long edge parallel and the short edge perpendicular to the bench, and tap it against a towel. Then quickly rinse the smears with a stream of PBS from a wash bottle while the slide is held horizontally with the long edge tipped downward. Alternatively, slides can be washed by dipping them two or three times in a staining jar of PBS, followed by transfer to a second jar where they are allowed to stand for 10 min. Slides must

be rinsed completely to ensure that all excess conjugate or serum is removed.

e. Mounting

The pH of the mounting fluid tends to decrease due to oxidation of the glycerol and absorption of atmospheric CO_2. An acid pH will cause a marked decrease in fluorescence; therefore, the pH should be checked monthly. When adding mounting fluid, use a very small drop and take care to avoid forming bubbles. Also, avoid forming bubbles when the cover glass is added.

f. Preservation

To preserve a slide, ring the cover glass with clear nail polish and store the slide at 2 to 8°C. Avoid exposure to light.

g. Drying stained smears

If slides are blotted, use lint-free paper to avoid addition of extraneous material, which might increase artifacts. Be sure to use a clean blotting surface each time to avoid carrying over organisms from one smear to another. In general, either blotting or air drying can be used for smears prepared from organism suspensions. Smears prepared directly from clinical specimens (for example, tissue impressions or sediments from cerebrospinal fluid) should be air dried.

B. Direct FA Procedures

1. Group A Streptococci

The presumptive identification of group A streptococci is the most widely used immunofluorescence procedure. The test may be performed on smears made directly from throat swabs, but recovery of streptococci from these swabs is poor, and the method is not recommended.[18] Sensitivity is vastly improved when the FA test is performed on the centrifuged sediment from a 2- to 5-hr broth culture of the swab. In fact, sensitivity by this method equals or exceeds the sensitivity of routine cultures prepared directly from the swab on blood agar,[22] and the delay imposed by the broth culture is insignificant in most clinical laboratory settings. Finally, the FA test can be substituted for the classical capillary precipitin test for serological identification of group A streptococci isolated from cultures.

Procedure for Detection and Identification of Group A Streptococci

Purpose:
To detect and to identify group A streptococci in throat swabs and cultures.

Principle:
Throat swabs are incubated for 2 to 5 hr in Todd-Hewitt broth, and bacteria are recovered from the broth by centrifugation. A smear of the bacteria is prepared and is stained with a fluorescein-labeled antibody specific for group A *Streptococcus.* The smears are examined with a fluorescence microscope to detect stained bacteria. The following procedure was described by Moody and associates[22] and was later modified to increase specificity.[11,18]

Specimen:

1. Throat swabs
2. Colonies of β-hemolytic streptococci

Reagents:

1. Phosphate buffered saline (PBS), pH 7.5

Na_2HPO_4 (MW 141.96)	1.24 g
$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	0.179 g
NaCl (MW 58.4)	8.5 g
Water	1000 ml

Mix ingredients until solution is complete. Check for a pH of 7.4 to 7.6 and adjust with 1 mol/liter NaOH if necessary.

2. Phosphate buffered saline (PBS), pH 8.5

K_2HPO_4 (MW 174.18)	1.16 g
KH_2PO_4 (MW 136.09)	0.907 g
0.85% NaCl	200 ml

Dissolve K_2HPO_4 and KH_2PO_4 in separate 100-ml portions of 0.85% NaCl solution. Mix 10 parts of the K_2HPO_4 solution (0.067 mol/liter) with 1 part of the KH_2PO_4 (0.067 mol/liter) solution.

3. Glycerol mounting medium

PBS, pH 8.5	1 part
Glycerol	9 parts

Stir (do not shake) glycerol and buffer. Let stand for bubbles to disappear. Check pH monthly, and discard if pH is less than 8.0.

4. FA test reagent for group A *Streptococcus*
Reconstitute with sterile, distilled water and titrate as described above (p. 255) to achieve maximal fluorescence. To eliminate cross-reactions with groups C and G streptococci and *Staphylococcus aureus,* add 1 ml of group C *Streptococcus* antiserum (rabbit) (Wellcome Reagents Ltd., cat. no. ZJ03) per milliliter of conjugate in the working dilution. For example, to prepare a 1:25 working dilution of conjugate, dilute 1 ml of conjugate with 23 ml of sterile PBS, pH 7.5, and add 1 ml

of group C *Streptococcus* antiserum. The conjugate must be diluted with PBS before adding the unlabeled antiserum against group C. (If the two antisera are mixed before dilution with PBS, the fluorescence intensity of stained smears is less.)
5. Fluorescein-labeled rabbit globulin (Difco Laboratories, cat. no. 2379–56–2). This reagent is used as an optional control for nonspecific staining. Prepare at the same dilution as the test reagent.
6. Ethanol (95% v/v)

Medium:
Todd-Hewitt broth (Difco Laboratories, cat. no. 0492).

Procedure:

1. Place the throat swab in 1 ml of Todd-Hewitt broth and incubate at 35°C for 2 to 5 hr.
2. Remove swab from the broth. The swab may be used for additional cultures.
3. Centrifuge the broth for 5 min at 1000 × *g* to pack the cells.
4. Carefully decant the supernatant fluid, resuspend the pellet in 1 ml of PBS, pH 7.5, and then centrifuge again for 5 min at 1000 × *g*.
5. Decant the supernatant fluid and place the tube in a rack for 2 to 3 min to allow residual fluid and cells to collect in the bottom of the tube.
6. Mix the cells thoroughly in the residual fluid. Colonies taken directly from blood agar should be suspended in a few drops of PBS.
7. Prepare two test areas on a clean (25 mm by 75 mm) microscope slide. Small circles drawn with a ballpoint paint applicator (Vogart Crafts Corp.) work well for defining the test areas and labeling the slides. Use a capillary pipette to spread a small drop of the sediment or cell suspension from the above step in one test area. The other test area can be used for a second specimen or optional control. If the control is desired, spread cells from one specimen on both test areas.
8. Allow the test areas to air dry, then immerse the slide in 95% ethanol for 1 min to fix the cells. Rinse the slide in PBS and allow to air dry or carefully blot dry. After the slides are thoroughly dried, they may be stored at −20°C or lower and stained at a later date. The slides should be used immediately after the first thawing to avoid erratic staining reactions. Do not thaw and refreeze the slides for use at a later date.
9. Add a small drop of anti-*Streptococcus* group A conjugate to one test area and spread with an applicator stick as described above. The optional fluorescein-labeled rabbit globulin is spread on the second test area.

10. Allow the slide to stand at room temperature for 30 min in a humid chamber.
11. Shake off excess reagent from the slide and place it in a staining jar rack. Rinse briefly in a jar of PBS, pH 7.5, then transfer to a second jar of the PBS, and let stand for 10 min.
12. Rinse the slide by immersion once or twice in distilled water and blot the slide gently to dry.
13. Add a drop of glycerol mounting medium to each test area before adding a cover glass.
14. Examine the test areas with a fluorescence microscope using the oil immersion objective.

Interpretation:
The presence of group A streptococci is indicated by morphologically typical cells showing 3+ or 4+ fluorescence (Figure 4.3–1; see color insert in the center of the book).

---

2. Yersinia pestis

The direct FA technique and the FA inhibition test are excellent aids in the rapid presumptive identification of *Y. pestis.* The Plague Laboratory of the Centers for Disease Control recommends that the FA inhibition test be used as a control to assess the specificity of the direct test.[23,25,33,35] This is necessary because organisms of other than *Y. pestis* are sometimes stained by the *Y. pestis* conjugate, particularly in impression smears prepared from animal carcasses or form cadavers.

Procedure for Presumptive Identification of *Yersinia pestis*[25]

Purpose:
To provide a rapid presumptive identification of *Yersinia pestis.*

Principle:
Appropriate specimens are stained with fluorescein-labeled antiserum to *Y. pestis.* To assess the specificity of the direct FA procedure, the specimen is also stained with a mixture of unlabeled and labeled antisera to *Y. pestis.* The unlabeled antiserum competitively inhibits binding of the labeled antiserum to *Y. pestis,* thereby causing a marked decrease in fluorescence intensity. Nonspecific binding of the labeled antiserum is inhibited to a lesser extent, which results in a smaller decrease in fluorescence.

Specimen:
Pure cultures, material aspirated from buboes, blood smears, sputum, and impression smears of organs obtained from man or animals may be tested.

Reagents:

1. PBS, pH 7.5, and glycerol mounting medium are as described for staining group A streptococci (p. 258).
2. Fluorescein-labeled antiserum to *Y. pestis* (Centers for Disease Control).
3. Unlabeled antiserum to *Y. pestis* (Centers for Disease Control).
4. Fluorescein-labeled normal rabbit globulin (Difco Laboratories, cat. no. 2379–56–2).
5. Unlabeled normal rabbit serum (Difco Laboratories, cat. no. 2423–50–4).

Procedure:

1. Prepare smears from a suspension of cells in PBS or from the specimen directly. Three smears are required for the test. Use two slides with two test areas each.
2. Allow the test areas to air dry, then heat fix gently.
3. Cover one smear with one drop of the *Y. pestis* conjugate diluted 1:2 with unlabeled normal rabbit serum. Cover a second smear with one drop of *Y. pestis* conjugate diluted 1:2 with unlabeled antiserum to *Y. pestis.* The third smear should be covered with one drop of fluorescein-labeled normal rabbit globulin diluted 1:2 with normal rabbit serum.
4. Incubate slides for 30 min at room temperature in a humid chamber.
5. Shake excess conjugate from slides and rinse briefly in a jar of PBS, then transfer to a second jar of PBS and let stand for 10 min.
6. Rinse slides by immersion once or twice in distilled water and let air dry.
7. Add a drop of glycerol mounting medium before adding a cover glass.
8. Examine with a fluorescence microscope using the oil immersion objective.

Interpretation:
Presumptive identification of *Y. pestis* requires that the organisms show 3+ to 4+ fluorescence on the smear stained with *Y. pestis* conjugate diluted with unlabeled normal rabbit serum. The smear stained with the mixture of labeled and unlabeled antisera to *Y. pestis* should show negative to 1+ fluorescence. The smear stained with labeled normal rabbit globulin should not show fluorescence.

Antisera to *Y. pestis* cross-react with *Yersinia pseudotuberculosis,* and

these organisms cannot be differentiated by the above FA test. Occasional false-positive stains can also occur with other organisms, for example *Escherichia coli* and *Pasteurella multocida*. Conventional methods must be used, therefore, for definitive identification.

---

3. Legionella

The direct FA test for *Legionella* is a rapid and specific method to diagnose Legionnaires' disease and related diseases by examination of lung tissue or lower respiratory tract fluids, such as sputum, transtracheal aspirates, bronchial washings, and pleural fluid.[5,6,13,16,19] The test can also be used to identify organisms isolated by culture. The sensitivity of the test varies with the specimen source. Examination of lung tissue has the highest sensitivity, and in a study of tissue obtained at autopsy, nearly all cases were positive.[16] Examination of lower respiratory tract fluids has been shown to be effective in detecting approximately 50% of the cases, and of these fluids, sputum provided the highest sensitivity.[13] Transtracheal aspirates and bronchial washings were less sensitive, presumably due to dilutional effects of the saline used to obtain these specimens. Regardless of the specimen source, cultural and conventional histopathological techniques should be used to supplement and to confirm the direct FA test.[17,19]

Direct FA Test for *Legionella*[17,19]

Purpose:
To detect and to identify *Legionella pneumophila* and related species in clinical specimens or cultures.

Principle:
Appropriate specimens are stained with fluorescein-labeled antiserum to *L. pneumophila* or related species. The antisera used in screening tests are polyvalent and have activity against *L. pneumophile* groups 1 through 6 and most of the other *Legionella* species that have been implicated in human disease. Positive specimens are retested using group-specific antisera.

Specimen:
Specimens of lung tissue, sputum, transtracheal aspirates, bronchial washings, and pleural fluid may be tested. Smears prepared from laboratory cultures may also be identified by this method. All initial processing of specimens and preparation of smears must be done in a biological safety cabinet.

Reagents:

1. Carbonate-buffered mounting medium

Na_2CO_3 (MW 105.99)	5.3 g
$NaHCO_3$ (MW 84.00)	4.2 g
Water	200 ml
Glycerol	9 ml

   Dissolve 5.3 g of Na_2CO_3 in 100 ml of water. Dissolve 4.2 g of $NaHCO_3$ in 100 ml of water. Add 4.4 ml of the Na_2CO_3 solution to 100 ml of the $NaHCO_3$ solution to give a 0.5 mol/liter carbonate buffer at pH 9.0. Adjust pH with additional Na_2CO_3 solution if necessary. Mix 1 ml of the carbonate buffer, pH 9.0, with 9 ml of glycerol. Check pH monthly and discard if pH is less than 8.5.
2. PBS, pH 7.5, is prepared as described for staining group A streptococci (p. 258).
3. Fluorescein-labeled rabbit antisera against *L. pneumophila* groups 1 through 6, *L. bozemanii, L. dumoffii, L. gormanii, L. micdadei,* and *L. longbeachae* groups 1 and 2 are available from Zeus Technologies. Three polyvalent pools with activity against these 12 species or serogroups are also available. Alternative suppliers for antisera and controls are BioDx and Litton Bionetics. Antisera are reconstituted with buffers provided by the manufacturer to give working dilutions directly. Titration is not necessary. Polyvalent antisera from Zeus Technologies contain rhodamine-labeled normal rabbit serum as a blocking agent for nonspecific binding. Alternatively, other suppliers may include Evan's blue as a blocking agent in their antisera.
4. Fluorescein-labeled normal rabbit serum (Zeus Technologies, cat. no. 1205). Reconstitute with buffer provided by manufacturer to give working dilution directly.
5. Neutral formalin (10%), pH 7.0

Formaldehyde stock solution	100 ml
Na_2HPO_4 (MW 141.96)	6.5 g
$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	4.0 g
Water	900 ml

   Formaldehyde stock solution is the commercially available solution that contains 37% w/v or 40% w/w of formaldehyde. Mix the ingredients and check to be sure the pH is 6.8 to 7.2.
6. Neutral formalin (1%)
   Dilute 1 ml of 10% neutral formalin to 10 ml with water.

Preparation of Smears:

During fixation and staining, slides from different specimens must be processed separately to ensure that organisms present in one specimen are not transferred to another specimen. Make two smears on each slide.

Prepare sufficient slides for testing with polyvalent antiserum and, if necessary, individual typing antisera (usually 5 slides). Sterility should be maintained when processing tissues and body fluids to ensure suitability for subsequent cultures.

1. Smears from Formalin-Fixed Tissue
Transfer each tissue block to a sterile petri dish and select one or more areas for testing. In the lung, areas of dense gray or reddish consolidation are selected. With a sharp scalpel, cut through these areas to produce new tissue faces for scraping. Grasp the tissue with forceps and holding the scalpel at a right angle to the tissue face, scrape it to produce a fine puree of tissue particles. (The lung tissue of victims of legionellosis is usually quite friable. If the tissue is rubbery or spongy, a positive test is unlikely.) Smear the particles of tissue and tissue fluids into 1.5-cm circles on microscope slides, using the scalpel blade to make the smears. Allow the smears to air dry. Gently heat fix.

2. Smears from Fresh Tissue (Autopsy or Biopsy)
Transfer each tissue block to a sterile petri dish and select one or more areas for testing. Using a scalpel or scissors, cut through these areas to produce new tissue faces, and using forceps, press and squeeze the tissue against sterile microscope slides. Tissue that is very moist and may produce smears that are too thick may be blotted first on sterile gauze. Air dry and heat fix the impressions. Then place the slides in a jar of 10% neutral formalin for 10 min. Remove the slides and rinse gently with distilled water from a wash bottle to remove excess formalin. Allow the smears to air dry.

3. Preparation of Tissue Sections
Legionella bacteria maintain their serological integrity through histopathological processing and can be easily demonstrated in tissue sections if reasonably numerous. However, they are not as easily demonstrated in sections as in scrapings of formalin-fixed lung or imprints of fresh lung tissue. This is because many of the bacteria are intracellular, they lie at many different levels in the section, and because they are shrunken in size by the histological processing.

To prepare tissue sections, cut the sections as thin as possible (4 μm or less). Fix the sections for approximately 15 min at 48 to 60°C. Deparaffinize the fixed sections by two washes with xylene, followed by two washes each with absolute ethanol, 95% ethanol, and water. Allow the mounted sections to air dry.

4. Smears from Lower Respiratory Tract Sections (Sputum, Transtracheal Aspirates, and Bronchial Washings) and Pleural Fluids
Prepare smears in the usual manner. After air drying and heat fixing the preparations, proceed with fixation in 10% neutral formalin as de-

scribed above for tissue impressions. Pleural fluids tend to form a fibrin clot on the slide. Thin smears should be made and handled carefully during processing to avoid dislodging the entire film from the slide.

5. Culture Smears

Make lightly turbid suspensions of bacterial cultures in 1% neutral formalin and prepare smears. Allow the test areas to air dry, then heat fix.

Procedure:

1. Stain all preparations by covering the smear nearest the labeled end of the slide with one to two drops of the fluorescein-labeled normal rabbit serum (control conjugate).
2. Cover the second smear with one to two drops of the polyvalent or grouping conjugate.
3. Incubate slides for 20 min at room temperature in a humid chamber.
4. Shake excess conjugate from each slide by tapping against a towel. Quickly and gently rinse smears with a stream of PBS from a wash bottle while holding each slide horizontally with the long edge tipped downward. Prevent a specific conjugate from coming into contact, even momentarily, with a control smear. Then immerse each slide in a jar of PBS, pH 7.5, and let stand for 10 min. Slides from different specimens must be washed in separate jars.
5. Rinse slides by immersion once or twice in distilled water and let air dry.
6. Add a small drop of carbonate-buffered mounting medium to each smear and cover with a cover glass.
7. Examine with a fluorescence microscope using the low-power objective first to select areas where organisms may be present before switching to the oil immersion objective.

Interpretation:

The bacteria will be visible as single short rods or small intra- or extracellular clumps of organisms showing strong peripheral staining with darker centers. The smear stained with the control conjugate should not have brightly staining bacteria present.

In clinical specimens, except sputum, the following criteria are used to evaluate the test results:

Result	Report
≥25 strongly fluorescing bacteria per smear	FA positive
<25 strongly fluorescing bacteria per smear	Numbers only
0 strongly fluorescing bacteria per smear	FA negative

In many FA positive specimens, an average of 50 or more *Legionella* per field may be observed.

In sputum, organisms are never numerous. Thus, the observation of five or more brightly stained small rods, morphologically typical of *Legionella,* is considered a positive result.

When positive results are obtained using the polyvalent antiserum, additional smears should be stained using the grouping antisera and results reported accordingly.

Comments:
Direct FA staining of scrapings or of sections of formalin-fixed lung, of fresh lung imprints, or of cultures or pleural fluids is rather straightforward. Organisms in culture are usually longer rods than those seen in tissues. In older cultures long filaments, swollen rods, and other bizarre forms may be seen.

Interpretation of the results of staining lower respiratory tract specimens is more difficult. Tissue and white blood cells may be highly autofluorescent. Bacteria such as staphylococci and streptococci may fluoresce due to natural antibodies in the serum of the immunized rabbit or to nonspecific reaction of the IgG molecule with cell wall components such as protein A. One strain of *Pseudomonas fluorescens* has been found which is brightly and specifically stained by the working dilution of a *Legionella* conjugate. It is necessary to be familiar with the morphology and staining characteristics of *Legionella* if false-positive diagnoses are to be avoided. Also, relatively few *Legionella* are seen in lower respiratory tract specimens so that a smear should not be called negative until at least a 5-min search has been made.

Because of the size of the particles of tissue obtained by scraping formalin-fixed lung, many particles are lost from the slide during processing. The free bacteria and tissue cells will, however, remain on the slide to give a very good test substrate.

---

4. FA Staining for Other Bacteria

The direct FA staining procedure described for group A streptococci is representative of the procedures that are used for other bacteria. Specifications for the variations in this procedure required for other species are described in Table 4.3–2.

Legionella pneumophila Antibody

The indirect FA test is the most widely used test for the antibodies to *L. pneumophila,* and, in principle, it can be applied to detection of anti-

Table 4.3–2. Specifications for Direct FA Tests of Common Bacteria

Organism	Fixative	Incubation time (min)	Comment[a]
Haemophilus influenzae	Heat	15	Can use on smears prepared directly from CSF.
Neisseria gonorrhoeae	None	7	Use only a light suspension (McFarland 0.5) of organisms to prepare smear; rinse stained slides with water rather than PBS; a smear made from a boiled suspension of *Enterobacter cloacae* is tested as an additional negative control.
Neisseria meningitidis	Heat	15	
Brucella	Heat	30	
Listeria	95% ethanol, 1 min	30	
Bordetella pertussis	Heat	30	Incubate nasopharyngeal specimen or culture isolate in 0.2 ml of 1% casamino acids for 1 hr at 35°C; prepare smears from the culture fluid.

[a] All tests include staining a control smear with fluorescein-labeled normal rabbit globulin. A positive control organism is tested each day or concurrently with each test. Tests are invalid if the stain with normal rabbit globulin shows fluorescence or if the positive control fails to fluoresce.

bodies to any of the *Legionella* species.[32] At present, antigens prepared specifically for the test are available commercially only for *L. pneumophila* serogroups 1 through 6. Antigens for other *Legionella* species are available as controls for direct immunofluorescent testing. Use of these antigens in an indirect test may be possible, but one should be cautioned that they have not been standardized for this purpose and interpretative criteria have not been set. Alternative tests using either microagglutination of killed organisms or the enzyme-linked immunosorbent assay technique have been reported.[14]

The indirect FA test for antibody to *Legionella* appears to be specific. When possible, however, the test should be used in conjunction with attempts to isolate from or to demonstrate the organism in tissue or lower respiratory tract secretions. As with any serological test, the most convincing evidence of recent infection is a fourfold rise in titer between the acute phase of illness (within the first week after onset of fever) and

the convalescent phase (3 to 6 weeks after onset of fever).[17,20] Current data show that antibody titers of 1:32 and 1:64 in the absence of detectable disease are common,[17,20,32] and in some studies, titers of ≥1:128 have been found in apparently healthy people.[28,32] To be diagnostic, therefore, the fourfold rise in antibody titer must be to at least 1:128. A single or standing titer of ≥1:256 is considered presumptive evidence of prior infection with *Legionella.*

Legionella pneumophila Indirect FA Test[17,20]

Purpose:
To determine the presence and titer of antibody to *Legionella* in serum.

Principle:
Smears made from heat-killed *Legionella* are overlaid with dilutions of the serum to be tested. After incubation and washing to remove nonbound antibodies, the smears are stained with fluorescein-labeled antiserum against human globulins. The titer of antibody in the test serum is established as the reciprocal of the highest dilution which results in at least 1+ fluorescence of the bacteria.

Specimen:
Acute and convalescent sera should be tested together. Convalescent serum alone may be tested. Acute serum usually should not be tested alone.

Reagents:

1. *Legionella pneumophilia* antigens (heat killed) are available from BioDx, Zeus Technologies, and Litton Bionetics. BioDx also has available formalin-killed antigens. Polyvalent antigen pools are available that contain different mixtures of antigens. The following procedure assumes an antigen that does not contain chicken yolk sac (NYS) antigens and does not require NYS as a diluent. Reagents provided by the Centers for Disease Control and Litton Bionetics differ in that they require use of 3% NYS as a diluent.
2. Fluorescein-labeled antihuman globulin (BioDx, cat. no. 1524). Reconstitute with 1.5 ml of distilled water. Dilute further with pH 7.5 PBS as listed on package label.
3. *Legionella pneumophila* positive-control primate serum (BioDx, cat. no. 7501 through 7506). Reconstitute with 0.5 ml of distilled water. The control serum should give a titer within one twofold dilution of the titer stated on each vial.
4. Negative-control primate serum (BioDx, cat. no. 7500). Reconstitute

with 0.5 ml of distilled water. Dilute 1:64 with pH 7.5 PBS before use.

5. Gram-negative antigen blocking fluid.[31] Prepare an overnight culture of *Escherichia coli* 055:B5 on plain agar. (The serogroup is apparently unimportant.) Suspend growth in 2.5 ml of pH 7.5 PBS to a density of McFarland 3. Boil for 30 min. Centrifuge for 20 min at 900 × *g* and filter supernatant through a 0.45 μm-pore-size filter.
6. Twelve-well microscope slides with acetone-resistant masking (Cel-line Associates). Slides must be scrupulously clean, or else antigen will not adhere.
7. PBS, pH 7.5 (p. 258).
8. Carbonate-buffered mounting medium is prepared as described for the direct FA test for *Legionella,* (p. 263).

Procedure:
Since a small number of positives are expected, all specimens are screened first at dilutions of 1:64 and 1:128. Those specimens that are positive at one of these dilutions and matching acute phase specimens, if appropriate, are titered to an end point.

1. Shake the antigen solution vigorously to ensure an even suspension. Using a Pasteur pipette, flood each test area with antigen suspension, then aspirate the excess back into the pipette before moving to the next test area in succession. Alternatively, a bacteriological loop may be used to apply approximately 2 μl of antigen suspension to each test area.
2. Allow the smears to air dry for 30 min, then place the slides in acetone for 15 min to fix the cells.
3. Allow slides to air dry.
4. Preparation of serum dilutions
   Add 50 μl of PBS, pH 7.5, to the wells of a 96-well microtitration plate. Prepare twofold dilutions by adding 50 μl of each specimen or control serum to the first well of each row. After mixing, transfer 50 μl from the first well to the next well and mix. Continue this sequence until the serum has been diluted 1:1024 using ten wells. Alternatively, an initial 1:16 dilution can be made by adding 10 μl of each serum to 150 μl of PBS, which is then followed by twofold dilutions, as described above.
5. Add a small drop of the 1:64 and 1:128 dilutions of each serum to separate test areas. Add the 1:256 through 1:1024 dilutions of the positive control serum to separate test areas. Add the diluted negative-control primate serum to one test area to serve as a conjugate control.
6. Incubate slides for 30 min at 35°C in a humid chamber.
7. Shake off excess conjugate from slides and rinse briefly in a jar of

PBS, then transfer to a second jar of PBS and let stand for 10 min. Gently blot dry.
8. Add a small drop of antihuman conjugate to each well.
9. Incubate slides for 30 min at 35°C in a humid chamber.
10. Shake off excess conjugate from slides and rinse briefly in a jar of PBS, then transfer to a second jar of PBS and let stand for 10 min.
11. Rinse briefly with distilled water and gently blot dry.
12. Add a drop of carbonate-buffered mounting medium before adding a cover glass.
13. Examine with a fluorescence microscope.
14. Positive sera for which end point titers are to be obtained are processed as above using all dilutions of each serum. To test for specificity, positive sera are also diluted 1:16 using the Gram-negative antigen fluid, with subsequent dilutions made in PBS.[31] The titers determined with and without blocking fluid should be within one, twofold dilution of each other. To avoid transfer of antibody from well to well, slides bearing strongly positive sera (titers ≥1:1024) should be rinsed separately.

Interpretation:
Record the brightness of staining as described above. The intensity of staining of the bacteria may vary from one microscopic field to another. The intensity of staining recorded is based on an overall appraisal of the smear. The serum titer is the reciprocal of the highest dilution of the serum giving at least 1+ fluorescence staining of at least half the *Legionella* in each field. Results should not be reported unless the positive control serum gives the expected titer within one twofold dilution.

III. Counterimmunoelectrophoresis

A. General Considerations

Counterimmunoelectrophoresis (CIE) is an immunological technique for the detection of soluble antigen in various fluids. In clinical microbiology, CIE has been used widely for the detection of bacterial antigens in cerebrospinal fluid (CSF), serum, and urine. The presence of detectable antigen has been shown to correlate well with the presence of disease and other laboratory findings suggestive of infection. Since soluble antigen is being detected, a positive CIE result may be obtained with a specimen that is negative by both Gram stain and culture. A detailed description of the principles and practice of CIE is presented elsewhere.[1]

B. Examination of CSF

CIE Procedure for CSF[1,3]

Purpose:
To detect soluble antigens of *Streptococcus pneumoniae*, group B streptococci, *Haemophilus influenzae*, and *Neisseria meningitidis* in CSF.

Principle:
Almost all bacterial antigens are negatively charged in slightly alkaline (pH 8.2 to 8.6) media. Under the same conditions, rabbit antibody (IgG) is neutral or only slightly charged. In CIE, two opposing wells are placed in an agarose-coated slide (Figure 4.3–2). One well is filled with the fluid

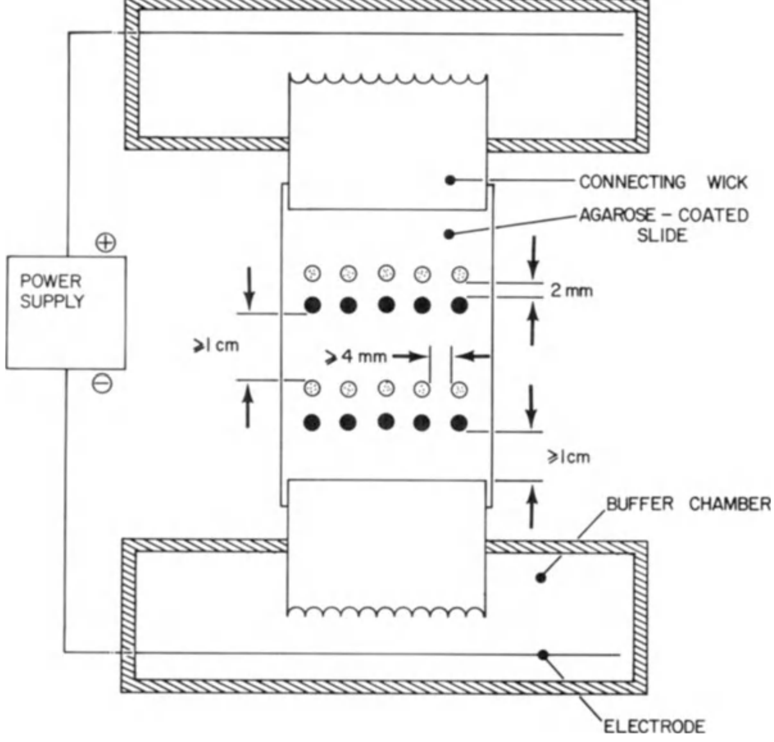


Figure 4.3–2. Diagram of basic CIE procedure showing two rows of pairs of wells. Speckled circle, well in which antiserum is placed; filled circle, well in which fluid to be tested for antigen is placed. Wells are 3 mm in diameter. (From Anhalt et al.[1] Used by permission.)

to be tested for antigen, and the other is filled with rabbit antibody specific for the antigen being tested. An electric field is placed across the slide such that the antigen, which is pulled toward the positive electrode, will enter a zone of reaction between the two wells. The antibody, which is carried in an opposite direction by the normal flow of buffer from the positive to the negative electrode, also enters the zone of reaction. With specific antigen and antibody, the resulting antigen-antibody complex forms a precipitin line between the two wells. By varying the antibody, a test can be made for several bacterial antigens.

Specimen:
CSF is the preferred specimen. If filtration or centrifugation is used to collect bacteria for culture, the cell-free filtrate or the supernatant from these procedures can be used when specimen is scarce.

Reagents:

1. Barbital buffer, 0.05 mol/liter, pH, 8.60

5,5′-Diethylbarbituric acid (barbital, Veronal) (MW 184.2)	6.2 g
Sodium 5,5′-diethylbarbiturate (sodium barbital, Veronal sodium) (MW 206.2)	34.2 g
Calcium lactate pentahydrate (MW 308.3)	1.6 g
Sodium azide (MW 65)	2.0 g
Water	4.0 liters

   Dissolve the ingredients by gently heating with continuous stirring. Let the solution cool to room temperature and adjust pH to 8.60 ± 0.02 with 1 mol/liter of NaOH if necessary. Store buffer at 4°C until used. Buffer is stable for at least 6 months. Use of this buffer may result in formation of precipitates in the buffer chambers. These precipitates can be removed by washing with dilute (1 mol/liter) acid.

2. *m*-Carboxyphenylboronic acid (CPB)-barbital buffer (CPB buffer), 0.03 mol/liter CPB, 0.05 mol/liter barbital, pH 8.60 (double strength)

CPB (MW 165.9)	1.99 g
Sodium 5,5′-diethylbarbiturate	4.12 g
Calcium lactate pentahydrate	0.16 g
Sodium hydroxide (1 mol/liter)	12 ml
Water	200 ml (q.s.)

   CPB can be obtained commercially (Calbiochem-Behring Corp., cat. no. 217401) or prepared by the method of Anhalt and Wee.[2] Dissolve the ingredients by gently heating with continuous stirring. Let the solution cool to room temperature and adjust pH to 8.60 ± 0.02 with 1 mol/liter of NaOH if necessary. Store buffer at 4°C. Buffer is stable

for at least 6 months. Note that this buffer is prepared double strength. The final concentration in the agarose gel contains 0.03 mol/liter of CPB and 0.05 mol/liter of barbital. Because of the expense of CPB and the fact that a gel buffered with barbital will almost always be tested concurrently, electrophoresis which uses this buffer is done in a discontinuous system. That is, CPB-barbital buffer is used in preparation of the gel, and the electrode chambers are filled with a buffer containing only barbital. Thus, the same electrophoresis chamber may be used for barbital-buffered and CPB-barbital–buffered agarose gels.

3. Preparation of 0.75% (w/v) agarose in barbital buffer
   Dissolve 1.5 g of agarose (Seakem HGT, Marine Colloids, Inc.) in 200 ml of barbital buffer with continuous stirring while heating. There should be no suspended solids left in the solution, but be careful to avoid overheating. Dispense 6 ml aliquots into 16 by 125 mm screw-capped tubes. Tighten caps when tubes are cool and store at 4°C. Use within 6 months. This amount of agarose, after melting, is sufficient for a slide 50 mm by 75 mm.
4. Preparation of 0.75% (w/v) agarose in CPB buffer
   Dissolve 0.75 g of agarose in 50 ml of distilled water by heating as described above. Dispense 1.5 ml aliquots into 16 by 125 mm screw-capped tubes. When cool, add 1.5 ml of CPB-barbital buffer (double strength) to each. Tighten caps and store at 4°C. Use within 6 months. This amount of agarose, after melting and mixing, is sufficient for a slide 25 mm by 50 mm.
5. Antisera
   Antisera are used undiluted as received from the manufacturer. Suitable antisera are as follows: polyvalent pneumococcal antiserum (Statens Seruminstitut, Copenhagen, Denmark, cat. name, Omniserum); *Haemophilus influenzae* type b antiserum (prepared at Mayo Clinic); *Neisseria meningitidis* antisera to group A, group B, group C, polyvalent groups X-Z and W-135 (Burroughs Wellcome Co.; cat. no ZM37, ZM38, ZM39, and ZM34, respectively); and *Streptococcus* group B antiserum (Burroughs Wellcome Co., cat. no. ZJ02). These antisera are color coded by adding a small amount (10 to 20 μl) of 1% (w/v) solutions of methylene blue to polyvalent pneumococcal antiserum and safranin O to *H. influenzae* type b antiserum. Acriflavine may be used as a third color if desired.
6. Antigen controls
   Purified antigens are not available commercially for use as controls. Working standards can be prepared by making a heavy suspension of an appropriate organism in PBS, pH 7.5. The suspension is refrigerated overnight, and an antimicrobial agent, such as Merthiolate, is added to a final concentration of 0.01% (w/v). Cells are then removed by a combination of centrifugation and filtration. The resulting solution is titrated using twofold dilutions to an end point. The dilution that

is one twofold dilution less than the end point is used as a sensitivity control for checking new lots of antisera. One of these controls, usually *H. influenzae* type b, is tested concurrently with each specimen. Aliquots of the undiluted and diluted standard can be stored at −60°C for future reference. Because some antigen preparations are unstable, only a minimal amount of working dilution should be stored at 4°C. Alternatively, dilutions of a commercially available vaccine can be used.

7. Preparation of slide with barbital-buffered agarose
   Place a tube of barbital-buffered agarose in boiling water until the agarose is completely melted. Wash a 50 mm by 75 mm glass slide in 70 to 95% ethanol. Drain the slide and wipe dry with lint-free disposable wipes. Pour agarose onto the slide on a level surface. Use a disposable Pasteur pipette to spread the agarose over the entire surface of the slide. After the agarose gels, store the slide at 4°C in a humid chamber. The slide can be used after it has been cooled for 15 min at 4°C. Do not use a slide that has been stored for more than 3 days.
8. Preparation of slide with CPB-buffered agarose
   Place a tube of CPB buffer and agarose in boiling water until the agarose is completely melted. Invert the tube several times to ensure complete mixing of the contents. Clean a 25 mm by 75 mm glass slide and pour the melted agarose onto the slide as described above. These slides are stored and are prepared at the same intervals as the slides with barbital-buffered agarose.
9. Electrophoresis apparatus
   The apparatus used at the Mayo Clinic was made in-house. It can accommodate two slides, each 50 mm by 75 mm, and has buffer chambers with a working capacity of 80 ml each (Figure 4.3–2). Any unit of similar design should also work. A regulated power supply (Lambda Electronics, model LL-905) is used to provide a voltage of 70 volts to the cell, which results in a voltage gradient of 6 to 10 V/cm measured in the agarose.

Procedure:

1. Fill the buffer chambers of the electrophoresis cell with the appropriate volume of barbital buffer.
2. Prepare strips of Telfa pad (Kendall Co.) to be used as connecting wicks. The strips are cut so that they are the same width as the slide on which they are to be used. To facilitate folding of the strips over the edges of the agarose slides, slash across the strips about 15 mm from one edge with a #11 surgical blade.
3. Use a template and a 3-mm-diameter needle to punch the desired pattern of holes in the agarose slides. Also mark one corner of each

slide to aid in orientation. When a double row of pairs of wells is needed, the rim of the outer wells in each row should be approximately 25 mm from the edge of the slide. A single row of pairs of wells is punched centrally. For screening CSF, prepare a double row of pairs of wells on a slide with barbital-buffered agarose (Figure 4.3–2) and two pairs of wells in a single row on a slide with CPB-buffered agarose.

4. Remove gel plugs by suctioning with a Pasteur pipette.
5. Use disposable capillary micropipettes (40 μl size, Sherwood Medical Industries) to fill wells to the brim with the appropriate specimen or antiserum. Rely on gravity flow and capillary action rather than positive pressure to fill the wells, and avoid splashing the solutions or overflowing the wells. Routinely, CSF is tested against antisera to *H. influenzae,* pneumococci (Omniserum) *N. meningitidis* group A, *N. meningitidis* group C, and *N. meningitidis* groups X-Z and W-135 (polyvalent) using a slide with barbital-buffered agarose. Antiserum to *Streptococcus* group B is added when the CSF is from a child less than 3 months old. An *H. influenzae* type b antigen is also tested as a control. The slide with CPB-buffered agarose is used for testing with antiserum to pneumococci (Omniserum), and on this slide, antigen from a type 7 pneumococcus serves as a control. After filling each antiserum well, use a rubber bulb to express excess antiserum in the capillary into the antiserum vial. Perform these steps as rapidly as possible to prevent drying of wells and evaporation and concentration of antisera in the vials.
6. Place the prepared slide(s) in the electrophoresis cell. Use strips of Telfa pad to connect the agarose gel to the buffer chambers, overlapping the gel about 1 cm. Be sure the strips are saturated with buffer to ensure good electrical contact.
7. Switch on the power supply to the electrophoresis cell and check to be sure the voltage and current are correct. Using two slides, one with barbital-buffered agarose and one with CPB-buffered agarose, the current should be 20 to 25 mA. If the voltage at the power supply is constant, a current that is too low will indicate poor electrical contacts, which most frequently occur between the gel and the buffer-saturated wicks. A current that is too high may be due to improper buffer, to placement of the contact wicks too closely together on the gel, or to a short circuit. A short circuit can occur, for example, when the back surface of the glass slide is wet.
8. Turn off the power supply after 60 min and remove slides. Flood slides with a solution of sodium ethylmercurithiosalicylate (Merthiolate or thimerosal) at a concentration of 0.025% (w/v) in water.
9. Examine the slides with oblique or dark-field illumination using a 4× or 10× magnifying lens to help detect faint precipitin lines. Slides should be examined immediately, after cooling for at least 15 min at 4°C, and after cooling overnight at 4°C.

Interpretation:
A true precipitin line is usually straight or only slightly arced and is located between the antigen and corresponding antibody well. In some cases, substances in a specimen or in the antiserum may form a hazy zone or highly curved precipitin arc around the antigen or antibody well. A precipitin arc around the antibody well has been a particular problem with antisera to *N. meningitidis* and may be differentiated from a truly positive reaction by the fact that it is highly curved and may extend to both sides of the well. A final test may be made by dilution of the specimen, in which case the nonspecific precipitin arc is usually unaffected, whereas specific reactions will become less intense and may form nearer the antigen well. Sometimes a nonspecific precipitin line near the antibody well can be eliminated by heating the antiserum at 56°C for 30 min before use to inactivate complement.

Comments:

1. In many cases, the selection of antisera used in a test can be guided by the findings in a Gram stained smear of the specimen. Commercial antiserum is relatively insensitive for detection of *N. meningitidis* group B. This antiserum is used only when the Gram stained smear shows gram-negative cocci are present. Conversely, when many organisms are present in the Gram stained smear, the CSF is diluted 1:20 in saline or PBS, pH 7.5, and both the undiluted and diluted specimen are tested against appropriate antisera. In cases where the Gram stained smear is positive, preliminary examination of the slide should be done 30 min after CIE is set up. For this preliminary reading, *do not* flood the slide, and if negative, continue electrophoresis for the remaining 30 min. Then examine the slides as described above.
2. Flooding the slide before examination serves two purposes. First, by filling empty wells, scattered light around these wells is decreased, which facilitates detection of faint precipitin lines near a well. Second, immersion in an aqueous solution for 15 min to several hours removes soluble substances, such as protein and buffer, which may interfere with formation or detection of a precipitate. Nonspecific reactions that may be confused with antigen–antibody precipitates may also be removed. The antimicrobial agent is added as a safety measure, since flooding of the slide may cause contamination with viable bacteria. The antimicrobial agent, however, does not inactivate hepatitis virus.
3. Blood can interfere with CIE. Therefore, grossly bloody specimens must be centrifuged to obtain a clear supernatant for CIE examination.

C. Examination of Urine

Urine can be tested for bacterial antigens using the same technique and antisera as are used for CSF. The testing of urine is particularly useful

when microscopic examination of the CSF is very suggestive of bacterial meningitis, but CIE is negative.[15] A positive result with urine alone, however, may represent bacteremia or another source of antigen besides meningitis. For optimal results, urine should be concentrated before being tested by CIE. Several procedures have been described to concentrate urine.[1] The following procedure was developed in our laboratory and has been shown to be comparable to the other procedures (Anhalt, J. P., Wold, A. D.: Unpublished).

Procedure for Concentration of Urine

Purpose:
To concentrate the antigen present in urine prior to testing by CIE.

Principle:
Urine is passed through an ultrafiltration membrane using centrifugal force. The high molecular weight antigens are retained by the filter and are thereby concentrated into a small volume.

Specimen:
A minimum of 5 ml of urine is required. If urine is turbid, centrifuge and draw off the clear supernatant for concentration.

Supplies:

1. Centriflo ultrafiltration membrane cone (Amicon Corp., model no. CF25). The cones must soak in water a minimum of 1 hr before use.
2. Special support and centrifuge tube for membrane cone (Amicon Corp., model no. CST1).

Procedure:

1. Add 5 ml of urine to a presoaked Centriflo ultrafiltration membrane cone.
2. Place the filled cone in the special support and centrifuge tube.
3. Centrifuge for 10 min at $1000 \times g$.
4. If the volume of concentrated urine in the cone is inadequate for CIE (about 0.05 to 0.1 ml will be required), add two or three drops of *unconcentrated* urine to the cone. If excess unconcentrated urine is not available, use urine that has collected in the centrifuge tube.
5. Vortex the cone while it is still in the adapter. Be sure the small amount of concentrated urine rides up the walls of the cone to wash down any concentrated material that may be adherent to these walls.
6. Perform the CIE test on the concentrate.

D. Identification of Bacterial Isolates

Many bacteria can be identified or serotyped by testing a suspension of the organism in saline by CIE. Streptococci other than *S. pneumoniae* may require a short incubation in Todd-Hewitt broth or some method of extraction to obtain detectable levels of antigen.

References

1. Anhalt, J. P., Kenny, G. E., and Rytel, M. W. *Cumitech 8:* Detection of microbial antigens by counterimmunoelectrophoresis. Coordinating ed. Gaven, T. L. Washington, D.C., American Society for Microbiology, 1978.
2. Anhalt, J. P., and Wee, S. H. Counterimmunoelectrophoresis of pneumococcal antigens: Alternative buffers for the detection of types VII and XIV. Program and Abstracts, 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, American Society for Microbiology, 1976, abstract 312.
3. Anhalt, J. P. and Yu, P. K. W. Counterimmunoelectrophoresis of pneumococcal antigens: Improved sensitivity for the detection of types VII and XIV. *J. Clin. Microbiol.* **2**:510, 1975.
4. Biegeleisen, J. Z., Jr., Mitchell, M. S., Marcus, B. B., Rhoden, D. L., and Blumberg, R. W. Immunofluorescence techniques for demonstrating bacterial pathogens associated with cerebrospinal meningitis. I. Clinical evaluation of conjugates on smears prepared directly from cerebrospinal fluid sediments. *J. Lab. Clin. Med.* **65**:976, 1965.
5. Broome, C. V., Cherry, W. B., Winn, W. C., Jr., and MacPherson, B. R. Rapid diagnosis of Legionnaires' disease by direct immunofluorescent staining. *Ann. Intern. Med.* **90**:1, 1979.
6. Chandler, F. W., Hicklin, M. D., and Blackmon, J. A. Demonstration of the agent of Legionnaires' disease in tissue. *N. Engl. J. Med.* **297**:1218, 1977.
7. Cherry, W. B., and Moody, M. D. Fluorescent-antibody techniques in diagnostic bacteriology. *Bacteriol. Rev.* **29**:222, 1965.
8. Collins, J. H., and Kelly, M. T. Comparison of Phadebact coagglutination, Bactogen latex agglutination, and counterimmunoelectrophoresis for detection of *Haemophilus influenzae* type b antigens in cerebrospinal fluid. *J. Clin. Microbiol.* **17**:1005, 1983.
9. Coonrod, J. D., and Rylko-Bauer. Latex agglutination in the diagnosis of pneumococcal infection. *J. Clin. Microbiol.* **4**:168, 1976.
10. Crosson, F. J., Winkelstein, J. A., and Moxon, E. R. Enzyme-linked immunosorbent assay for detection and quantitation of capsular antigen of *Haemophilus influenzae* type b. *Infect. Immun.* **23**:617, 1978.
11. Difco Technical Information. *Fluorescent Antibody Reagents and Procedures.* Detroit, Mich., Difico Laboratories, 1973.
12. Dirks-Go, S. I. S., and Zanen, H. C. Latex agglutination, counterimmunoelectrophoresis, and protein A coagglutination in the diagnosis of bacterial meningitis. *J. Clin. Pathol.* **31**:1167, 1978.
13. Edelstein, P. H., Meyer, R. D., and Finegold, S. M. Laboratory diagnosis of Legionnaires' disease. *Am. Rev. Respir. Dis.* **121**:317, 1980.

14. Farshy, C. E., Klein, G. C., and Feeley, J. C. Detection of antibodies to Legionnaires disease organism by microagglutination and micro-enzyme-linked immunosorbent assay tests. *J. Clin. Microbiol.* **7**:327, 1978.
15. Feigin, R. D., Wong, M., Shackelford, P. G., Stechenberg, B. W., Dunkle, L. M., and Kaplan, S. Countercurrent electrophoresis of urine as well as of CSF and blood for diagnosis of bacterial meningitis. *J. Pediatr.* **89**:773, 1976.
16. Hicklin, M. D., Thomason, B. M., Chandler, F. W., and Blackmon, J. A.: Pathogenesis of acute Legionnaries' disease pneumonia. *Am. J. Clin. Pathol.* **73**:480, 1980.
17. Jones, G. L., and Hebert, G. A. (eds.). *"Legionnaires'" the disease, the bacterium and methodology.* Atlanta, Center for Disease Control, Department of Health, Education, and Welfare, October, 1978.
18. Jones, G. L., Hebert, G. A., and Cherry, W. B. *Fluorescent Antibody Techniques and Bacterial Applications.* Atlanta, Center for Disease Control, Department of Health, Education, and Welfare, April, 1978.
19. Legionella *Direct Fluorescent Antibody Reagents.* Atlanta, Center for Disease Control, Department of Health, Education, and Welfare, March, 1979.
20. Legionella *Indirect Fluorescent Antibody Research Reagents.* Atlanta, Center for Disease Control, Department of Health, Education, and Welfare, August, 1979.
21. Leinonen, M., and Käyhty, H. Comparison of counter-current immunoelectrophoresis, latex agglutination, and radioimmunoassay in detection of soluble capsular polysaccharide antigen of *Haemophilus influenzae* type b and *Neisseria meningitidis* of groups A or C. *J. Clin. Pathol.* **31**:1172, 1978.
22. Moody, M. D., Siegel, A. C., Pittman, B., and Winter, C. C. Fluorescent-antibody identification of group A streptococci from throat swabs. *Am. J. Public Health* **53**:1083, 1963.
23. Moody, M. D., and Winter, C. C. Rapid identification of *Pasteurella pestis* with fluorescent antibody. III. Staining *Pasteurella pestis* in tissue impression smears. *J. Infect. Dis.* **104**:288, 1959.
24. O'Reilly, R. J., Anderson, P., Ingram, D. L., Peter, G., and Smith, D. H. Circulating polyribophosphate in *Haemophilus influenzae* type b meningitis. Correlation with clinical course and antibody response. *J. Clin. Invest.* **56**:1012, 1972.
25. *Recommended Methods for the Use of FITC Conjugated Globulins in the Diagnosis of Pasteurella pestis by the Direct FA and the FA Inhibition Test.* Atlanta, Center for Disease Control, Department of Health, Education, and Welfare, November, 1970.
26. Rytel, M. W. Rapid diagnostic methods in infectious disease. *Adv. Intern. Med.* **20**:37, 1975.
27. Thirumoorthi, M. C., and Dajani, A. S. Comparison of staphylococcal coagglutination, latex agglutination, and counterimmunoelectrophoresis for bacterial antigen detection. *J. Clin. Microbiol.* **9**:28, 1979.
28. Tsai, T. F., and Fraser, D. W. The diagnosis of Legionnaires' Disease (editorial). *Ann. Intern. Med.* **89**:413, 1978.
29. Ward, J. I., Siber, G. R., Scheifele, D. W., and Smith, D. H. Rapid diagnosis of *Haemophilus influenzae* type b infections by latex particle agglutination and counterimmunoelectrophoresis. *J. Pediatr.* **93**:37, 1978.
30. Whittle, H. C., Tugwell, P., Egler, L. J., and Greenwood, B. M. Rapid bacterio-

logical diagnosis of pyogenic meningitis by latex agglutination. *Lancet* **2**:619, 1974.

31. Wilkinson, H. W., Farshy, C. E., Fikes, B. J., Cruce, D. D., and Yealy, P. Measure of immunoglobulin G-, M-, and A-specific titers against *Legionella pneumophila* and inhibition of titers against nonspecific, gram-negative bacterial antigens in the indirect immunofluorescence test for legionellosis. *J. Clin. Microbiol.* **10**:685, 1979.
32. Wilkinson, H. W., Reingold, A. L., Brake, B. J., McGiboney, D. L., Gorman, G. W., and Broome, C. V. Reactivity of serum from patients with suspected legionellosis against 29 antigens of Legionellaceae and Legionella-like organisms by indirect immunofluorescent assay. *J. Infect. Dis.* **147**:23, 1983.
33. Winter, C. C., and Moody, M. D. Rapid identification of *Pasteurella pestis* with fluorescent antibody. I. Production of specific antiserum with whole cell *Pasteurella pestis* antigen. *J. Infect. Dis.* **104**:274, 1959.
34. Winter, C. C., and Moody, M. D. Rapid identification of *Pasteurella pestis* with fluorescent antibody. II. Specific identification of *Pasteurella pestis* in dried smears. *J. Infect. Dis.* **104**:281, 1959.

Section 4.4

Antimicrobial Susceptibility Tests of Aerobic and Facultatively Anaerobic Bacteria

John P. Anhalt, Ph.D., M.D.
John A. Washington II, M.D.

I. Overview

A. General Considerations

The selection of appropriate antimicrobial therapy is the only decision made in medical practice that requires consideration of the pharmacology of a drug in different species, the patient and the microorganism, simultaneously. The pharmacological considerations in the patient include toxicity and factors that influence the concentration and duration of drug at the site of infection, including bioavailability, distribution, protein binding, and rate and route of excretion. Also to be considered are how well a particular dosage form will be tolerated by the patient, whether the drug will have an adverse effect because of an underlying disease or condition, and whether the antimicrobial agent will affect other therapy the patient is receiving. For example, rifampin and griseofulvin increase, whereas chloramphenicol and sulfonamides decrease, the dosage requirements for oral anticoagulants.

Concurrently, it is necessary to consider the concentration of an antimicrobial that is effective in preventing the growth or in killing the microorganism and, possibly, the rate at which these actions occur. Because laboratory tests of antimicrobial activity cannot duplicate the conditions that exist in an infection, the results from tests with a particular isolate must be considered in context with the results usually obtained with other members of the same species and with clinical experience.

A complete understanding of the pharmacology of an antimicrobial,

however, is not sufficient for selecting appropriate therapy. The natural history and pathology of the infection, as well as the immune status of the patient, must also be considered. These factors make it difficult to measure accurately the efficacy of an antimicrobial. For example, many patients who receive inappropriate or no antimicrobial therapy will recover from serious infections, whereas others who receive presumably appropriate antimicrobial therapy will not.[18]

Of the many factors that influence the efficacy and affect the choice of antimicrobial therapy, the laboratory can routinely measure the concentrations of antimicrobial required to inhibit or kill organisms *in vitro* and the drug concentrations attained in serum or other fluids (Section 12.1). Susceptibility tests that measure the inhibitory effects of antimicrobials are described below. Tests of the killing effects of antimicrobial are described in Section 12.2.

B. Definitions of Susceptibility

Susceptibility test results can be reported either quantitatively or qualitatively. Quantitative results are expressed as the minimal concentration of an antimicrobial that inhibits organism growth (minimal inhibitory concentration or MIC), and qualitative results are expressed as susceptible, intermediate, or resistant. Similar interpretative labels are also often applied to quantitative results. A tentative standard for dilution susceptibility testing categorizes quantitative results even further as being susceptible, moderately susceptible, resistant, and conditionally susceptible.[25]

The simplest definition of a susceptible organism is one for which the MIC with a particular drug is less than the drug's attainable concentration (usually a half to a quarter of the peak concentration) in serum or other fluid with normal doses.[18] This definition, though clinically useful, is incomplete and must be modified to account for cases in which populations of resistant organisms that have arisen from mutation or acquisition of extra-chromosomal resistance factors can be differentiated from populations of uniformly sensitive organisms. In routine susceptibility testing, for example, staphylococci that produce β-lactamase are considered resistant to penicillin; however, many will have MICs that are less than attainable serum levels. Similarly, the definition must account for those cases in which MICs are less than attainable serum concentrations but in which clinical results do not show that the drug is effective. Bearing these conditions in mind, the four interpretative categories of susceptibility can be defined as shown in Table 4.4–1.[25] The moderately susceptible category shown in the table is usually included in the intermediate or indeterminate category of susceptibility in the three-category system. This category also functions to prevent major interpretative discrepancies that may

Table 4.4–1. Definitions of Susceptibility Based on MIC

Category	Definition
Susceptible	Readily inhibited by levels attainable with usual dosage and route of administration; implies the drug may be used for effective therapy
Moderately susceptible	Inhibited only by levels achieved with high dosage
Resistant	Resistant to usually achievable levels
Conditionally susceptible	Inhibited only by levels achieved where the drug is concentrated (e.g., urine)

arise from minor technical variations and allows for the fact that zones of inhibition determined by diffusion tests and MICs determined by dilution tests do not correlate precisely (p. 296).

C. Indications for Testing

Susceptibility tests are indicated for organisms that are presumed to be causative agents of an infection and for which the susceptibility cannot be predicted from knowledge of their identity or by performance of simple tests, such as tests for production of β-lactamase by *Haemophilus influenzae.* Susceptibility tests should not be done on normal flora that are not implicated in an infection.

D. Test Variables

Common variables that affect the outcome of susceptibility tests are medium, inoculum, incubation conditions, and antimicrobial stability.

1. Medium

Mueller-Hinton is the medium recommended for use in the United States for susceptibility testing of rapidly growing aerobic and facultatively anaerobic bacteria.[6,8,15,16,25,26,42,44] Components of this medium that significantly affect the activity of antimicrobials include its content of thymidine (trimethoprim and sulfonamides) and free divalent cations (tetracyclines, polymyxins, and aminoglycosides). A study of several susceptibility testing media available in the United Kingdom showed that Mueller-Hinton agar from Difco Laboratories seemed to be free of trimethoprim antagonists.[1] A far more complex problem is that of the relationship between divalent cations in the medium and aminoglycoside activity, particularly against

Pseudomonas aeruginosa. While in broth, the activity of aminoglycosides against *P. aeruginosa* is inversely related to the concentrations of Ca^{2+} and Mg^{2+}, the situation in agar is not so simple.[9,20,29,30,43,46] In this medium, aminoglycoside activity cannot be accurately predicted by measurement of the total cation concentration because variable proportions of these ions are bound and do not diffuse freely.[20,46] Activity has been related to the concentration of divalent cations in a soluble fraction of the gel,[20] but simply adding calculated amounts of calcium and magnesium salts to agar may not give the expected result. Deviation occurs, because the extent of binding is affected by other variables, such as temperature and length of heating during autoclaving.[46] It is mandatory that suitable control strains be tested to establish the acceptability of each lot number of medium used.

Mueller-Hinton broth contains negligible amounts of Ca^{2+} and Mg^{2+}; therefore, significant differences can occur between MICs of aminoglycosides against *P. aeruginosa* determined in broth and in agar. It has been suggested, therefore, that broth be supplemented with physiological levels of Ca^{2+} and Mg^{2+}.[25,40] In general, strains of *P. aeruginosa* that are resistant on the basis of plasmid-determined aminoglycoside-modifying enzymes will be resistant when tested in either broth or agar. The major difficulty is with strains that have a lower level of resistance on the basis of relative impermeability to aminoglycosides[11] and that may be resistant when tested on agar or in broth supplemented with cation but susceptible when tested in broth without added cation.[27] Recent Surveys of the College of American Pathologists and a tentative standard of the National Committee for Clinical Laboratory Standards (NCCLS)[25] have stressed the need for cation supplementation of Mueller-Hinton broth to final concentrations of 25 mg/liter of Mg^{2+} and 50 mg/liter of Ca^{2+}. This recommendation is not based so much on a significant incidence of false susceptibility using unsupplemented media, but rather on a desire to establish interlaboratory comparability of susceptibility test results.

Although the laboratory clearly has little or no control over composition of media or of batch-to-batch variations in media, the pH of each batch must be checked to ensure that it is in the range of 7.2 to 7.4. An increase in acidity will cause a decrease in the apparent activity of aminoglycosides and erythromycin, whereas the activity of tetracycline and methicillin will increase. With a change toward alkalinity, the converse situation will occur.[14] To support growth of fastidious bacteria, defibrinated or chocolatized sheep blood may be added to Mueller-Hinton agar and will not significantly affect the activity of antimicrobials exclusive of trimethoprim and sulfonamides. Alternatively, Mueller-Hinton agar supplemented to contain 1% hemoglobin and 1% Supplement XV (Difco Laboratories) or IsoVitaleX (BBL Microbiology Systems) can be used for testing *Haemophilus.*[40] When it is necessary to test the activity of trimethoprim or sulfonamides, lysed horse blood can be substituted for the sheep blood.

2. Inoculum

Differences in inoculum size probably contribute the greatest day-to-day variability in susceptibility test results. The inoculum should be adjusted so that its turbidity matches that of a No. 0.5 McFarland barium sulfate standard[21] prepared by adding 0.5 ml of 0.048 *M* $BaCl_2$ (1.175% w/v $BaCl_2 \cdot H_2O$) to 99.5 ml of 0.36 *N* H_2SO_4 (1% v/v).[2] This standard should be agitated on a Vortex mixer prior to use and should be replaced every 6 months unless it is kept in heat sealed tubes in which it lasts almost indefinitely.[45] A useful device for standardizing the inoculum, by visual comparison with a McFarland barium sulfate standard, was described by Stemper and Matsen.[38]

It is important to use a pure inoculum because mixtures of different organisms confuse and confound the results.[35] Direct susceptibility testing of certain kinds of clinical specimens has been evaluated in several studies. In general, the presence of mixtures of organisms and the lack of a standardized inoculum have limited the reliability of this approach.[12,35,47] An exception occurs with blood and spinal fluid cultures. In these situations, single isolates are common, and an aliquot of the culture broth can be used to prepare a standardized inoculum.[19] Direct testing in these cases can provide more rapid results; these results need not be confirmed by subsequent testing of isolated organisms unless the inoculum used for direct testing proves to be a mixed culture.

3. Incubation

Incubation of media should be at 35°C. Bacteria grow as well and as rapidly at this temperature as at 37°C. More important, however, is that incubation at 35°C is required to detect methicillin-resistant staphylococci within the usual incubation time of 16 to 18 hr. Prolongation of the incubation time will decrease the reproducibility of results. For routine susceptibility testing, incubation in an atmosphere containing CO_2 should be avoided because CO_2 will alter the pH of the medium and has been shown to affect the activity of some antimicrobials.[14] Results of tests with fastidious organisms, for which the inoculum cannot be standardized satisfactorily and for which capneic or anaerobic incubation, prolonged incubation, and specially enriched media are used, must be interpreted with caution.

4. Selection of Antimicrobials for Testing and Reporting

The selection of antimicrobials for routine testing should be restricted to class representatives in instances where the in vitro or in vivo activity of analogues within a class does not differ significantly from that of a representative. For example, the tetracycline analogues are adequately

Table 4.4–2. Routine Reporting Protocol of Antimicrobial Susceptibility Tests

Antimicrobial[a]	Streptococci	Staphylococci	Gram-negative isolates	Gram-positive bacilli
Amikacin			X	
Ampicillin	X		X	X
Cefazolin[b]		X[c]	X	X
Cefoperazone[b]			X	
Cefoxitin[b]			X	
Cefuroxime[b]			X	
Ceftizoxime[b]			X	
Chloramphenicol[d]	X	X	X	
Clindamycin		X[e]		X
Erythromycin	X	X		X
Gentamicin	X[f]	X[f]	X	
Mezlocillin			X	
Moxalactam[b]			X	
Nalidixic acid[g]			X	
Nitrofurantoin[g]	X		X	
Oxacillin		X		X
Penicillin	X	X		X
Piperacillin			X	
Tetracycline[h]			X	
Trimethoprim/ sulfamethoxazole[g,i]		X	X	
Trimethoprim[g]			X	

[a] Selection of the class representatives for third-generation cephalosporins and antipseudomonal penicillins is undergoing evaluation.

[b] If MIC of cefazolin is ≤8 μg/ml, only cefazolin is reported. If MIC of cefazolin is >8 μg/ml and MIC of cefuroxime or cefoxitin is ≤8 μg/ml, then cefazolin, cefuroxime, and cefoxitin are reported. Cefoperazone, ceftizoxime, and moxalactam are reported only when organism is resistant to cefazolin, cefuroxime, and cefoxitin.

[c] Do not report if oxacillin MIC is ≥4 μg/ml for staphylococci.

[d] Reported only for isolates from blood, spinal fluid, eye, and stool cultures.

[e] Do not report a clindamycin MIC ≤4 μg/ml if erythromycin MIC is >4 μg/ml for staphylococci.

[f] Test and report only for isolates from cultures of eyes (scraping, intraocular, etc.).

[g] Reported only for isolates from urine cultures.

[h] Tetracycline is not tested against *Proteus.*

[i] Report for *Shigella* isolates from stools.

represented by testing tetracycline itself. The activity of the penicillinase-resistant penicillins is adequately represented by testing methicillin, oxacillin, or nafcillin. The appropriate class representative selected will vary by institution and should reflect the drugs included in that institution's formulary. For example, among the so-called first-generation cephalosporins, either cephalothin or cefazolin can be used as a class representative.

Because cefazolin has slightly greater activity against some Enterobacteriaceae,[7] it should not be used as the class representative unless it is the drug principally in use. Conversely, to use cephalothin might underestimate the activity of cefazolin and lead physicians to use more expensive second- and third-generation drugs.

Testing of unnecessary antimicrobials should be avoided because of cost; however, in most procedures, it is more cost effective to test routinely a fixed set of antimicrobials against all organisms belonging to relatively large groups (e.g., groups based on the Gram stain properties), rather than tailor the selection for a particular isolate. In this situation, antimicrobials may be tested unavoidably against bacteria for which they are contraindicated as therapy. For example, ampicillin, oxacillin, and penicillin may be tested against gram-positive bacteria; however, ampicillin results should not be reported for staphylococci because penicillinase producing organisms may appear falsely sensitive, and the report might lead to inappropriate therapy. The routine reporting protocol used at the Mayo Clinic is shown in Table 4.4–2.

E. Quality Control

With all the variables involved in antimicrobial susceptibility tests, quality control is essential and should include daily performance testing of reference strains of bacteria. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 have been recommended for disk-diffusion tests.[26] The same strains can be used for quantitative tests; however, substitution of *S. aureus* ATCC 29213 for *S. aureus* ATCC 25923 and addition of *Streptococcus faecalis* ATCC 29212 have been recommended to provide a selection of organisms that have MICs more appropriate for quality control of these tests.[25] *Pseudomonas aeruginosa* ATCC 27853 develops resistance to carbenicillin after repeated subcultures, and fresh cultures from storage should be substituted at appropriate intervals or whenever resistance develops.[25] Routine susceptibility testing at the Mayo Clinic uses a smaller number of dilutions than are used in reference methods and some broth dilution procedures. The control strains used are *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. faecalis* ATCC 29212, and *P. aeruginosa* (Mayo strain).

To minimize the frequency of problems related to lot-to-lot variation of agar media, samples of several lots are tested with 30 to 60 strains of *P. aeruginosa* against gentamicin. When a lot is found that gives approximately the same MICs as the lot in use, a large quantity is purchased.

Stock cultures of reference strains should be stored under conditions that will minimize the possibility of mutation and subsequent change in susceptibility pattern. Several acceptable methods are available and include lyophilization and freezing in a stabilizer medium.[25] The method

used at the Mayo Clinic is as follows: 20 ml of sterile horse serum is added to 20 ml of an 18 hr Mueller-Hinton broth culture of a control strain; 0.5-ml portions of the mixture are pipetted into small screw-cap glass vials and frozen at −20°C. Each afternoon, one vial is thawed and a loopful of the suspension is streaked onto a blood agar plate which is incubated overnight at 35°C. Colonies from this plate are then tested in the same manner as those isolated from cultures of clinical material.

II. Preparation of Antimicrobial Solutions

A. Stock Solutions*

Antibiotic powders that are designated for preparation of standards can be obtained directly from the manufacturers or from the U.S. Pharmacopeia Convention Inc. (12601 Twinbrook Parkway, Rockville, MD 20852). Preparations intended for clinical purposes should not be used for in vitro tests because they are less precisely standardized. Containers of each powder should bear a label stating the potency, expressed in micrograms of activity or in international units (IU) per milligram of powder, and an expiration date. Store the powders according to the manufacturer's directions. Avoid unnecessary storage of powders under refrigeration because this increases the likelihood that the powders will absorb water vapor and change in potency.

Powders are weighed on an analytical balance and dissolved to yield the required concentration based on labeled "activity" or "potency." The following formulas may be used to determine the amount of powder or diluent necessary for a stock solution:

$$\text{Weight (mg)} = \frac{\text{volume (ml)} \times \text{concentration } (\mu\text{g/ml})}{\text{potency } (\mu\text{g/mg})}$$

or

$$\text{Volume (ml)} = \frac{\text{weight (mg)} \times \text{potency } (\mu\text{g/mg})}{\text{concentration } (\mu\text{g/ml})}$$

In most cases, it is advisable to weigh a portion of antimicrobial roughly exceeding that needed and to calculate the volume of solvent required. This procedure avoids multiple adjustments of the powder during weighing and minimizes time and exposure to atmospheric moisture. Weighing a predetermined amount of powder is advisable when an organic solvent is used to dissolve the antimicrobial, and the solution is diluted to a fixed volume in a volumetric flask.

* Adapted from Anhalt and Washington.[2]

Solvents and diluents for common antimicrobials are listed in Table 4.4–3. These recommendations are based on an effort to use a minimal number of solvents or buffers and may not give optimal stability. In general, ethanol can be substituted for methanol, dimethylsulfoxide for dimethylformamide, and phosphate buffer (0.1 mol/liter, pH 6) for water to dissolve β-lactam antimicrobials to improve stability (water should be used as diluent).

Table 4.4–3. Solvents and Diluents for Stock Solutions of Antimicrobials

Antimicrobial	Solvent	Diluent
Amikacin	Water	Water
Amoxicillin[a]	Phosphate buffer, 0.1 *M*, pH 8	Phosphate buffer, 0.1 *M*, pH 6; water
Amphotericin B	Dimethylformamide	Water
Ampicillin[a]	Phosphate buffer, 0.1 *M*, pH 8	Phosphate buffer, 0.1 *M*, pH 6; water
Bacitracin	Water	Water
Carbenicillin	Water	Water
Cefamandole	Water	Water
Cefazolin	Water	Water
Cefoperazone	Phosphate buffer, 0.1 *M*, pH 6	Water
Cefotaxime	Water	Water
Cefoxitin	Water	Water
Ceftazidime	10% w/w Na_2CO_3 in water	Water
Ceftizoxime	Water	Water
Cefuroxime	Water (final pH $\leq$7.0)	Water
Cephalothin[b]	Phosphate buffer, 0.1 *M*, pH 6	Water
Chloramphenicol	Methanol	Water
Clindamycin	Water	Water
Cloxacillin	Water	Water
Colistin (polymyxin E)	Water	Water
Cycloserine	Water	Water
Erythromycin[c]	Methanol	Phosphate buffer, 0.1 *M*, pH 8
Ethambutol	Water	Water
Flucytosine	Water	Water
Gentamicin	Water	Water
Imidazoles	Dimethylformamide	Water
Isoniazid	Water	Water
Kanamycin	Water	Water
Methicillin	Water	Water
Metronidazole	Water	Water
Mezlocillin	Water	Water

Table 4.4–3 (*continued*)

Antimicrobial	Solvent	Diluent
Moxalactam	Water or HCl[d]	Water
Nafcillin	Water	Water
Nalidixic acid[e]	NaOH, 0.1 *M*	Water
Nitrofurantoin (sodium)	Water	Water
Oxacillin	Water	Water
p-Aminosalicylic acid	Water	Water
Penicillin G	Water	Water
Piperacillin	Water	Water
Polymyxin B or E	Water	Water
Rifampin	Methanol	Water
Spectinomycin	Water	Water
Streptomycin	Water	Water
Sulfonamides[e]	NaOH, 0.1 *M*	Water
Tetracycline	Water	Water
Tobramycin	Water	Water
Ticarcillin	Water	Water
Trimethoprim[f]	HCl, 0.05 *M*	Water
Trimethoprim lactate	Water	Water
Vancomycin	Water	Water

[a] Dilute with water after the first 1:10 dilution with phosphate buffer at pH 6.0.

[b] Other cephalosporins can be dissolved in water or phosphate buffer at pH 6. Should cephradine fail to dissolve in water, add a small amount (e.g., 20 μl) of 5 to 10% $NaHCO_3$ solution. Phosphate buffer at pH 6 should be used to dissolve cephaloglycin and cefaclor.

[c] Alcohol solutions of erythromycin are unstable due to formation of esters and should be diluted immediately. Solutions in acetone are more stable and can be stored. Water has also been used as a diluent.

[d] If using the sodium salt, dissolve contents of preweighed vial in water and dilute to appropriate concentration. For the diammonium salt, dissolve salt at 20,000 μg/ml in 0.08 *M* HCl. Let stand 1½ to 2 hr at room temperature. Dilute to give 10,000 μg/ml stock solution with 0.1 *M* phosphate buffer, pH 8. Swirl while adding the phosphate buffer to avoid exposing the drug to local concentrations at pH 8. Alternatively, a 1,000-μg/ml stock solution can be prepared by dissolving the diammonium salt at 10,000 μg/ml in 0.04 *M* HCl. Let stand 1½ to 2 hr and dilute with 9 volumes of 0.1 *M* phosphate buffer at pH 6.

[e] Use 1.0 ml of 0.1 mol/liter NaOH per 10 mg of antimicrobial to dissolve. Dilute with water.

[f] Use 1.0 ml of 0.05 mol/liter HCl per 10 mg of trimethoprim to dissolve. Dilute with water. Solution is stable indefinitely at 4°C.

Stock solutions containing high concentrations of antimicrobials do not usually need to be sterilized; however, sterile water or buffers should be used in their preparation. Membrane filtration should be used when it is necessary to sterilize an antimicrobial solution to minimize loss due to adsorption. Rifampin and amphotericin B solutions should not be filtered. Antimicrobials can also adsorb to the walls of glass vessels, so that polypropylene or polyethylene vials and labware are recommended for preparation and storage of solutions containing low concentrations. Ad-

sorption to surfaces is decreased in the presence of protein or broth media.

Stability of antimicrobial solutions varies greatly, and data regarding maximum storage times at −20°C or −60°C are generally lacking. Stock solutions of most antimicrobials will remain stable for at least 6 months at −20°C and longer at −60°C in concentrations of 1000 μg/ml or greater. Ampicillin and amoxicillin are unstable, particularly in concentrations greater than 1000 μg/ml, and should be stored no longer than 6 weeks at −20°C or 6 months at −60°C. Cefaclor and rifampin are even less stable and should be prepared freshly with each use. Antimicrobials are often more unstable in serum than in buffer. The β-lactam antimicrobials should not be stored in serum even in the frozen state. Once thawed for use, solutions of antimicrobials should never be refrozen.

B. Buffers

Preparation of various pH phosphate buffers, each at a concentration of 0.1 mol/liter, is described in Table 4.4–4. Analytical grade chemicals and distilled or deionized water should be used. Solutions may be sterilized by autoclaving at 121°C for 15 min with very little change in pH. When necessary, the pH should be adjusted by addition of 1 mol/liter NaOH before, or 0.1 mol/liter H_3PO_4 after, dilution to the final volume (1.0 liter). Phosphate buffers can deteriorate rapidly from microbial growth and should not be stored longer than 6 months.

III. Testing Procedures

A. General Considerations

Susceptibility testing is usually carried out by either the disk-diffusion technique or a dilution method. In recent years disk elution-methods

Table 4.4–4. Preparation of 0.1 mol/liter Phosphate Buffers

	Amount (g) dissolved in water to make 1.0 liter of solution	
Desired pH (±0.2)	KH_2PO_4 (MW 136.09)	K_2HPO_4 (MW 174.18)
6	11.9	2.2
7	5.2	10.8
8	0.90	16.3

have gained acceptance in automated devices, such as Autobac (General Diagnostics) and MS-2 (Abbott Laboratories).

Dilution tests are performed by inoculating a standardized suspension of the organism into serial dilutions of the antimicrobial agent prepared in broth or in agar. The primary purpose of these tests is to obtain a quantitative result that is expressed as the MIC. Such results may be helpful in determining the degree of susceptibility of an organism, thereby facilitating decisions regarding dosage and the route of administration of an antimicrobial agent, or they can provide a more precise estimate of the activity of an antimicrobial agent against an organism classified as intermediately susceptible by the disk-diffusion test (Table 4.4–1). In the latter instance, infections may be successfully and preferentially treated with ordinary dosages of relatively nontoxic drugs at sites where these drugs are highly concentrated, as in the urinary tract. Dilution tests are suggested to confirm resistance to aminoglycosides as determined by the disk-diffusion test. Finally, dilution tests are generally recommended for determining the susceptibility of fastidious organisms.

Table 4.4–5. Concentrations of Antimicrobials Tested by Agar Dilution at the Mayo Clinic

	Concentration (μg/ml)										
Antimicrobial	0.03	0.12	0.5	1	2	4	8	16	32	64	128
Amikacin							X	X			
Ampicillin					X		X	X			X
Cefazolin							X	X			X
Cefoperazone								X	X		
Cefoxitin							X	X			X
Ceftizoxime							X	X	X		
Cefuroxime							X	X			X
Chloramphenicol							X	X			
Clindamycin			X	X		X					
Erythromycin			X	X		X					
Gentamicin				X	X	X					
Mezlocillin								X	X	X	X
Moxalactam							X	X	X		
Nalidixic acid								X			
Nitrofurantoin									X		
Oxacillin				X	X						
Penicillin	X	X		X			X				
Piperacillin								X	X	X	X
Tetracycline				X		X	X				
Trimethoprim/ sulfamethoxazole			0.5/ 9.5		2/ 38						
Trimethoprim							X				

The performance of dilution tests has been facilitated by the use of replicating devices for delivering inocula onto agar[37] or into broth in microdilution trays. Further simplification of the agar dilution method has been achieved by using expanded dilution steps (Table 4.4–5) that are selected to discriminate between resistant and susceptible strains (Table 4.4–6). It should be emphasized, however, that the proper interpretation of the results of dilution tests requires a substantial fund of knowledge about the pharmacology of antimicrobial agents and the mechanisms of microbial resistance.

For most purposes the qualitative results provided by the disk-diffusion susceptibility test are sufficient. The principle of this test is that the diameter of a zone of inhibition produced about an antimicrobial-impregnated paper disk applied to an agar surface containing a fresh standardized inoculum relates approximately linearly to the logarithm of the MIC. By determining the MIC values and zone diameters for a large number of organisms with the same antimicrobial, it is possible to construct a regression line by using the formula of least squares (Figure 4.4–1). Based on the principles described above for defining the MIC for a susceptible strain, the zone diameter correlate of this MIC can be extrapolated from the regression line. There are usually a number of organisms with MIC values above that defined as susceptible and below that defined as resistant. These organisms are termed intermediately susceptible. While it is often possible to establish zone diameter interpretative criteria reliably by inspecting the regression line for any given antimicrobial, greater objectivity and reliability is achieved with the error rate-bounded method of classification described by Metzler and DeHaan.[23] This method requires definition of susceptibility according to the MIC values and pharmacokinetics of the drug and specification of the limits for false-resistant and false-susceptible classifications. In general, the recommended limit for false-susceptible classification is 1%, while that for false-resistant classification, a relatively less serious error clinically, is 5%. The rate of intermediate susceptibility is also kept small.

B. Dilution Tests

1. Agar Dilution

Agar dilution has been in routine use for susceptibility testing at the Mayo Clinic for at least 30 years,[17] and it has been recommended as a reference method by an international collaborative study (ICS) sponsored by the World Health Organization (WHO).[14] Details of the reference method have been published elsewhere.[14,25,44] While strict adherence to the details of the reference method is essential when performing reference work, assessing the accuracy and reliability of new susceptibility

Table 4.4–6. Clinically Oriented MIC Guidelines[a]

Antimicrobial	Minimal inhibitory concentration (μg/ml)			
	Susceptible	Moderately susceptible	Resistant	Conditionally susceptible[b]
Amikacin	≤8	16	>16	—
Ampicillin[c]:				
Gram-negative bacilli	≤8	16	>16	≤128
Enterococci	≤8	—	>8	≤128
Non-enterococcal streptococci and *Haemophilus influenzae*[d]	≤2	—	>2	—
Cefazolin[e]	≤8	16	>16	≤128
Cefoperazone	≤16	32	>32	—
Cefotaxime	≤8	16–32	>32	—
Cefoxitin	≤8	16	>16	≤128
Ceftizoxime	≤8	16–32	>32	—
Cefuroxime	≤8	16	>16	≤128
Chloramphenicol	≤8	16	>16	—
Clindamycin	≤0.5	1–4	>4	—
Erythromycin	≤0.5	1–4	>4	—
Gentamicin	≤2	4	>4	—
Mezlocillin	≤16	32–64	>64	≤128
Moxalactam	≤8	16–32	>32	—
Nalidixic acid	≤16	—	>16	—
Nitrofurantoin	≤32	—	>32	—
Oxacillin	≤2	—	>2	—
Penicillin:				
Staphylococci[f]	≤0.03	—	>0.12	—
Enterococci	≤8	—	>8	—
Non-enterococcal streptococci and other Gram-positive organisms	≤0.12	1	>1	—
Piperacillin	≤16	32–64	>64	≤128
Tetracycline	≤1	2–8	>8	—
Trimethoprim/sulfamethoxazole	≤0.5/9.5	1/19–2/38	>2/38	—
Trimethoprim	≤8	—	>8	—
Vancomycin	≤4	8	>8	—

[a] Based primarily on NCCLS Tentative Standard [M7-T (modified)]: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. National Committee for Clinical Laboratory Standards, 1983. Changes have been made for some antimicrobials to account for testing practices and usage at the Mayo Clinic.

[b] Describes strains that are normally considered resistant. Uncomplicated infections with these strains at sites where drug is highly concentrated, as in the lower urinary tract, might be amenable to treatment provided no alternative and more active drug is available and accepta- ▷

test techniques, and evaluating the in vitro activity of new antimicrobial agents, certain deviations from this method can be made for routine testing purposes without significant alteration of the results. With these alterations the agar dilution method represents the most economical approach for the large laboratory determining the susceptibility of at least 30 organisms daily, particularly when it is linked with a replica plating method of identification.[10]

Agar Dilution Method

Purpose:
To determine quantitatively the susceptibility of a bacterial isolate.

Principle:
A standardized inoculum of the organism is applied to the surface of agar plates containing various dilutions of antimicrobials. The lowest concentration of each antimicrobial that completely inhibits growth of the organism is recorded as the MIC for that drug. This procedure is slightly modified from methods recommended by the WHO-ICS[14] and the NCCLS.[25]

Specimen:
The inoculum must be prepared from a pure culture or well isolated colonies of an organism.

Materials and reagents:

1. McFarland No. 0.5 and No. 1.0 barium sulfate turbidity standards (p. 285).[2]
2. Mueller-Hinton agar (BBL Microbiology Systems) prepared with an

---

Footnotes to Table 4.4–6 continued

ble. When no value is listed, either a value for conditional susceptibility is not recognized or an appropriate concentration is not tested. These values are reported for isolates from the lower urinary tract only.

[c] Includes amoxicillin and ampicillin analogues.

[d] *Haemophilus influenzae* that produce β-lactamase are resistant to ampicillin. Rare strains that do not produce β-lactamase may also be resistant.

[e] Applies to other first generation cephalosporins, such as cefaclor, cephradine, cephalexin, cefadroxil, and cephalothin.

[f] All penicillinase-producing staphylococci are resistant to penicillin. An MIC $\leq$0.03 μg/ml usually implies lack of penicillinase production, and an MIC >0.12 μg/ml implies enzyme production. Strains with MICs of 0.06 or 0.12 μg/ml may or may not produce the enzyme, and resistance can be determined by direct measurement of enzyme production.

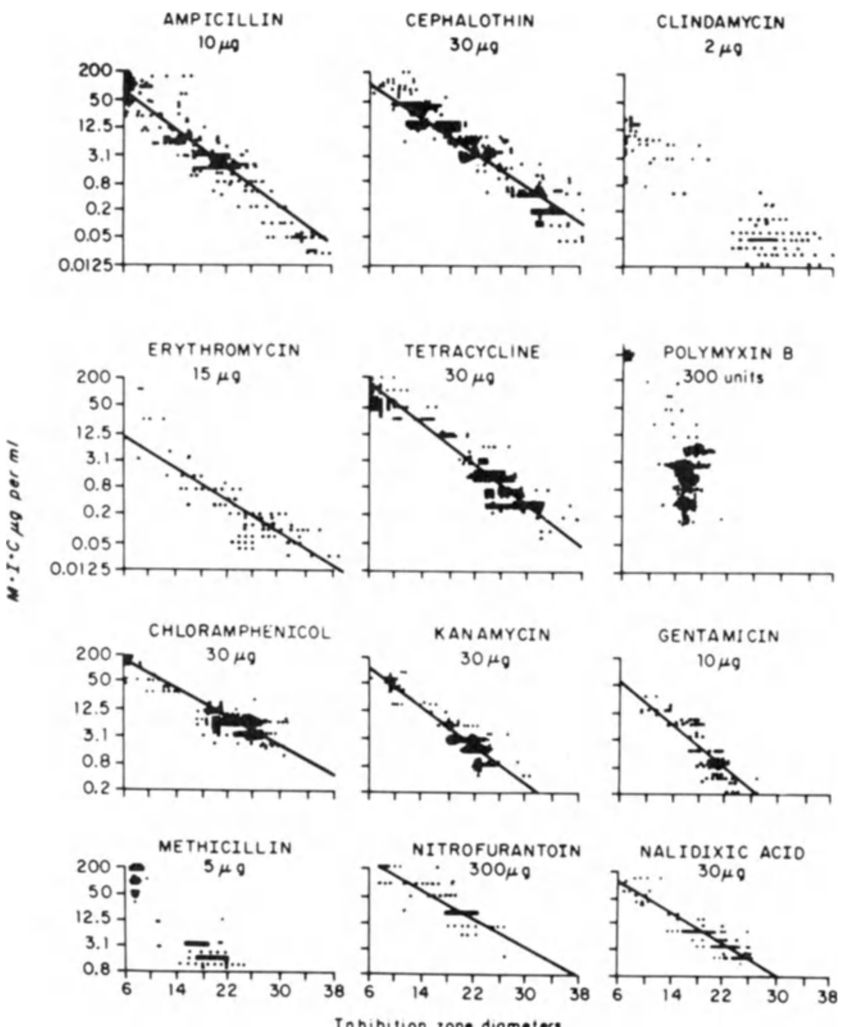


Figure 4.4–1. Regression lines for various antimicrobials showing a linear relationship between inhibition zone diameter and the logarithm of the MIC. (From Matsen, J. M., and Barry, A. L. Susceptibility Testing: Diffusion Test Procedures. *In* Lennette, E. H., Spaulding, E. H., and Truant, J. P., *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., 1974. Used by permission.)

additional 15 g/liter of agar is adjusted to pH 7.4 and is dispensed into screw-capped bottles containing 100 ml or 200 ml each.

3. Mueller-Hinton broth (MHB), Trypticase soy broth (TSB), or Todd-Hewitt broth (BBL Microbiology Systems).
4. Inocula-replicating apparatus that delivers 0.1 µl of inoculum per spot using pins approximately 1 mm in diameter.

Media:
The NCCLS and WHO-ICS reference methods for agar dilution tests recommend that media containing various concentrations of antimicrobial be prepared by adding 1 part of an appropriate dilution of antimicrobial to 9 parts of molten agar.[14,25] A suggested dilution protocol for use with these methods is shown in Table 4.4–7. This approach is easy to follow and is not subject to the same extent to the cumulative error that occurs with serial dilution. For routine work, the ratio of antimicrobial solution to agar can be as high as 1:100, and the volume of agar used per 100 mm petri dish (either round or square) can be reduced from 25 ml to 10 ml. These measures provide savings in the amount of antimicrobial and agar used without affecting the accuracy or reproducibility of results, provided the agar and antimicrobial are mixed thoroughly.[36] Further economy is accomplished by limiting the concentrations tested to those included in Table 4.4–5. An example of a protocol for preparing agar dilutions using these economy measures is shown in Table 4.4–8.

To prepare media for testing, screw-capped bottles or tubes containing molten Mueller-Hinton agar are allowed to equilibrate to 50°C in a water bath. Supplements for growth of fastidious organisms are added if necessary, followed by the appropriate volume of antimicrobial solution. After thorough mixing, the agar is poured into 100-mm round petri dishes on a level surface and allowed to harden. Routinely, 100 ml or 200 ml of agar is distributed into 6 or 12 petri dishes, respectively. The plates may be used immediately or stored at 4°C. For routine and most reference

Table 4.4–7. Antimicrobial Dilution Protocol for Agar Dilution Method[a]

Antimicrobial solution		+ Water (ml)	= Concentration (μg/ml)	Final concn at 1:10 dilution in agar (μg/ml)
Volume (ml)	Concentration (μg/ml)			
6.4	2000	3.6	1280	128
2	1280 (from above)	2	640	64
1	1280 (from above)	3	320	32
1	1280 (from above)	7	160	16
2	160 (from above)	2	80	8
1	160 (from above)	3	40	4
1	160 (from above)	7	20	2
2	20 (from above)	2	10	1
1	20 (from above)	3	5	1.5
1	20 (from above)	7	2.5	0.25
2	2.5 (from above)	2	1.2	0.12
1	2.5 (from above)	3	0.6	0.06
1	2.5 (from above)	7	0.3	0.03

[a] Adapted from Ericsson and Sherris.[14]

Table 4.4–8. Sample Antimicrobial Dilution Protocol to Obtain Expanded Dilution Steps

Desired final concentration in agar (μg/ml)	Volume (ml) of antimicrobial solution added to 200 ml agar				
	20,000 μg/ml	10,000 μg/ml	1000 μg/ml	100 μg/ml	10 μg/ml
256	2.56				
128		2.56			
64		1.28			
32			6.4		
16			3.2		
8			1.6		
4			0.8		
2			0.4		
1			0.2		
0.5				1	
0.25				0.5 or	5.0
0.12				0.24 or	2.4
0.06				0.12 or	1.2
0.03					0.6

work,[25] plates can be stored for up to 1 week, during which time less than 1 to 5% of activity is lost.[33] Plates that have been refrigerated should be permitted to equilibrate to room temperature before inoculation.

Although 100-mm-square petri dishes offer more spots than 100-mm-round dishes, as well as the convenience of a grid embossed on the bottom of the dish for identifying the location of each organism, the round dish is considerably cheaper. When using a round dish, a nondiffusable dye, such as India ink, can be added to one inoculum well to help maintain proper orientation of the dish.

Procedure:

1. Portions of four or five discrete representative colonies of the organism to be tested are inoculated into 2 ml of indole broth. It is preferable not to use colonies growing on colistin-nalidixic acid agar (CNA). To prepare an inoculum for a direct susceptibility test on positive cultures of blood or cerebrospinal fluid, add an aliquot of the culture to 2 ml of broth. (The susceptibility tests in these instances must be repeated in standard fashion after isolated colonies are obtained in subcultures only if the inoculum could not be standardized on initial testing or a mixture of organisms is isolated.) Each of the four control strains that were plated out on the previous day (p. 287) is inoculated into broth.

Each inoculated tube must be properly labelled as to the source and type of organism.

2. Incubate the inoculated tubes for 2 to 3 hr at 35°C.
3. After incubation, each tube is agitated on a Vortex mixer. The turbidity of the broth culture should be between that of a No. 0.5 and a No. 1 McFarland standard, which corresponds to a bacterial density of about 10^8 CFU/ml. If the turbidity of the broth culture is too low, more colonies of the organism may be added, or preferably, the broth may be reincubated. If the turbidity is excessive, the broth culture should be diluted with broth until its turbidity is in the proper range. If necessary, and when there are a sufficient number of isolated colonies of the same morphology, one may suspend these directly in broth to the correct turbidity. This last procedure should be followed when testing *Haemophilus* or meningococci.
4. Each tube of adjusted inoculum is agitated on a Vortex mixer, and an aliquot is transferred using a Pasteur pipette to a well in the seed plate. Each well should be ½ to ¾ full. One well is filled with India ink to serve as a marker for orientation.
5. The agar plates containing antimicrobials are inoculated using a replica plating device (Figure 4.4–2). This step should be performed within 30 min of adjusting the inocula. Each rod of the replica plating device delivers 0.1 μl of inoculum per spot, which provides an inoculum of about 10^4 CFU. Each plate must be inspected to make certain that

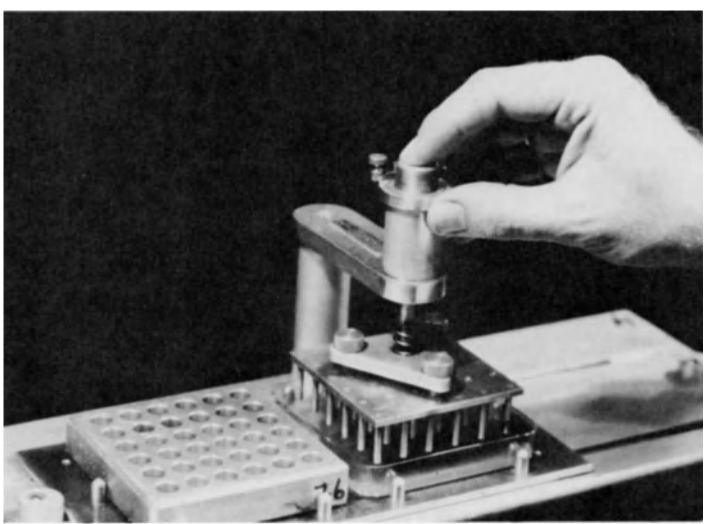


Figure 4.4–2. Inocula-replicating apparatus with seed plate at left containing 36 reservoirs. Plate with medium containing antibiotic is being inoculated. (From Washington, J. A., II. *Mayo Clin. Proc.* **44:**811, 1969. Used by permission.)

there is a drop of inoculum for each filled well of the seed plate. If there is a skip, the organism should be retested; the agar should not be restamped.

6. Incubate all plates at 35°C for 16 to 18 hr without added CO_2, except that plates inoculated with meningococci or *Haemophilus* and blood agar plates with penicillin are incubated in an atmosphere of 5 to 10% CO_2. Agar plates with anaerobic bacteria are incubated promptly in an anaerobic incubator (p. 373).
7. After use, the seed plates and inoculating prongs are soaked overnight in 70% ethanol, scrubbed clean with a brush, and wrapped in a cloth towel. This pack is placed into a large glass petri dish and autoclaved.

Controls:
Each plate is inoculated with the control organisms described above. Acceptable limits for MIC values are shown in Table 4.4–9. In addition, two agar plates (one with and one without 5% lysed horse blood) without

Table 4.4–9. Acceptable MIC Values for Control Organisms

	Acceptable MIC (μg/ml) for:			
Antimicrobial	*Escherichia coli* ATCC 25922	*Staphylococcus aureus* ATCC 29213	*Pseudomonas aeruginosa* (Mayo strain)	*Streptococcus faecalis* ATCC 29212
Amikacin	8	8	8	16
Ampicillin	8	2	>128	2
Cefazolin	8	8	>128	8 or 16
Cefoperazone	16	16	16	—
Cefoxitin	8	8	>128	—
Cefuroxime	8	8	>128	—
Chloramphenicol	4	8	>16	8
Clindamycin	>4	0.5	>4	≥4
Erythromycin	>4	0.5 or 1	>4	1 or 4
Gentamicin	1	1	1	2 or 4
Mezlocillin	16	16	32	—
Moxalactam	8	8 or 16	16	—
Nalidixic acid	16	>16	>16	>16
Netilmicin	1	1	1	—
Nitrofurantoin	32	32	>32	32
Oxacillin	>2	1	>2	>2
Penicillin	>1	≥1	>1	≥1
Piperacillin	16	16	16	—
Tetracycline	1	1	≥8	>8
Trimethoprim	8	8	>8	—
Trimethoprim/ sulfamethoxazole	0.5/9.5	0.5/9.5	≥2/38	—

antimicrobials are inoculated to confirm the viability of the isolate being tested, determine if blood supplementation might be required for growth of some bacteria, and assist in detection of contaminants. To further facilitate detection of contaminants, each well is subcultured at the conclusion of the procedure to half of a blood agar plate.

Interpretation:
The plates are examined using reflected or transmitted light after incubation. The MIC is interpreted according to WHO-ICS and proposed NCCLS recommendations.[14,25] It is the lowest concentration of antimicrobial resulting in complete inhibition, a very fine barely visible haze, or no more than a single colony. The MIC of each antimicrobial against each control strain is recorded and checked for correspondence with previously established values (Table 4.4–9). Any deviation in MIC among the controls requires careful analysis of the possible causes of the deviation, for example, contamination of the inoculum or improper preparation or deterioration of the antimicrobial agent. If contamination of the control can be excluded, it is likely that repeat testing of all organisms with a new set of plates will be required. The MIC and identity of each isolate are recorded along with the date of testing and the site of inoculation on the agar plate.

Comments:

1. When testing *Haemophilus,* the agar should be supplemented to contain 1% hemoglobin and 1% IsoVitaleX (BBL Microbiology Systems), Supplement XV (Difco Laboratories), or 5% lysed horse blood. Routinely, *H. influenzae* is tested for β-lactamase production and against ampicillin. Other antimicrobials are tested upon request in accord with the protocol shown in Table 4.4–10.
2. To test the susceptibility of meningococci, freshly prepared Mueller-Hinton agar plates containing antimicrobial concentrations shown in Table 4.4–11 are used.
3. *Campylobacter* sp. are tested against the antimicrobial dilutions shown in Table 4.4–12. The test can be performed as described above, except plates are incubated in an atmosphere containing 5 to 7% O_2 and 4 to 8% CO_2 for 18 to 24 hr; a few strains may require an additional 24-hr incubation.
4. Tests with very few strains can be inoculated using a calibrated loop to deliver on the agar 1 μl of a 1:10 dilution of the inoculum broth described above.
5. Maximum efficiency and economy are derived from the agar dilution method when there is full utilization of the inoculum spots available. The costs per organism in this method exceed those of disk diffusion when fewer than 10 organisms are tested on a daily basis. When there

Table 4.4–10. Antimicrobial Testing Protocol for *Haemophilus influenzae,* type b

Antimicrobial	Comment
Ampicillin	Do β-lactamase test. If negative, test ampicillin at 0.13 to 4 μg/ml using 10^4 CFU/spot.
Cephalosporins	Test moxalactam instead.
Chloramphenicol	Test at 0.5 to 4 μg/ml.
Moxalactam	Test only if β-lactamase producer. Use concentrations of 0.06 to 8 μg/ml and inocula of 10^4 CFU/spot and 10^6 CFU/spot.[a]
Rifampin[b]	Test at 0.5 to 8 μg/ml.
Trimethoprim	Do MBC.[c]
Trimethoprim/sulfamethoxazole	Do MBC of trimethoprim; do not test combination.

[a] For broth-dilution testing, use inocula of 10^5 CFU/ml and 10^7 CFU/ml.
[b] Most isolates have MICs ≤1 μg/ml; resistant organisms usually have MICs ≥25 μg/ml.
[c] Efficacy for eradication of carrier state correlates with an MBC ≤2 μg/ml.

Table 4.4–11. Concentrations of Antimicrobials to be Tested against *Neisseria meningitidis*

	Concentration (μg/ml)							
Antimicrobial	16	8	4	2	1	0.5	0.25	0.12
Sulfonamide	X	X	X	X	X	X	X	
Rifampin					X	X	X	X
Minocycline				X	X	X	X	

Table 4.4–12. Testing Protocol for *Campylobacter* sp.

Organism	Antimicrobial	Concentrations (μg/ml)
C. jejuni[a]	Tetracycline	1,4,8
	Erythromycin	0.1,1,4
C. fetus subsp. *fetus*	Tetracycline	1,4,8
	Erythromycin	0.1,1,4
	Gentamicin	1,2,4
	Ampicillin	2,8,16
	Chloramphenicol	4,8,16

[a] *C. jejuni,* if isolated from a source other than feces, is tested against the same antimicrobials as *C. fetus* subsp. *fetus.*

are fewer than 10 organisms or when it is necessary to test a single isolate separately (e.g., at night or on weekends) an agar microdilution technique can be used. In this procedure, agar containing each dilution of antimicrobial used routinely is pipetted into the wells of a microdilution tray. This is done most conveniently at the same time plates for routine testing are prepared. Each of the agar-filled wells is inoculated with 10 μl of a 1:100 dilution of an inoculum broth prepared as described above. Incubation and interpretation are the same as for the routine procedure. Each time a batch of plates is prepared (weekly), four plates are used to test the usual control organisms.

2. Broth Dilution

Dilution tests may be carried out in broth either as an alternative to agar for determining the MIC or as a means of determining the minimal bactericidal concentration (Section 12.2). The method is rather impractical and cumbersome for routine purposes unless adapted to a microdilution technique. Detailed descriptions of broth dilution techniques are available.[16,25,44] The MICs by broth microdilution for gram-negative bacilli are often a two-fold dilution lower than those obtained by broth macrodilution.[5] The microdilution procedure may also fail to detect some β-lactamase–producing staphylococci, and penicillin-susceptible organisms should be confirmed by testing for the absence of β-lactamase.[40]

C. Disk-Diffusion Tests

The use of a single, high-content risk for determination of antimicrobial susceptibility was first described in Sweden by Ericsson et al.[13] In 1954 and, subsequently, in the United States by Bauer et al.[8] This procedure, as well as a seeded agar overlay method described by Barry et al.[4] has been approved for clinical laboratory use in the United States by the Food and Drug Administration[15] and by the National Committee for Clinical Laboratory Standards.[26] Disk-diffusion tests are not used at the Mayo Clinic, except for research, and the following descriptions are intended only to highlight certain aspects of the methodology. Detailed procedures are published elsewhere and should be followed exactly to obtain accurate and reproducible results.[6,26]

1. Preparation of Agar Plates

Bottles containing molten Mueller-Hinton agar are allowed to equilibrate to 50°C in a water bath. If necessary, 5% defibrinated sheep, human, or horse blood may be added to the agar or the blood may be chocolatized. The pH of the medium should be between 7.2 and 7.4. The medium is poured into sterile petri dishes on a level surface to a depth of 4 mm

and allowed to harden. The medium is allowed to dry for 15 to 30 min before use or storage at 4°C. Agar plates to be stored for longer than 7 days should be wrapped in plastic to prevent excessive evaporation of moisture. Sterility tests of agar plates should include incubation of several samples of each lot at 35°C for 24 to 72 hr.

2. Preparation of Inoculum

a. Standard Method

Portions of four or five well-isolated colonies, which are similar morphologically, are touched in turn with a sterile wire loop and inoculated into a tube containing 5 ml of a broth medium, such as Trypticase soy broth. This medium is incubated at 35°C until the turbidity matches that of a No. 0.5 McFarland standard.

A sterile cotton swab on a wooden stick is dipped into the broth suspension of organisms within 15 min after the turbidity is adjusted. Excess inoculum is removed by rotating the swab several times against the wall of the tube above the fluid level. The entire surface of the agar is then evenly streaked in three directions.

b. Agar Overlay Method

Portions from four or five isolated colonies are inoculated into 0.5 ml of brain heart infusion broth and incubated at 35°C for 4 to 8 hr. A 1 μl calibrated loopful of the broth culture is transferred to 9.0 ml of a 1.5% aqueous solution of molten agar that has been allowed to equilibrate from 1 to 8 hr in a 45° to 50°C heating block. After mixing the seeded agar by gentle inversion, it is poured and spread over the surface of a Mueller-Hinton agar plate (150 by 15 mm) that has been allowed to equilibrate to room temperature.

3. Disk Placement

Within 15 min after the surface of the agar has been inoculated, antimicrobial disks are applied with sterile forceps or a dispenser and are tamped onto the agar with sterile forceps. The space between disks must be sufficient to preclude overlapping of zones of inhibition, and the disks must be no less than 10 to 15 mm from the edge of the dish.

4. Incubation

The plates are inverted and incubated, within 30 min of inoculation, at 35° to 35°C for 16 to 18 hr. Incubation in an atmosphere containing CO_2 will invalidate the results of the test.

5. Interpretation

The diameter of each zone of inhibition is measured to the nearest millimeter through the underside of the plate using a ruler, calipers, or template with reflected light and the unaided eye. Swarming by *Proteus* is ignored, as is slight growth with sulfonamides. When the agar contains blood, zones should be measured from the surface of the agar. Zone diameters are interpreted as shown in Table 4.4–13.

D. Disk-Elution Tests

Antimicrobial agents have been shown to elute completely from paper disks within 30 min at 36°C when agitated.[22] This means of delivery of antimicrobials into broth has been used for automated susceptibility testing systems, such as the MS-2 (Abbott Laboratories) and Autobac (General Diagnostics),[41] as well as for testing anaerobic bacteria.[40]

E. Detection of β-Lactamase

The primary mechanism by which staphylococci, *Haemophilus influenzae,* and *Neisseria gonorrhoeae* are resistant to penicillins is by their elaboration of β-lactamases that hydrolyze the β-lactam ring to produce penicilloic acid. While β-lactamase is usually the major mechanism of producing resistance to penicillins among gram-positive cocci, β-lactamases are only one of several factors contributing to resistance to penicillins and cephalosporins by gram-negative bacilli.[39] Other factors include the outer membrane of gram-negative bacteria which constitutes a hydrophobic barrier layer and crypticity which refers to the interaction of the cell-bound β-lactamase and the barrier function of the cell envelope.[39]

The detection of β-lactamase constitutes a rapid means of determining the susceptibility of organisms, such as those named above, to penicillinase-susceptible penicillins (e.g., penicillin G or V). The methods most commonly used are those in which penicilloic acid is detected by its ability to reduce iodine or alter pH, and those in which an electron shift in a chromogenic cephalosporin gives rise to colored products. The iodometric and acidimetric methods are easy to use but are also less sensitive than the chromogenic method. Many variations of these approaches have been published, only a few representative methods of which will be described in detail in this section.

Table 4.4–13. Zone Diameter Interpretative Criteria and Approximate Minimum Inhibitory Concentration (MIC) Correlates[a]

Antimicrobial agent	Disk content (μg)	Zone diameter (mm)			Approximate MIC correlates (μg/ml)[b]	
		Resistant	Intermediate or moderate susceptibility	Susceptible	Resistant	Susceptible
Amikacin[c]	30	≤14	15–16	≥17	≥32	≤16
Ampicillin[d]						
vs gram-negative						
enteric organisms	10	≤11	12–13	≥14	≥32	≤8
vs *Haemophilus*	10	≤19	—	≥20	≥4	≤2
vs enterococci	10	≤16	≥17	—	≥16	—
Nonenterococcal						
streptococci	10	≤21	22–29	≥30	≥4	≤0.12
Cefazolin	30	≤14	15–17	≥18	≥32	≤8
Cefoperazone	75	≤15	16–20	≥21	≥64	≤16
Cefoxitin	30	≤14	15–17	≥18	≥32	≤8
Ceftizoxime	30	≤10	11–19	≥20	—	—
Cefuroxime	30	≤14	15–17	≥18	≥32	≤8
Cephalothin	30	≤14	15–17	≥18	≥32	≤8
Chloramphenicol	30	≤12	13–17	≥18	≥25	≤12.5
Clindamycin	2	≤14	15–16	≥17	≥2	≤1
Erythromycin	15	≤13	14–17	≥18	≥8	≤2
Gentamicin[c]	10	≤12	13–14	≥15	≥8	≤4
Mezlocillin	75	≤12	13–15	≥16	≥256	≤64
Moxalactam	30	≤14	15–22	≥23	≥64	≤8
Nalidixic acid	30	≤13	14–18	≥19	≥32	≤12
Nitrofurantoin	300	≤14	15–16	≥17	≥100	≤25

Oxacillin						
vs staphylococci[e]	1	≤10	11–12	≥13	—	≤1
vs pneumococci[f]	1	≤19	—	≥20	—	≤0.06
Penicillin G						
vs staphylococci	10 units	≤28	—	≥29	β-lactamase	≤0.1
vs *N. gonorrhoeae*	10 units	≤19	—	≥20	β-lactamase	≤0.1
vs enterococci	10 units	≤14	≥15	—	≥16	—
vs other gram-positive cocci	10 units	≤19	20–27	≥28	4	≤2
Piperacillin	100	≤14	15–17	≥18	≥256	≤64
Tetracycline	30	≤14	15–18	≥19	≥16	≤4
Trimethoprim	5	≤10	11–15	≥16	≥16	≤4
Trimethoprim/ sulfamethoxazole	1.25/23.75	≤10	11–15	≥16	≥8/152	≤2/38
Vancomycin	30	≤9	10–11	≥12	—	≤5

[a] Adapted from National Committee for Clinical Laboratory Standards,[26] Thornsberry et al.,[40] Thornsberry and Hawkins,[42] and manufacturer's prescribing information. See reference 26 for additional antimicrobials and comments.

[b] These correlates are not meant to be used for interpretative criteria with dilution testing.

[c] Zone sizes obtained with aminoglycosides are very medium–dependent, particularly when testing *P. aeruginosa.*

[d] Class disk for ampicillin, amoxicillin, bacampicillin, cyclocillin, and hetacillin.

[e] Oxacillin is preferred as the class representative of the β-lactamase resistant penicillins, because it is more stable on storage and results have been standardized for testing pneumococci. Cloxacillin disks should not be used, because they fail to detect methicillin-resistant staphylococci.

[f] Oxacillin results predict penicillin susceptibility of pneumococci and are preferred.

Chromogenic Cephalosporin Test[24,28]

Purpose:
To detect β-lactamase.

Principle:
Nitrocefin (Glaxo) in solution is normally yellow. Upon opening of the β-lactam ring by a β-lactamase, conjugation of the dinitrostyryl group at position 3 with the dihydrothiazine ring is increased, thereby producing a change in color to red.

Reagent:

Nitrocefin solution

Nitrocefin (Glaxo)	5 mg.
Dimethyl sulfoxide	0.5 ml
Phosphate buffer, 0.1 mol/liter, pH 7.0	9.5 ml

Dissolve nitrocefin in dimethyl sulfoxide and dilute to 10 ml with phosphate buffer to provide a final concentration of 500 μg/ml. The solution is dispensed in 0.2 ml portions and frozen at −20°C until ready for use.

Procedure:

1. Saturate a small piece of filter paper with the nitrocefin reagent. Place the saturated paper in a closed petri dish to prevent it from drying out too rapidly.
2. Smear a small loopful or portion of a colony to be tested on the filter paper.

Interpretation:
An organism that produces β-lactamase will change the yellow color to red in the paper. Most reactions occur within 30 sec, but tests are finally read after 15 min.

Comment:
Disks impregnated with nitocefin are available commercially as Cefinase™ (Becton Dickinson, cat. no. 31617). These disks are used essentially as described above. An alternative chromogenic cephalosporin, PADAC™ (Calbiochem-Behring), changes from purple to yellow upon action of a β-lactamase.[34] This substrate is very sensitive to β-lactamases from gram-negative bacteria, but it should not be used to detect β-lactamase production from staphylococci.[3]

Iodometric Slide Test[32]

Purpose:
To detect β-lactamase.

Principle:
Starch and iodine react in solution to produce a purple color. Penicilloic acid, which is formed by the action of β-lactamase on penicillin, reacts with iodine making it unavailable for reacting with starch. The presence of β-lactamase is indicated by decolorization of (or failure to form) the starch-iodine complex.

Reagents:

1. 0.4% starch solution
   Dissolve 0.4 g of soluble starch in 100 ml of water. Sterilize by autoclaving. Store in refrigerator.
2. Iodine solution
   Dissolve 1.5 g of potassium iodine (KI) and 0.3 g of iodine in 100 ml of 0.1 mol/liter phosphate buffer, pH 6.4 (prepared by mixing 60 ml of pH 6 buffer and 40 ml of pH 7 buffer). Mix and let stand 15 min until solution turns dark brown.
3. Penicillin solution
   Add 1 ml of sterile water to a vial of penicillin (10^6 units). Withdraw the entire volume and freeze aliquots of 0.15 ml in small (1 dram) vials.

Procedure:

1. Add 1.1 ml of iodine solution to a vial of penicillin solution.
2. On a flamed slide, emulsify the organism to be tested in a drop of the penicillin-iodine solution forming a heavy suspension.
3. Add one drop of the starch solution.

Interpretation:
The mixture will turn purple initially. A positive test is indicated by clearing of the purple within 5 min. The entire mixture does not have to clear. Definite clear areas around clumps of organisms are sufficient for a positive test.

Comment:
Once the iodine and penicillin solutions are mixed, they must be used within 1 hr to avoid falsely positive results. This test requires a larger sample and is less sensitive than the nitrocefin test.

Acidimetric Test[31]

Purpose:
To detect β-lactamase.

Principle:
Opening of the β-lactam ring of penicillin produces penicilloic acid, which is more acidic than penicillin. Phenol red is used to indicate the production of acidity by the test organism in the presence of penicillin.

Reagents:

Phenol red solution
Dilute 2 ml of a 0.5% solution of phenol red with 16.6 ml of water. Add this solution to a vial containing 20×10^6 units of penicillin G. Then add 1 mol/liter NaOH dropwise until the solution just turns purple (pH 8.5). Aliquots can be stored at −20°C for up to 1 week.

Procedure:

1. Dip one end of a capillary into the phenol red solution and allow the capillary to fill a distance of 1 cm.
2. Scrape the filled end of the capillary across a colony of bacteria to be tested so that they form a plug in the end of the tube.
3. Incubate the tube at room temperature for 1 hr.

Interpretation:
The production of β-lactamase is indicated if the solution turns yellow.

References

1. Amyes, S. G. B., and Smith, J. T. Trimethoprim antagonists: Effect of uridine in laboratory media. *J. Antimicrob. Chemother.* 4:421, 1978.
2. Anhalt, J. P., and Washington, J. A., II. Appendix 1. Preparation and storage of antimicrobic solutions. *In* Lennette, E. H., Balows, A., Hausler, W. J., Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 495.
3. Anhalt, J. P., and Nelson, R. Failure of Padac test strips to detect staphylococcal β-lactamase. *Antimicrob. Agents Chemother.* 21:993, 1982.
4. Barry, A. L., Garcia, F., and Thrupp, L. D. An improved single-disk method for testing the antibiotic susceptibility of rapidly-growing pathogens. *Am. J. Clin. Pathol.* 53:149, 1970.
5. Barry, A. L., Jones, R. N., and Gavan, T. L. Evaluation of the Micro-Media system for quantitative antimicrobial drug susceptibility testing: A collaborative study. *Antimicrob. Agents Chemother.* 13:61, 1978.

6. Barry, A. L., and Thornsberry, C. Susceptibility testing: Diffusion test procedures. *In* Lennette, E. H., Balows, A., Hausler, W. J., Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980. p. 463.
7. Barry, A. L., Thornsberry, C., Jones, R. N., Fuchs, P. C., Gavan, T. L., and Gerlach, E. H. Reassessment of the "class" concept of disk susceptibility testing. *Am. J. Clin. Pathol.* **70**:909, 1978.
8. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**:493, 1966.
9. Beggs, W. H., and Andrews, F. A. Role of ionic strength in salt antagonism of aminoglycoside action on *Escherichia coli* and *Pseudomonas aeruginosa. J. Infect. Dis.* **134**:500, 1976.
10. Brown, S. D., and Washington, J. A., II. Evaluation of the Repliscan system for the identification of *Enterobacteriaceae. J. Clin. Microbiol.* **8**:695, 1978.
11. Bryan, L. E., and Van Den Elzen, H. M. Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: A model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrob. Agents Chemother.* **12**:163, 1977.
12. Ellner, P. D., and Johnson, E. Unreliability of direct antibiotic susceptibility testing on wound exudates. *Antimicrob. Agents Chemother.* **9**:355, 1976.
13. Ericsson, H., Högman, C., and Wickman, K. A paper disk method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents. *Scand. J. Clin. Lab. Invest. 6, Suppl.* **11**:1, 1954.
14. Ericsson, H. M., and Sherris, J. C. Antibiotic sensitivity testing: Report of an international collaborative study. *Acta Pathol. Microbiol. Scand.* (*B*) *Suppl.* **217**:1, 1971.
15. Food and Drug Administration. Rules and regulations: Antibiotic susceptibility discs. *Fed. Register* **37**:20525, 1972.
16. Gavan, T. L., and Barry, A. L. Microdilution test procedures. *In* Lennette, E. H., Balows, A., Hausler, W. J., Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 459.
17. Herrell, W. E., and Heilman, F. R. Subacute bacterial endocarditis. *In* Thomas, L. (ed.), *Rheumatic Fever: A Symposium.* Minneapolis, University of Minnesota Press, 1952, p. 265.
18. Hewitt, W. L., and McHenry, M. C. Blood level determinations of antimicrobial drugs. *Med. Clin. N. Am.* **62**:1119, 1978.
19. Johnson, J. E., and Washington, J. A., II. Comparison of direct and standardized antimicrobial susceptibility testing of positive blood cultures. *Antimicrob. Agents Chemother.* **10**:211, 1976.
20. Kenny, M. A., Pollock, H. M., Minshew, B. H., Casillas, E., and Schoenknecht, F. D. Cation components of Mueller-Hinton agar affecting testing of *Pseudomonas aeruginosa* susceptibility to gentamicin. *Antimicrob. Agents Chemother.* **17**:55, 1980.
21. McFarland, J. The nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J. Am. Med. Asso.* **49**:1176, 1907.
22. McKie, J. E., Borovoy, R. J., Dooley, J. F., Evanega, G. R., Mendoza, G.,

Meyer, F., Moody, M., Packer, D. E., Praglin, J., and Smith, H. Autobac 1—A 3-hour, automated antimicrobial susceptibility system: II. Microbiological studies. *In* Hedén, C.-G. and Illéni, T. (eds.), *Automation in Microbiology and Immunology.* New York, Wiley, 1975, p. 209.

23. Metzler, C. M., and DeHaan, R. M. Susceptibility tests of anaerobic bacteria: Statistical and clinical considerations. *J. Infect. Dis.* **130**:588, 1974.
24. Montgomery, K., Raymundo, J. R., and Drew, W. L. Chromogenic cephalosporin spot test to detect beta-lactamase in clinically significant bacteria. *J. Clin. Microbiol.* **9**:205, 1979.
25. National Committee for Clinical Laboratory Standards. Tentative standard M7-T. *Methods for Dilution Antimicrobial Tests for Bacteria that Grow Aerobically,* vol. 3, no. 2. Villanova, National Committee for Clinical Laboratory Standards, 1983.
26. National Committee for Clinical Laboratory Standards. Tentative standard M2-T3. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* vol. 3, no. 14. Villanova, National Committee for Clinical Laboratory Standards, 1983.
27. Nicas, T. I., and Bryan, L. E. Relationship between gentamicin susceptibility criteria and therapeutic serum levels for *Pseudomonas aeruginosa* in mouse infection model. *Antimicrob. Agents Chemother.* **13**:796, 1978.
28. O'Callaghan, C. H., Morris, A., Kirby, S. M., and Shingler, A. H. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. *Antimicrob. Agents Chemother.* **1**:283, 1972.
29. Pollock, H. M., Minshew, B. H., Kenny, M. A., and Schoenknecht, F. D. Effect of different lots of Mueller-Hinton agar on the interpretation of the gentamicin susceptibility of *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **14**:360, 1978.
30. Reller, L. B., Schoenknecht, F. D., Kenny, M. A., and Sherris, J. C. Antibiotic susceptibility testing of *Pseudomonas aeruginosa:* selection of a control strain and criteria for magnesium and calcium content in media. *J. Infect. Dis.* **130**:454, 1974.
31. Rosen, I. G., Jacobsen, J., and Rudderman, R. Rapid capillary tube method for detecting penicillin resistance in *Staphylococcus aureus. Appl. Microbiol.* **23**:649, 1972.
32. Rosenblatt, J. E., and Neumann, A. M. A rapid slide test for penicillinase. *Am. J. Clin. Pathol.* **69**:351, 1978.
33. Ryan, K. J., Needham, G. M., Dunsmoor, C. L., and Sherris, J. C. Stability of antibiotics and chemotherapeutics in agar plates. *Appl. Microbiol.* **20**:447, 1970.
34. Schindler, P., and Huber, G. Use of Padac, a novel chromogenic β-lactamase substrate, for the detection of β-lactamase producing organisms and assay of β-lactamase inhibitors/inactivators. *In* Brodbeck, U. (ed.), *Enzyme Inhibitors,* Weinheim, Verlag Chemie, 1980, p. 169.
35. Shahidi, A., and Ellner, P. D. Effect of mixed cultures on antibiotic susceptibility testing. *Appl. Microbiol.* **18**:766, 1969.
36. Snyder, R. J., Kohner, P. C., Ilstrup, D. M., and Washington, J. A., II. Analysis of certain variables in the agar dilution susceptibility test. *Antimicrob. Agents Chemother.* **9**:74, 1976.
37. Steers, E., Foltz, E. L., and Graves, B. S. An inocula replicating apparatus

for routine testing of bacterial susceptibility to antibiotics. *Antibiot. Chemother.* (*Basel*) 9:307, 1959.
38. Stemper, J. E., and Matsen, J. M. Device for turbidity standardizing of cultures for antibiotic sensitivity testing. *Appl. Microbiol.* **19**:1015, 1970.
39. Sykes, R. B., and Matthew, M. The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. *J. Antimicrob. Chemother.* **2**:115, 1976.
40. Thornsberry, C., Gavan, T. L., and Gerlach, E. H. *Cumitech 6:* New developments in antimicrobial agent susceptibility testing. Coordinating ed. Sherris, J. C. Washington, D.C., American Society for Microbiology, 1977.
41. Thornsberry, C., Gavan, T. L., Sherris, J. C., Balows, A., Matsen, J. M., Sabath, L. D., Schoenknecht, F. D., Thrupp, L. D., and Washington, J. A., II. Laboratory evaluation of a rapid, automated susceptibility testing system: Report of a collaborative study. *Antimicrob. Agents Chemother.* **7**:466, 1975.
42. Thornsberry, C., and Hawkins, T. M. *Agar Disc Diffusion Susceptibility Testing Procedure.* Atlanta, U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, 1977.
43. Washington, J. A., II, Snyder, R. J., Kohner, P. C., Wiltsie, C. G., Ilstrup, D. M., and McCall, J. T. Effect of cation content of agar on the activity of gentamicin, tobramycin, and amikacin against *Pseudomonas aeruginosa. J. Infect. Dis.* **137**:103, 1978.
44. Washington, J. A., II, and Sutter, V. L.: The dilution susceptibility test: Agar and macro-broth dilution procedures. *In* Lennette, E. H., Balows, A., Hausler, W. J., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 453.
45. Washington, J. A., II, Warren, E., and Karlson, A. G. Stability of barium sulfate turbidity standards. *Appl. Microbiol.* **24**:1013, 1972.
46. Waterworth, P. M. Uniformity in sensitivity test media. *J. Antimicrob. Chemother.* **4**:4, 1978.
47. Waterworth, P. M., and Del Piano, M. Dependability of sensitivity tests in primary culture. *J. Clin. Pathol.* **29**:179, 1976.

5

Anaerobic Bacteria

Jon E. Rosenblatt, M.D.

I. General Considerations

Anaerobes are bacteria that grow and metabolize only in an environment with a relative lack of oxygen. More specifically, anaerobes will not grow on the surface of agar plates in room air or in room air that has been augmented with as much as 10% CO_2. Most anaerobes grow well only in an atmosphere containing as little as 3 to 6 ppm O_2, although several species of *Actinomyces* and *Clostridium* are more aerotolerant, a characteristic that can, in fact, be used to help identify them. Establishing an anaerobic atmosphere will also generally lower the oxidation-reduction potential (E_h) of the medium used for growth of anaerobes. Although most anaerobes grow adequately at E_h levels around -10 mV, commonly employed anaerobic incubation methods reduce the media to much lower levels. Obligate anaerobes, which will not grow in air or CO_2, must be differentiated from facultatively anaerobic bacteria (most of the commonly recognized "aerobes") which grow well under anaerobic conditions, as well as in air or CO_2. Capnophilic (or "microaerophilic") bacteria (primarily streptococci) require room air enhanced with at least 4 to 5% CO_2 but also grow well in anaerobic incubation systems, which usually include excess CO_2. They can be differentiated from anaerobes by demonstrating their growth in environments that contain CO_2 (candle jar, CO_2 incubator) but are not anaerobic.

A. Taxonomy and Description of Anaerobic Bacteria

1. Clostridium

A taxonomic classification of clinically significant anaerobic bacteria is provided in Table 5–1. Anaerobic spore-forming gram-positive bacilli belong to the genus *Clostridium,* of which *C. perfringens* is the most commonly encountered and is responsible for gas gangrene (anaerobic myone-

Table 5–1. Classification of Clinically Significant Anaerobic Bacteria

- Spore-formers
  - *Clostridium* (gram-positive bacilli)
    - Lecithinase-producers
      - *C. perfringens*
      - *C. barati* (*C. paraperfringens*)
      - *C. bifermentans*
      - *C. novyi,* types A and B
      - *C. sordellii*
    - Non-lecithinase-producers
      - *C. butyricum*
      - *C. cadaveris*
      - *C. clostridiiforme*
      - *C. innocuum*
      - *C. ramosum*
      - *C. histolyticum*
      - *C. septicum*
      - *C. sporogenes*
      - *C. paraputrificum*
      - *C. tertium*
      - *C. difficile* (enterotoxin)
      - *C. botulinum* (neurotoxin)
      - *C. tetani* (neurotoxin)
- Non-spore-formers
  - Gram-positive bacilli
    - *Actinomyces*
    - *Arachnia*
    - *Propionibacterium*
    - *Bifidobacterium*
    - *Lactobacillus*
    - *Eubacterium*
  - Gram-negative bacilli
    - *Bacteroides*
    - *B. fragilis* group
      - *B. fragilis*
      - *B. distasonis*
      - *B. ovatus*
      - *B. thetaiotaomicron*
      - *B. uniformis*
      - *B. vulgatus*
    - *B. melaninogenicus* group
      - *B. asaccharolyticus*
      - *B. intermedius*
      - *B. melaninogenicus*
    - *B. bivius*
    - *B. capillosus*
    - *B. disiens*
    - *B. oralis*
    - *B. ruminicola* subsp. *brevis* and subsp. *ruminicola*
    - *B. ureolyticus*
    - *Capnocytophaga*[a]
    - *Fusobacterium*
      - *F. mortiferum*
      - *F. necrogenes*
      - *F. necrophorum*
      - *F. nucleatum*
      - *F. varium*
  - Gram-positive cocci
    - *Peptococcus niger*
    - *Peptostreptococcus*
      - *P. anaerobius*
      - *P. asaccharolyticus*
      - *P. magnus*
      - *P. micros*
      - *P. prevotii*
      - *P. tetradius*
  - Gram-negative cocci
    - *Veillonella parvula*

[a] Many strains are actually capnophilic.

crosis), as well as a variety of other infections. *C. perfringens, C. barati, C. bifermentans, C. novyi,* and *C. sordellii* produce lecithinase. *C. ramosum* is the second most frequent clinically isolated species and is unusually resistant to antimicrobials. Enterotoxin producing strains of *C. difficile* are thought to be the etiological agents of antibiotic induced colitis. *C. septicum* is often recovered from patients with malignancies. *C. botulinum* and *C. tetani* cause disease by toxin production.

2. Nonsporing Gram-Positive Bacilli

The nonsporing gram-positive bacilli are infrequently clinically significant, although actinomycetes can be recovered from orofacial sinus tracts, pulmonary infections, and brain abscesses. *Propionibacterium* is infrequently pathogenic and frequently contaminates blood cultures and other specimens. Occasionally, *Propionibacterium acnes* may be associated with infections involving implanted prosthetic devices, such as heart valves and total joint arthroplasties.

3. Nonsporing Gram-Negative Bacilli

The nonsporing gram-negative bacilli include the genera *Bacteroides* and *Fusobacterium.* A new genus, *Capnocytophaga,* has been proposed for those strains that formerly would have been identified as *Bacteroides ochraceus.*[16] Although many of these strains are actually capnophilic, they often are obligately anaerobic on initial isolation. Episodes of sepsis due to this organism have been reported in patients who are immunocompromised, especially granulocytopenic. The *Bacteroides fragilis* group of organisms and its species *B. fragilis* (formerly subspecies *fragilis*) are the most frequently isolated, clinically important anaerobes, both from all cultures and specifically from blood cultures (Table 5–2). The *Bacte-*

Table 5–2. *Bacteroides fragilis* Group Isolated from Clinical Specimens at the Mayo Clinic (1983)

Species[a]	Percent of group isolates from: All cultures	Blood cultures
B. fragilis	54	74
B. thetaiotaomicron	23	14
B. distasonis	9	5
B. vulgatus	9	5
B. ovatus or nonspeciated	6	3

[a] These organisms, formerly considered "subspecies" of *B. fragilis,* have now been given species status; however, because of similarities in their clinical manifestations and laboratory characteristics, they are often considered together as the "*B. fragilis* group."

roides melaninogenicus group has assumed increased clinical importance since 59% of strains produce β-lactamase and because some clinical infections have responded poorly to penicillin.[6] *Fusobacterium* is probably of less clinical significance than *Bacteroides* but is frequently isolated from anaerobic infections of the upper and lower respiratory tracts or those secondary to distal spread from a respiratory focus (e.g., brain abscess secondary to a lung abscess). Other genera include *Desulfomonas, Leptotrichia, Selenomonas,* and *Succinivibrio.* These organisms are often found as part of the normal oral flora but do not cause clinical infections often enough to warrant further consideration.

4. Anaerobic Cocci

Anaerobic gram-positive cocci belonging to the genera *Peptococcus* and *Peptostreptococcus* are frequently isolated from a variety of different infections and are, along with the *B. fragilis* group, *Clostridium,* and *Fusobacterium,* among the most clinically significant anaerobes. A recent major taxonomic revision based on DNA homology studies has resulted in the placement of all the former species of *Peptococcus* (except for *P. niger*) in the genus *Peptostreptococcus.*[8] In addition, a new species, *P. tetradius,* has been proposed to include those organisms formerly designated as *"Gaffkya anaerobia." P. magnus* is the most clinically important

Table 5-3. Anaerobic Bacteria Most Frequently Isolated from Clinical Specimens at the Mayo Clinic During 1983[a]

Organism	Number of isolates
B. fragilis group	458
P. magnus	176
B. melaninogenicus	127
C. perfringens	97
P. asaccharolyticus	72
V. parvula	55
B. ruminicola subsp. *brevis*	49
B. oralis	48
B. bivius	42
C. ramosum	41
E. lentum	41
F. nucleatum	38
P. anaerobius	35
P. micros	31
P. prevotii	30

[a] Excluding *P. acnes*

species of the anaerobic cocci.[3] The gram-negative cocci are not frequent causes of significant anaerobic infection. Although the genus *Veillonella* has been reclassified into three separate species, it is most practical to retain only the most important species, *V. parvula. Acidaminococcus fermentans,* also a gram-negative coccus, is infrequently isolated. Those anaerobic cocci isolated with some frequency and thought to participate in clinically significant infections are listed in Table 5–3, along with other commonly isolated anaerobic bacteria. More complete taxonomic tables of anaerobes can be found in reference texts and manuals.[15,23,25]

B. Infections Caused by Anaerobic Bacteria

1. General Considerations

Since anaerobic bacteria predominate in the indigenous flora of the skin, mucous membranes of the upper respiratory and genital tracts, and the lower gastrointestinal tract (Table 1–1), anaerobic infections usually result from the introduction of organisms from normally colonized, contiguous areas after trauma and/or inadequate blood supply have caused devitalization of tissues and conditions (oxygen lack, fall in E_h) favoring proliferation of anaerobic bacteria. Specimens for anaerobic culture must be collected from infected sites so as to avoid contamination with indigenous anaerobic flora which are indistinguishable from those producing disease.

Most anaerobic bacteria can invade and destroy infected tissues; however, *Clostridium botulinum* and *Clostridium tetani* cause disease by toxin elaboration rather than by tissue destruction. Ingestion of food containing *C. botulinum* toxin alone is sufficient to produce botulism. Neither of these species of *Clostridium* may be cultivable from the host, although *C. botulinum* may be recovered from contaminated foods, wounds, and the patient's stools. *C. difficile* causes antibiotic-associated diarrhea and colitis by elaboration of an enterotoxin.

2. Specific Infections

Familiarity with clinical infections which have been shown to involve anaerobic bacteria will aid the microbiologist in considering what specimens are appropriate for examination (Table 5–4). Virtually any intraabdominal or intrapelvic infection should be considered as being caused, at least in part, by anaerobic bacteria. Anaerobic bacteria are also well-recognized causative agents of brain abscesses and certain pulmonary infections, such as aspiration or necrotizing pneumonia, lung abscess, and empyema. Anaerobic bacteria are undoubtedly frequently involved in infections of the oral cavity, respiratory sinuses, middle ear, and soft tissues of the nose and throat. However, documentation of these infections

Table 5–4. Infections in Which Anaerobic Bacteria Most Frequently Are Important

Pleuropulmonary
Aspiration pneumonia
Empyema
Lung abscess
Necrotizing pneumonitis
Intraabdominal
Peritonitis
Liver abscess
Biliary tract
Subphrenic abscess
Intraabdominal abscess associated with bowel wall defect (surgical, trauma, appendicitis, diverticulitis, malignancies)
Female genital tract
Abscesses of Bartholin's glands or Skene's ducts
Endometritis or myometritis
Postpartum or postabortal sepsis
Pelvic inflammatory disease (nonvenereal); includes salpingitis, tuboovarian abscess, pelvic abscess
Bone, joint, and soft tissues
Osteomyelitis
Septic arthritis
Anaerobic cellulitis
Infected vascular gangrene
Clostridial myonecrosis (gas gangrene)
Other
Brain abscess
Sinusitis

is difficult because of difficulties in obtaining specimens from these areas that are free from contamination with indigenous flora.

Experience at the Mayo Clinic has also shown that anaerobic bacteria probably play a role in certain infections which have not previously been considered as being commonly caused by these organisms. We have found that 40% of positive bile cultures yielded anaerobic bacteria, predominantly *Bacteroides fragilis* rather than *Clostridium perfringens.* In addition, we have frequently isolated anaerobic bacteria, including *B. fragilis* and anaerobic gram-positive cocci, from synovial fluid, often when the septic arthritis is superimposed on significant underlying medical disease, such as rheumatoid arthritis, or following joint surgery. *Peptococcus magnus,* in particular, has caused infection of total joint arthroplasties. Recent reviews have emphasized the importance of anaerobic bacteria in bone and joint infections.[11,12] The classical clinical features of actinomycosis and clostridial gas gangrene are familiar to most clinicians and microbiologists; however, anaerobes are also frequently involved in more common

Table 5–5. Distribution of Strains in Clinically Significant Anaerobic Bacteremias at the Mayo Clinic During 1983

Group	Number	Percent
Bacteroides	48	59
B. fragilis group	44	54
Fusobacterium	5	6
Clostridium	21	25
C. perfringens	9	11
Gram positive cocci	3	4
Nonsporing gram-positive bacilli	5	6
Total	81	100

soft tissue infections, such as cellulitis, infective vascular gangrene, and decubitus ulcers.

Anaerobic bacteria, most frequently the *Bacteroides fragilis* group (Table 5–5), were isolated from approximately 8% of clinically significant bacteremias at the Mayo Clinic in 1983. The focus of infection is usually found in the gastrointestinal or female genital tracts. Approximately 30% of anaerobic bacteremias are polymicrobial.

While it may be true that anaerobic bacteria can be legitimately suspected of causing infection in virtually any part of the body, currently available information suggests that the bulk of specimens processed for anaerobic cultures should come from the type of infections which are listed in Table 5–4. The great majority of infections involving anaerobic bacteria are mixed. For these reasons, it is imperative to pay close attention to the Gram-stained smear of the specimen and to use media that are selective for anaerobic bacteria. Overgrowth by facultative anaerobes will make the isolation of anaerobes impossible unless inhibitory agents, such as antibiotics, are incorporated in the media.

II. Isolation of Anaerobic Bacteria

A. Clues to the Presence of Anaerobic Bacteria (Table 5–6)

A foul or feculent odor of the clinical specimen or cultures is virtually pathognomonic of the presence of anaerobes. Certain characteristic morphologies in the Gram-stained smear of the original specimen are suggestive of anaerobes. For instance, *Bacteroides fragilis* may be pleomorphic and stain irregularly, often appearing barrel shaped with bipolar staining (Figure 2–1j). *Fusobacterium* may appear as long, slender, gram-negative

Table 5–6. Clues to the Presence of Anaerobic Bacteria in Cultures

1. Foul or feculent odor of specimen or culture
2. Characteristic Gram stain morphologies
3. Characteristic colonies
4. Growth on LGV[a] plates after anaerobic incubation
5. Growth in the deep portion of a thioglycollate broth tube as well as abundant gas production

[a] Laked gentamicin-vancomycin blood agar

bacilli; *F. nucleatum* characteristically has pointed ends (Figure 2–1k) and *F. mortiferum* may have centrally located bulbous structures. Many clostridia demonstrate spores when Gram-stained, but *C. perfringens* usually does not. *C. perfringens* characteristically appears as a large boxcar shaped gram-positive bacillus (Figure 2–1i). The mere observation of multiple organisms with different morphologies in the Gram-stained smear can be a clue to the presence of anaerobes.

Dilution of a small specimen in transport medium often results in unrepresentative Gram-stained smears; however, this disadvantage can be circumvented by making smears directly at the time the specimen is obtained and before it is injected into the anaerobic transporter vial or by preparing smears in the laboratory with other portions of the same specimen which may have been sent in another container without diluent.

The morphological appearance of colonies on blood agar may also be helpful, e.g., black-pigmented *Bacteroides melaninogenicus*, pitting *Bacteroides ureolyticus*, or hemolytic (double zones) *Clostridium perfringens*. Gram-negative bacilli that grow on an anaerobically incubated laked blood agar plate containing gentamicin and vancomycin (LGV) are very likely to be *Bacteroides*. Likewise, growth only in the deep portion of a tube of thioglycollate broth, especially when abundant gas is produced, indicates anaerobes are present.

B. Incubation of Cultures

1. The Holding Jar

After specimen inoculation on primary plating media, the plates are stored in a holding jar until at least 10 to 12 plates have accumulated. The holding jar is an ordinary anaerobic jar with a loosely fitted, vented lid. Carbon dioxide flows from a tank through rubber tubing connected to the vent and through a rubber tube which extends to the bottom of the jar, thereby displacing O_2. Prior to its use, each empty jar is flushed (1 liter/min) with CO_2 for 3 to 4 min to displace most of the room air

and lower the O_2 concentration to approximately 1 to 2%. Subsequently, the flow of CO_2 may be decreased to 0.1 liter/min, and the loose fitting lid is simply removed when adding plates. After an arbitrarily set maximum holding time of two hours, the jars are sealed and their contents rendered anaerobic, regardless of the number of plates contained therein. Parallel studies of specimens have shown no loss of organisms on media held for a maximum of two hours compared with those immediately placed under anaerobic conditions (Mayo Clinic, unpublished data). The same holding jar system may be used to minimize loss of viability while transferring organisms from primary agar media or subcultures on the open bench for various testing procedures.

2. The Anaerobic Jar

The atmosphere in the jar may be made anaerobic either by using the simple and convenient commercially available GasPak H_2 and CO_2 generator envelopes (BBL Microbiology Systems) or by an evacuation-replacement method utilizing a vacuum pump and tank of oxygen-free gas. Both systems are efficient, provide approximately the same degrees of anaerobiosis within 30 to 60 min, and have shown equivalent growth of anaerobes from clinical specimens. Since the cost of GasPak envelopes has greatly increased in recent years, the evacuation-replacement system is more economical, despite the initial costs of a vacuum pump and tanks of gas. Although we have used the GasPak jars exclusively in our laboratory, there are at least two other types of jars that are commercially available (Oxoid USA, Inc., Columbia, MD; Scott Labs Inc, Fiskeville, RI) and function in a manner similar to the GasPak jar. Whichever system is used, anaerobiosis needs to be monitored by inclusion of an oxidation-reduction potential indicator and periodic demonstration of the ability of a fastidious anaerobic organism, such as *P. anaerobius,* to grow in the jar.

The jars are evacuated to a negative pressure of 25 in. of mercury, and the vacuum is then replaced with oxygen-free nitrogen. This cycle is repeated three times. After the fourth evacuation, the replacement usually consists of a mixture of 85% N_2, 10% H_2, and 5% CO_2. A methylene blue strip placed in each jar provides a monitor for the development and maintenance of anaerobic conditions during the period of incubation. The jar's components must be kept in good physical condition. Especially important are the rubber O-ring seals, clamps, and jar lids, which should be periodically inspected for cracks.

Failure to obtain anaerobiosis in jars is most often due to inactivation of the paladium-coated alumina catalyst pellets. The pellets, which are enclosed in a wire cage which screws into the jar lid, catalyze the combination of any O_2 in the jar with the H_2 to produce water and become inactivated after repeated use by moisture and H_2S produced by the

microorganisms. Sufficient wire cages containing catalysts should be available so that after each use they can be "reactivated" by heating in a 160°C oven for 90 to 120 min. Under these conditions, catalysts are probably usable for a number of years, although the manufacturers have recently established a 6 month expiration date for the product. In routine practice, we find it efficient to use the evacuation-replacement system during regular working hours and to use the GasPak envelopes at night and on weekends when the laboratory is only partially staffed.

3. Examination of Cultures after 24 hr of Incubation

On the basis of comparisons of yield of anaerobic bacteria after 24 and 48 hr of incubation, it is generally recommended that anaerobic jars be incubated at 35°C for 48 hr before primary cultures are removed and initially examined. Our own experience, however, shows an equivalent rate of recovery of anaerobic bacteria from clinical specimens when cultures were examined after 24 hr and then reincubated for an additional 24 hr compared with cultures that were incubated for an uninterrupted 48-hr period.[20] Since approximately 25% of isolates of *B. fragilis* can be detected after 24 hr of incubation, it is important to examine the primary cultures at that time and again after an additional 24 hr of incubation. Our procedure is to open the jars after 24 hr of incubation and examine the cultures. Cultures are replaced in the jar for the additional 24 hr of incubation. Laboratories may examine the cultures on an open bench, taking care to store them in the holding jar until they can be replaced in anaerobic conditions.

4. The Anaerobic Glove Box

The anaerobic glove box is one of several alternatives to anaerobic jars. Most glove boxes are more complex and expensive than jars, and none has proved to be more efficient than jars for culturing clinical specimens. The glove box may be likened to a large anaerobic jar in which reduced conditions are achieved through the use of oxygen-free hydrogen-containing gas mixtures and the presence of palladium or platinum catalysts. Work is performed within the glove box through glove portals so that all manipulations are performed without exposure to oxygen. Although this system may be advantageous when working with extremely oxygen-sensitive organisms, such as those found in the bowel, it is not advantageous when working with clinical specimens. In fact, the glove box may pose sufficient physical and psychological barriers to impair the efficiency of work and the yields from clinical specimens.

Studies in our laboratory have also shown considerably poorer recovery of anaerobic bacterial isolates from primary cultures incubated in the glove box, compared with the anaerobic jar.[20] Of a total of 265 isolates recovered, 41.5% were not isolated in the glove box, compared with

13.6% not isolated in the jar. These results were reinforced by the observation that a quality control strain of *P. anaerobius* grew poorly in the glove box, compared with florid growth in a jar that contained numerous other cultures. Further investigation demonstrated a "co-incubation" phenomenon in that the growth of certain anaerobic bacteria was enhanced by the presence of other organisms (especially *C. perfringens*), growing in the same jar. The exact mechanism of growth enhancement is not known, but it does not seem related to E_h, and cross-connected jar studies have suggested that a volatile gas is involved. It has been recommended that all anaerobic jars should be set up containing three blood agar plates heavily streaked with *C. perfringens* to promote maximum growth from clinical specimens. The efficacy of this procedure has not yet been demonstrated. At the present time in our laboratory, all primary culture plates are incubated in full anaerobic jars, and use of the glove box is limited to storage of cultures for one week (3 weeks for *Actinomyces*) before discarding and for incubation of biochemical tests and microdilution trays for antimicrobial susceptibility testing.

5. PRAS Media

The "roll-tube" method, which employs tubed, prereduced anaerobically sterilized (PRAS) media, has been described elsewhere.[15] This method is neither utilized in our laboratory nor recommended for clinical laboratories because of the expense and complexity of the equipment and media required and the inexperience of most technologists in working with colonies that have been inoculated on a thin layer of agar (roll-streak method) coating the inner surface of a tube.

The yield of anaerobes from clinical specimens has never been shown to be superior when this method has been compared with anaerobic jars. While there is little doubt that the growth of anaerobes is improved in PRAS media, satisfactory results can be obtained in most instances by using much less expensive and easily prepared non-PRAS media. Agar and broth media become sufficiently reduced during the early stages of anaerobic incubation to allow the growth of anaerobes. Blood agar plates that are sealed in mylar or other plastic bags and are stored under refrigeration will support the growth of anaerobes as well as freshly prepared plates. Our practice is to prepare batches of plates weekly and to store them in bags under refrigeration.

C. Isolation Procedures

1. Laboratory Workcard

An efficiently designed laboratory workcard will aid in following the logical progression of work and rapid isolation and identification of anaerobic

Clinic No. Name Spec. Date

Date Out Acc. No.

DATE							Thio Stain
Colony Media							
GRAM STAIN							Anaerobes
MORPH							
THIO							
SEC-TIONS	O_2						Aerobes
	CO_2						
	H_2						
SUB PLATES	O_2						
	CO_2						Original Stain
	H_2						
Date of SET ID							
REFERRAL							PD-1966

Fig. 5-1a

ACC. NO.					ORG. NO.	
QUAD'S			DISCS		MOROPHOLOGY	
O_2			CLS			
CO_2			ERY			
H_2			KM			
7′	ESC		PCN		CARBOHYDRATES	
	GEL		RF		1	ARA
8′	IND		VM		2	CEL
9′	NO_3				3	FRU
	CAT		FA		4	GLC
	BILE		UVL		5	INS
	EYA		PIG		6	LAC
	lec.		URE		7	MAL
	nag.				8	MNT
	lip.				9	MAN
	MOT				10	RAF
	SPORE				1′	RHA
GC					2′	SOR
					3′	S_pH
					4′	SUC
					5′	TRE
					6′	XY
ID						
						PD-1965

Fig. 5-1b

Figure 5–1. Workcards for anaerobic bacteriology. (a) Card for information about the patient, specimen, pertinent dates, macroscopic and microscopic descriptions of various colonial types, subculture details, and final identification of any aerobic and facultatively anaerobic bacteria isolated. Antimicrobial susceptibility testing results can be recorded on the back of this card. (b) Card for results of all tests used for identification of isolates.

bacteria by allowing technologists to trace the progress of identifying each colony type examined and to determine at any time where the work stands. Colonial and Gram-stained microscopic morphology must be adequately described so that the final results of the culture can be reviewed and compared with the kinds of organisms observed microscopically in the specimen. Figure 5–1 illustrates the workcards used in our laboratory. When all of the work on each culture is completed, both workcards are filed together. The supervisor or director of the laboratory should review the cards thoroughly to check for any inconsistencies in the work-up of a culture.

2. Subcultures to Determine Aerotolerance

When the primary cultures are initially inspected, growth on the selective (LGV, PEA) media is compared with that on the nonselective (Brucella blood agar or BBA) medium and that on a blood agar plate incubated in 5 to 10% CO_2. Bacteria with the same microscopic and colonial morphology which grow on the blood agar incubated in CO_2 and on the BBA incubated anaerobically are considered to be facultative anaerobes. The remaining organisms on BBA and all organisms growing on LGV should be considered probable anaerobes and subcultured to blood agar plates which are incubated both anaerobically and in 5 to 10% CO_2. Probable anaerobes should also be subcultured to a tube of thioglycollate broth. These procedures are carried out on an open laboratory bench. After 24 hr of incubation, subcultures are examined and those isolates which are considered anaerobes (growth only on the anaerobic subcultures) are Gram-stained and processed further. All primary cultures are incubated for a total of 7 days and then reexamined for the appearance of new organisms. The thioglycollate broth culture of the original specimen is Gram-stained to detect the presence of organisms not already recognized on plating media.

3. Subcultures for Capnophilic Streptococci

Some gram-positive cocci growing anaerobically on BBA primary isolation plates but not on those incubated in 5 to 10% CO_2 are not actually obligate anaerobes. The growth of these capnophiles (or "microaerophiles") is enhanced by the CO_2 in the anaerobic incubation environment, but they do not require anaerobiosis and grow perfectly well when subcultured to media incubated in 5 to 10% CO_2 (candle jar or CO_2 incubator). A scheme for determining the aerotolerance of gram-positive cocci growing anaerobically is shown in Figure 5–2. A second subculture is suggested because some apparently "anaerobic" cocci will not demonstrate their ability to grow in CO_2 (capnophilic) until after several subcultures. Capno-

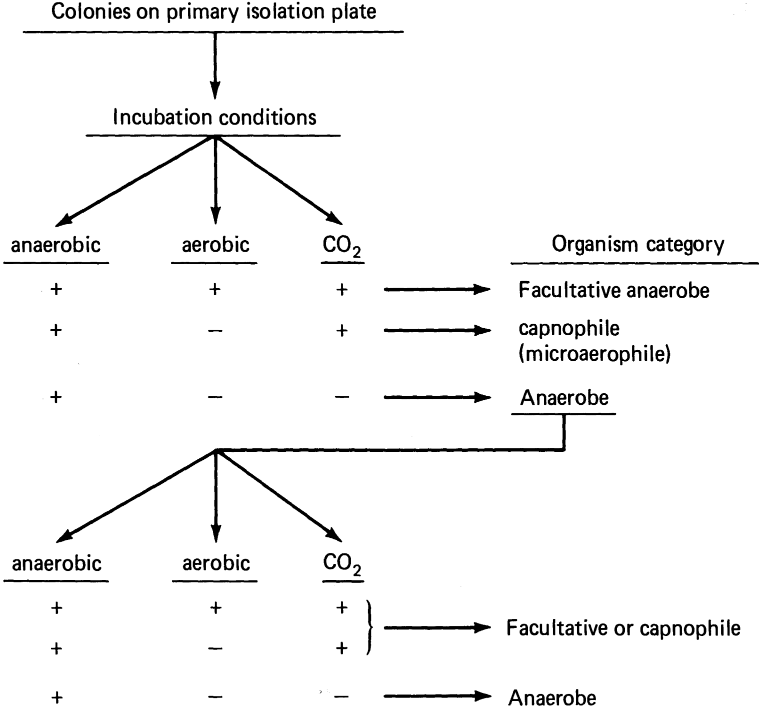


Figure 5–2. Subculture scheme to determine aerotolerance of gram-positive cocci.

philes are identified in the same manner as the viridans streptococci (p. 140).

4. Cultures for Actinomyces

Cultures for *Actinomyces* are inoculated onto brain heart infusion agar (BHIA) and into thioglycollate broth. If no anaerobic culture was specifically requested, BBA is also inoculated and incubated anaerobically. The BHIA is incubated for 48 hr in an anaerobic jar and transferred to the anaerobic glove box the next morning for prolonged incubation. The tube of thioglycollate broth is sealed by tightening its cap and incubated in air at 35°C. After 48 hr of incubation, the BHIA is examined for the presence of heaped, rough, lobate colonies typical of *Actinomyces.* If any are present, they are Gram-stained and also subcultured to test their aerotolerance. A Gram-stained smear is also prepared from the thioglycollate broth. If gram-positive branching rods are seen and sufficient growth is present, biochemical tests and gas liquid chromatography (GLC) for nonspore-forming gram-positive bacilli are performed. Negative cultures are examined *weekly* for a total of 3 weeks and, if still negative, discarded. A Gram-stained smear of the broth is examined before discarding it.

III. Identification of Anaerobic Bacteria

A. General Procedures

1. Principles of Identification

a. Adequate Growth

Accurate identification requires the presence of adequate growth in broth or on agar of a pure culture of a proved obligate anaerobe. Performance or interpretation of tests when growth is inadequate only causes confusing and/or misleading results which, in turn, require needless repetition of tests. *Do not inoculate or read tests unless adequate growth is present.*

b. Reporting Results

Reports should be provided as rapidly as possible, especially when clinically significant organisms are isolated from sites of major importance. When anaerobes are isolated from blood, brain abscesses, and synovial fluids, or when β-lactamase–producing Gram-negative bacilli are identified in any culture, reports should be phoned to the physician. Preliminary written reports should be provided as soon as any new or important information becomes available, even if it is only descriptive (e.g., "anaerobic gram-negative bacilli isolated, identification pending"). Final reports should be issued no later than 2 weeks from the time the specimen was received, since later reports are of little use to physicians. Some final reports may provide only incomplete identifications (e.g., "*Clostridium*, not speciated"); however, they should include as much useful information as possible (e.g., the unspeciated *Bacteroides* isolated is "not *B. fragilis*" or the gram-positive bacillus is "not a *Clostridium*" or if so, is "not a *C. perfringens*").

Despite published recommendations for specimen collection, a certain number of specimens reaching the laboratory do not warrant the time and expense of full identification of all isolates. Specimen labeling will often lead one to suspect that there has been contamination with normal flora. For instance, "perirectal abscesses" often grow numerous facultatively anaerobic and anaerobic bacteria and can be reported as "mixed fecal flora" as long as there is no single predominating organism. In a similar manner, we identify anaerobic bacterial isolates only by Gram stain morphology if more than three different morphotypes of anaerobic bacteria or more than a total of four different organisms are recovered from specimens from the following sites: peritoneal, intraabdominal, pelvic, buttock, sacral, or decubitus ulcer. Some of these specimens may also be reported simply as "mixed fecal flora" if numerous organisms

are isolated. Those anaerobic gram-negative bacilli, which are reported only by Gram stain morphology, are also tested for β-lactamase production using nitrocefin disks (Cefinase, BBL Microbiology Systems).

2. Identification Tests

Table 5–7 lists the tests which are used for identification of anaerobes and indicates which should be used for specific groups of organisms, based on their Gram-stained microscopic appearance. Figure 5–3 is a flow diagram for identifying anaerobes, based on their Gram-stained microscopic morphology and using the tests listed in Table 5–7. Some anaerobes may have gram-variable reactions; however, their antibiotic disk antibiogram patterns (p. 335) can be helpful in suggesting their most likely Gram reaction: gram-negative bacteria are generally inhibited by colistin but resistant to vancomycin, whereas gram-positive bacteria show the reverse pattern. Cocco-bacillary forms may make classification difficult, although this is rarely a problem with gram-positive bacteria. *Bacteroides melaninogenicus* may resemble gram-negative cocci, but young colonies of the former display brick-red fluorescence when exposed to ultraviolet light and subsequently develop black pigment. Some caution must be exercised since strains of *Veillonella* may also fluoresce brick red but colonies will not pigment.

B. Identification of the "*Bacteroides fragilis* Group"

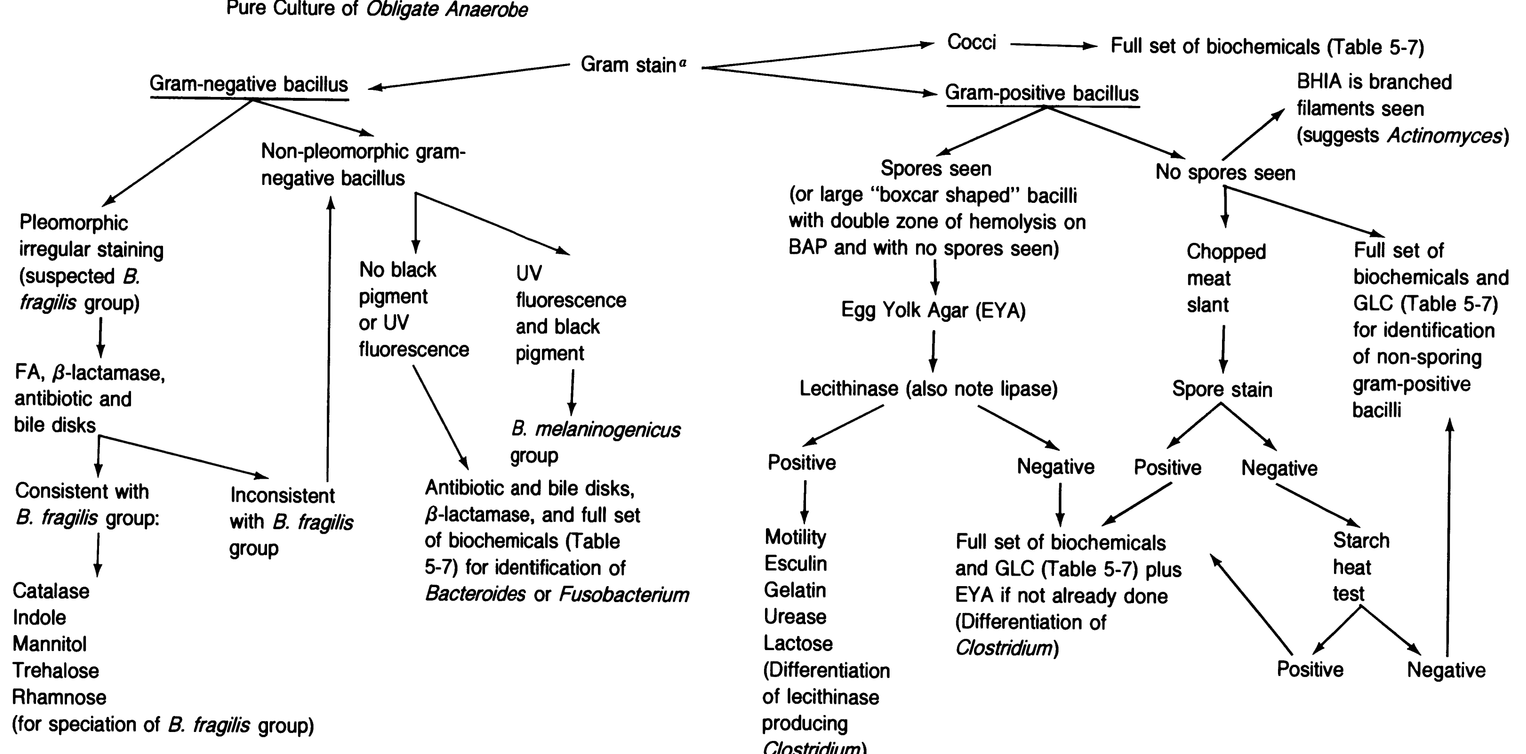
1. General Considerations

This group includes organisms formerly classified as subspecies of *B. fragilis* but now given species status: *B. fragilis, B. distasonis, B. thetaiotaomicron, B. vulgatus,* and *B. ovatus.* Some include *B. eggerthii* and *B. uniformis* as well. They are frequently grouped together because of similarities in clinical significance, specimen source, serological reactivity, and biochemical identification.

2. Growth Characteristics

These are pleomorphic gram-negative bacilli varying from short fat barrel shapes to long thin forms; staining is often irregular and can be bipolar (Figure 2–1). Colonies on blood agar are moderately sized (1–3 mm diameter), translucent, white to pearly gray, and nonhemolytic.

Approximately 25% of *B. fragilis* isolates will produce recognizable colonies after 24-hr anaerobic incubation and most will be apparent after 48 hr.



[a] If Gram reaction equivocal, note antibiotic disk pattern (colistin-S, vancomycin-R for gram-negatives and the reverse for gram-positives).

Figure 5–3. Flow scheme for identification of anaerobic bacteria.

Table 5–7. Tests Required for Identification of Anaerobic Bacteria

	Non-spore-forming gram-negative bacilli	Anaerobic cocci	*Clostridium*		Non-spore-forming gram-positive bacilli	*B. fragilis* group (pleomorphic gram-negative bacilli)
			Lecithinase−[a]	Lecithinase+[a]		
Motility	X		X	X	X	
Indole production	X	X	X		X	X
Nitrate reduction	X	X	X		X	
Esculin hydrolysis	X	X	X	X	X	X
Gelatin liquefaction	X	X	X	X	X	
Growth in bile + desoxycholate (or around bile disk)	X		X	X	X	X
Catalase	X	X	X		X	X
Antibiotic disks	X	X[b]	X		X	X
Growth on egg yolk agar lecithinase, lipase production			X	X	X	
Spores (stain, heat-starch)			X	X	X	
Urease				(X)[c]	X	
Gas chromatography (GC)	X[d,e]	X[e]	X		X[e]	
(Lactate, Threonine)	X[f]					

Table 5–7 (*continued*)

	Non-spore-forming gram-negative bacilli	Anaerobic cocci	*Clostridium*		Non-spore-forming gram-positive bacilli	*B. fragilis* group (pleomorphic gram-negative bacilli)
			Lecithinase–[a]	Lecithinase+[a]		
Carbohydrate fermentations						
Glucose	X	X	X		X	
Levulose	X		X		X	
Lactose	X	X	X	X	X	
Mannose	X	X	X		X	
Rhamnose	X		X		X	X
Trehalose	X		X		X	X
Mannitol	X		X		X	X
Maltose	X	X	X		X	
Sucrose	X	X	X		X	
Cellobiose	X	X	X		X	
Arabinose	X		X		X	
Inositol	X		X		X	
Raffinose	X		X		X	
Sorbitol	X	X	X		X	
Xylose	X	X	X		X	
Starch hydrolysis	X		X		X	

[a] As seen on EYA plates.
[b] Colistin and vancomycin only if Gram stain equivocal.
[c] Add urease to differentiate *C. sordellii* from *C. bifermentans.*
[d] Not done on easily identified *Bacteroides fragillis* and *Clostridium perfringens.*
[e] Do methylated derivatives.
[f] Done on suspected *Fusobacterium.*

3. Preliminary Tests

β-Lactamase Tests[2]

Purpose:
For presumptive identification of isolated colonies of members of the *Bacteroides fragilis* group and other β-lactamase–producing *Bacteroides.*

Principle:
Most members of the *B. fragilis* group and approximately 60% of other *Bacteroides* produce a β-lactamase that is readily detected by a chromogenic cephalosporin (nitrocefin). *B. melaninogenicus* produces a β-lactamase, which can be detected by both the chromogenic cephalosporin and starch-iodine tests. Fusobacteria generally do not produce β-lactamase (Table 5.8).

For details regarding Specimen, Reagents, and Procedure, see p. 308. There is now available commercially a nitrocefin-saturated paper disk method (Cefinase™, BBL Microbiology Systems).

Interpretation:
Positive and negative test results are as described on p. 308.

4. Definitive Tests

Antibiotic Disk Identification[25]

Purpose:
To identify presumptively members of the *Bacteroides fragilis* group and other anaerobic bacteria.

Table 5.8. Penicillin Susceptibility and β-Lactamase Production in *Bacteroides* Other Than *B. fragilis* Group[a]

Penicillin MIC (μg/ml)	Number tested	Number β-lactamase positive
≤0.78	11	3
1.56–100	30	30
Total	41	33
Additional isolates	559	331 (59%)

[a] Adapted from Edson et al.[6]

Principle:
Many anaerobic bacteria can be preliminarily or presumptively identified according to their patterns of inhibition by a special set of antibiotic-containing disks. These disks should be used solely for the identification of anaerobes and not for predicting therapeutic efficacy of antimicrobials. *B. fragilis* group organisms are inhibited only by erythromycin and rifampin. Most other gram-negative anaerobic bacteria are resistant to vancomycin only, whereas most gram-positive anaerobic bacteria are resistant to colistin only. Other specific inhibitory patterns will be discussed with the appropriate organisms.

Specimen:
An 18-hr supplemented thioglycollate broth culture of the test organism.

Disks:

Antibiotic disks	(*BBL Microbiology Systems*)
Colistin	10 μg
Erythromycin	60 μg
Kanamycin	1000 μg
Penicillin	2 U
Rifampin	15 μg
Vancomycin	5 μg

Procedure:

1. Moisten a swab by dipping it into the broth culture.
2. Use the swab to streak the surface of blood agar in a 100-mm petri dish.
3. Apply six antibiotic disks and a bile disk (p. 337) to the agar surface either manually (making sure that the disks are evenly spaced) or with a mechanical dispenser (BBL Microbiology Systems).
4. Incubate the plates anaerobically at 35°C.
5. Examine and measure the zone diameters of inhibition after 18 hr or after good growth and clear zones of inhibition have appeared (up to 48 hr).

Interpretation:

Inhibition: Zone diameter ≥10 mm.
No inhibition: Zone diameter ≤9 mm.

Bile Tolerance Test[5]

Purpose:
To differentiate the *Bacteroides fragilis* group, which is bile tolerant, from other anaerobic gram-negative bacilli that are not.

Principle:
Members of the *B. fragilis* group are, with the exception of the infrequently isolated but easily distinguished *Fusobacterium mortiferum* group, the only anaerobic gram-negative bacilli that are capable of growing in the presence of bile.

Specimen:
An 18-hr supplemented thioglycollate broth culture of the test organism.

Reagent:

Bile solution

Oxgall (Difco Laboratories)	1 g
Distilled water	1 ml

Sterilize at 121°C for 15 min.

Bile disks
Saturate paper disks (Schleicher and Schuell, no. 740 E) with the bile solution (one drop from a Pasteur pipette or approximately 20 to 25 μl per disk). Store at −20°C in a desiccator.

Procedure:

1. Apply the bile disk to the center of the agar surface containing the antibiotic disks (p. 335).
2. Incubate the plates anaerobically at 35°C.

Interpretation:

Bile tolerance: Growth to the disk margin. Members of the *B. fragilis* group will be inhibited only by erythromycin and rifampin (Figure 5–4).

Bile intolerance: Inhibition of growth surrounding the disk.

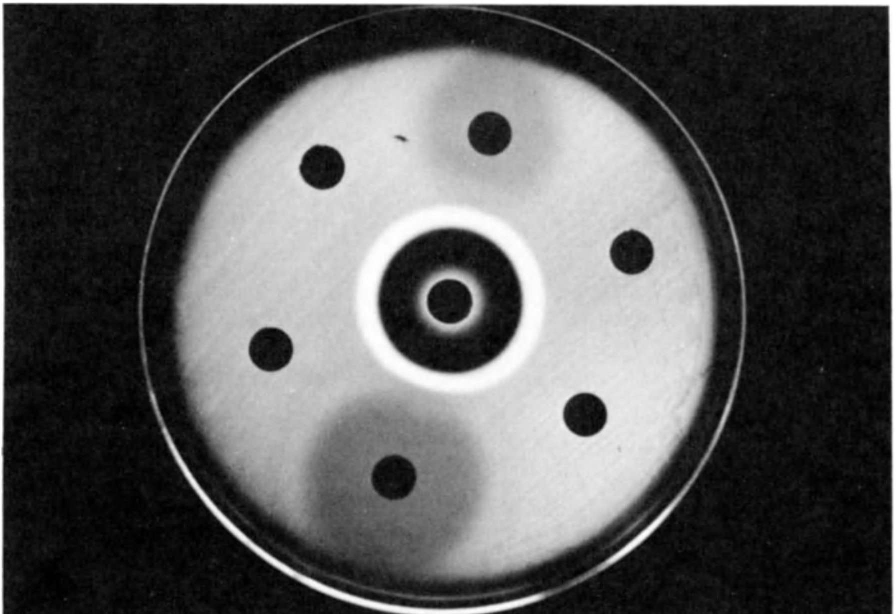


Figure 5–4. Bile antibiotic disk identification test with *Bacteroides fragilis* demonstrating inhibition by erythromycin and rifampin but not by bile (center disk). Bile has hemolyzed blood and darkened the medium which is responsible for the concentric light and dark rings around the disk.

Catalase Test[14]

Purpose:
To separate members of the *Bacteroides fragilis* group, which usually produce catalase, from other anaerobic gram-negative bacilli that do not.

Principle:
Catalase is produced by 80 to 90% of members of the *B. fragilis* group. The only other anaerobic bacteria which can produce catalase are *Propionibacterium, Veillonella, Eubacterium lentum,* and *Peptococcus.* The principle of the test is explained elsewhere (p. 134).

Specimen:
Isolated colonies on blood agar that has been exposed to air for at least 30 min.

Reagent:
3% H_2O_2 (We have not found 15% H_2O_2 in Tween-80 to increase the sensitivity of the test).

Procedure:
Emulsify an isolated colony in a drop of 3% H_2O_2.

Interpretation:

Positive test: Evolution of gas bubbles.
Negative test: No gas bubbles.

Indole Test[25]

Purpose:
To detect the production of indole from tryptophan by certain species of anaerobic bacteria.

Principle:
Indole production is a useful characteristic for differentiating certain anaerobic bacteria, including species within the *Bacteroides fragilis* group where *B. ovatus* and *B. thetaiotaomicron* are indole positive and the other species are negative. Interestingly enough, this characteristic is thought to correlate with increased resistance of these two species to antimicrobials. The principle of the test is explained elsewhere (p. 197).

Specimen:
Thioglycollate broth culture.

Reagent:
Ehrlich's reagent (p. 199).

Medium:
Indole-nitrate broth (BBL Microbiology Systems, cat. no. 11299), supplemented with vitamin K_1 (0.1 $\mu g/ml$) and hemin (5 $\mu g/ml$).*

Procedure:

Broth test

1. Inoculate 2 ml of the indole broth.
2. Incubate anaerobically at 35°C for 48 hr or until good growth has occurred.
3. Transfer 2 ml to another tube for testing.
4. Add 1 ml of xylene, shake well, and allow to stand for 2 min.
5. Add 0.5 ml Ehrlich's reagent.

* Broth may also be used for nitrate reduction test (see p. 221 for principle, reagents, and procedure).

Interpretation:

Positive test: Pink or fuchsia ring in upper organic layer within 10 min.
Negative test: Yellow ring in upper organic layer.

Commercial kit test, see page 341.

Esculin Hydrolysis[25]

Purpose:
To assist in the differentiation of anaerobic gram-negative bacilli.

Principle:
Esculin is generally hydrolyzed by the *Bacteroides fragilis* group, *B. oralis, B. ruminicola, B. melaninogenicus* subsp. *melaninogenicus,* and *Fusobacterium mortiferum.* The principle of the test is explained elsewhere (p. 148).

Specimen:
Thioglycollate broth culture.

Medium:
Esculin broth.

Reagent:
1% ferric ammonium citrate.

Procedure:

Broth test

1. Inoculate esculin broth.
2. Incubate anaerobically for 48 hr or until good growth has appeared.

Interpretation:

Positive test: Loss of fluorescence under long wave UV light (365 nm) or appearance of black color following addition of a few drops of reagent.
Negative test: Fluorescence under UV light or lack of development of black color following addition of reagent.

Commercial kit test
See page 341.

Carbohydrate Fermentation[25]

Purpose:
To speciate members of the *Bacteroides fragilis* group.

Principle:
The principles involved in carbohydrate fermentation are explained elsewhere (p. 207). The production of acid (pH <6.0) can be detected with a pH electrode, with indicator solutions in broth, or with a commercially prepared kit. A flow chart for the identification of anaerobic gram-negative bacilli is included in Figure 5–3.

Procedure:

Broth test

1. Inoculate each tube containing carbohydrate fermentation broth with a few drops of an overnight broth culture of the test organism.
2. Incubate anaerobically at 35°C either in air with the tubes' caps tightly closed or in an anaerobic jar or glovebox with the caps loosened. Reactions should not be read before good growth has occurred, which is usually after 2 days, but 3 to 5 days may be necessary. An acidic (<6.0) pH change may be determined with a pH meter or by using bromthymol blue (which is greenish-blue in an alkaline medium and yellow in an acid medium. These color changes are not always clearcut, making it necessary to measure the actual pH).

Commercial kit test
Commercially prepared kits (Minitek, BBL Microbiology Systems; API-20A, Analytab Products, Inc.) may be used instead of broth tests for determining carbohydrate fermentation reactions. The Minitek kit, which consists of substrate-impregnated paper disks placed into the wells of plastic trays, is used in this laboratory for most biochemical tests excluding the one for indole. The manufacturers' instructions must be carefully followed when using kits.

C. Identification of *"Bacteroides melaninogenicus* Group"

1. General Considerations

Anaerobic gram-negative coccobacilli forming black pigmented colonies on blood agar formerly were all classified as *B. melaninogenicus.* This "group" has now been divided into three species, *B. asaccharolyticus,*

B. intermedius, and *B. melaninogenicus*. For all practical purposes in clinical anaerobic bacteriology, a black pigmented gram-negative bacillus may be termed "*B. melaninogenicus.*" A phenotypically similar organism (which, however, does not produce black pigment), *B. oralis*, is also included in this group.

2. Growth Characteristics

Growth and pigmentation are optimal on Schaedler blood agar but adequate on Brucella blood agar (BBA). Rabbit blood, especially when lysed, is superior to sheep blood. Colonies are small (1 to 3 mm) after 48 hr incubation, and pigmentation may require three to 7 days, progressing from light brown to black. Young, nonpigmented, colonies exposed to ultraviolet light (365 nm) fluoresce brick red (except *B. oralis*). The fluorescence disappears as pigment appears.

3. Preliminary Tests

The *B. melaninogenicus* group may be definitively identified with the appearance of black or brown pigmentation. Young, nonpigmented colonies that fluoresce and are β-lactamase positive in the nitrocefin test (p. 308) starch-iodine slide test (p. 309) may also be definitively identified as belonging to the *B. melaninogenicus* group.

4. Definitive (Differential) Tests

B. melaninogenicus group speciation and *B. oralis* identification may be performed with the biochemical tests outlined in Table 5–7. *B. asaccharolyticus* and *B. intermedius* produce indole but do not hydrolyze esculin, and the former is the only member of this group that does not ferment glucose and starch. *B. oralis* is biochemically similar to *B. melaninogenicus*, but is nonpigmented. *B. melaninogenicus* does not produce indole but does ferment glucose, while *B. intermedius* does not ferment glucose.

Gas Liquid Chromatography (GLC)

Purpose:
To identify anaerobic bacteria.

Principle:
Patterns produced by GLC analysis of volatile fatty acids and nonvolatile organic acids formed by glucose fermentation may be of considerable aid in the identification of many different anaerobic bacteria, including

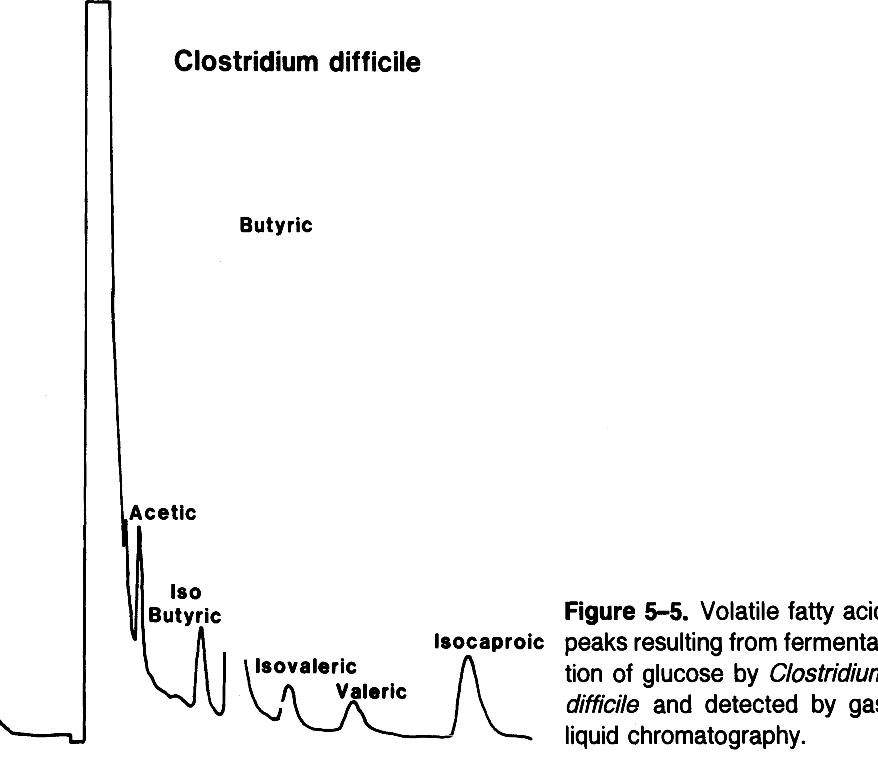


Figure 5–5. Volatile fatty acid peaks resulting from fermentation of glucose by *Clostridium difficile* and detected by gas liquid chromatography.

the *B. melaninogenicus* group. Major (or large) chromatographic peaks (Figure 5–5) of acetic acid and minor (small) peaks of isobutyric, isovaleric, lactic, and succinic acids are produced by members of this group. *B. asaccharolyticus* also produces minor peaks of propionic and butyric acids, helping to differentiate it from the rest of the group. By convention, the acids are abbreviated by using their first (Acetic = A) or first two (isobutyric = ib) letters, while capitalized or small letters indicate major or minor peaks, respectively.

Specimen:
24- to 48-hr supplemented thioglycollate broth culture.

Procedure:

1. Inject 0.1 ml of the broth culture into commercially prepared (Gibco Laboratories; Carr-Scarborough Microbiologicals, Inc.) vials or tubes of prereduced anaerobically sterilized (PRAS) peptone-yeast-glucose (PYG) broth medium.
2. Incubate in air at 35 to 37°C for 2 to 5 days, depending on the appearance of good growth.

3. Prepare the ether extracts of volatile fatty acids and methylated derivatives of nonvolatile acids as follows:
   a. Ether extract:
      (1) Pipet 1 ml of PYG culture into tube. Add 0.2 ml of 50% H_2SO_4, 0.4 g NaCl and 1 ml ethyl ether; stopper tube.
      (2) Mix ethyl ether and culture by inverting the tube gently about 20 times.
      (3) Centrifuge briefly to break the ether-culture emulsion.
      (4) Pipet the ether layer off the aqueous layer carefully so as not to contaminate the ether with water. Add anhydrous $MgSO_4$ to equal about ¼ the volume of ether in the tube, stopper, and let stand about 5 min. The $MgSO_4$ removes dissolved water from the ether.
   b. Methyl derivatives:
      (1) Pipet 1 ml of the PYG culture into test tube.
      (2) Add 2 ml methanol and 0.4 ml of 50% H_2SO_4. Stopper, and heat at 60°C in a temperature block or water bath for 30 min.
      (3) Add 1 ml water and 0.5 ml chloroform, replace stopper, mix by gentle inversion of the tube 20 times. Centrifuge the tube briefly to break the emulsions.
      (4) Remove the chloroform layer directly from underneath the aqueous layer.
4. Inject 14 μl of the extracts into the chromatograph columns and record the patterns.
5. Before injecting ether extracts, recondition the column by injecting 14 μl of methanol.

Note: Traces of the free (unmethylated) acid may be detected in the methylated samples when very large amounts of volatile fatty acids are produced. These have the same elution time as they do in the volatile acid chromatograms.

Details of GLC design, function, and operation are given in the references provided and the manufacturers' instructions. Virtually any GLC machine can be used, but the simplest (employing a thermal conductivity detector) is best for the average clinical laboratory. A compact and reliable machine, including all of the necessary equipment and standards, is marketed by DODECA (Model 700 Anaerobe Identification System, Fremont, CA). The Virginia Polytechnic Institute Anaerobe Laboratory Manual provides complete GLC identification patterns. GLC analysis of standard solutions of fatty acids and uninoculated peptone-yeast-glucose broth should be performed for comparison with test cultures.

D. Identification of Other Clinically Significant *Bacteroides*

1. General Considerations

Only *B. ruminicola, B. bivius, B. disiens,* and *B. ureolyticus* will be discussed here. Other *Bacteroides* species are infrequently isolated in the clinical laboratory, although certain recently described species (*B. splanchnicus* and *B. capillosus*) do seem to play a role in infection. Refer to the Wadsworth and VPI Anaerobe Laboratory Manuals for details on their identification.[15,25]

2. Bacteroides ureolyticus

a. Growth Characteristics

B. ureolyticus is a small gram-negative bacillus with rounded ends. Colonies characteristically grow down into agar to produce pitting, although this characteristic may require as long as 7 to 10 days to develop. Pinpoint colonies with spreading edges grow on blood agar in 48 to 72 hr.

b. Preliminary Tests

Identification prior to the appearance of pitting is based on determining characteristics similar to those of species of *Bacteroides* other than those in the *B. fragilis* group (Table 5–9). These strains display the following characteristics in preliminary tests:

β-Lactamase (p. 308): Negative
Bile tolerance (p. 337): Inhibition
Antibiotic disks (p. 335): Resistant to vancomycin only

c. Definitive Tests

Esculin hydrolysis (p. 340): Negative
Indole production (p. 339): Negative
Nitrate reduction (p. 339): Positive
Carbohydrate fermentation (p. 341): Negative
GLC analysis of PYG fermentation (p. 342): minor acetic and lactic and major succinic acid peaks are produced

Table 5–9. Identification of Anaerobic Gram-Negative Bacilli: Genus *Bacteroides*[a]

Organism	Gram stain	Colonial morphology	β-Lactamase[b]	Bile tolerance[c]
B. fragilis group	Pleomorphic, irregular staining	2–3 mm diam., translucent, gray	+ (N)	G
B. fragilis				
B. distasonis				
B. vulgatus				
B. ovatus				
B. thetaiotaomicron				
B. melaninogenicus can be definitively identified on basis of Gram stain and black pigment)	Small, coccobacillary	1–2 mm diam., brown-black pigment; fluoresce red under UV light before pigment appears	+ (N) + (SI)	I
B. intermedius				
B. asaccharolyticus				
B. oralis	Small coccobacillary	1–2 mm diam. nonpigmented	+/− (N)	I
B. ureolyticus	Small bacilli, rounded ends	Colonies pit the agar but may take 7–10 days	− (N)	I
B. ruminicola	Long pleomorphic	0.5–2.0 mm	+/− (N)	I
B. bivius	Short, in chains with different sizes	Circular colonies with entire edge	Mostly + (N)	I
B. disiens	Varying lengths, from short and plump to more elongated	Same as *B. bivius*	Mostly + (N)	I

[a] Most common isolates.
[b] N, nitrocefin test; SI, starch-iodine slide test for presence of β-lactamase.
[c] G, growth; I, inhibition of growth around a bile disk on a BBA.
[d] S, susceptibility; R, resistance; rif, rifampin; eryth, erythromycin; vanco, vancomycin; kana, kanamycin.

Disk antibiogram[d]	Catalase	Esculin hydrolysis	Indole	Nitrate	Carbohydrates fermented[e]	GLC[f]
S-rif, eryth	+	+				A, P, l, S
			–	–	glc	
			–	–	glc, tre	
			–	–	glc, rham	
			+	–	glc, man	
			+	–	glc, tre	
R-vanco only	–	+	–	–	glc, fruc, lac, mal, man, raf, suc, starch (both subspecies)	A, ib, iv, l, s (both subspecies)
		–	+	–	glc, suc	
R-vanco only	–	–	+	–	None	A, p, ib, b, iv, l, s
R-vanco only	–	+	–	–	glc, fruc, lac, mal, man, raf, suc, starch	A, ib, iv, l, S
R-vanco only	–	–	–	+	None	a, l, S
R-vanco, kana	–	+	–	–	arab, glc, fruc, lac, mal, raf, starch, suc, xyl	A, iv, l, S
R-vanco only	—	—	—	—	glc, lac, mal	A, iv, S
R-vanco only	—	—	—	—	glc, mal	A, iv, S

[e] glc, glucose; tre, trehalose; rham, rhamnose; man, mannose; fruc, fructose; suc, sucrose; lac, lactose; xyl, xylose; mal, maltose; raf, raffinose; arab, arabinose.

[f] GLC, gas liquid chromatographic analysis of glucose (PYG) fermentation end products; A, acetic; p, propionic; ib, isobutyric; b, butyric; iv, isovaleric; l, lactic; s, succinic (capitalized letters for major peaks and small letters for minor peaks).

3. Bacteroides ruminicola

a. General Considerations

Bacteroides ruminicola is the second most frequently isolated group of clinically significant anaerobic gram-negative bacilli after the *B. fragilis* group. Like *B. fragilis,* it is resistant to multiple antibiotics including the penicillins. This species has been subdivided into two subspecies, *brevis* and *ruminicola,* on the basis of requirement for heme. Because subsp. *brevis* is isolated much more frequently than subsp. *ruminicola,* we will be referring to the former only when the species *B. ruminicola* is used. *B. ruminicola* may resemble the *B. fragilis* group but can be differentiated on the basis of characteristics given in Table 5–9.

b. Growth Characteristics

B. ruminicola is a pleomorphic, long, gram-negative bacillus which grows on blood agar in 48 hr to produce low, convex, gray, translucent-to-opaque colonies with irregular margins.

c. Preliminary Tests

β-Lactamase production (p. 308): Positive or negative
Bile tolerance (p. 337): Inhibition of growth
Antibiotic disks (p. 335): Resistant to vancomycin, kanamycin and sometimes to penicillin

d. Definitive Tests

Esculin hydrolysis (p. 340): Positive
Indole production (p. 339): Negative
Nitrate reduction (p. 339): Negative
Carbohydrate fermentations (p. 341): Arabinose, glucose, fructose, lactose, maltose, raffinose, starch, sucrose and xylose are fermented
GLC analysis of PYG fermentation (p. 342): A major acetic and minor isovaleric, lactic and succinic peaks are produced

4. Bacteroides bivius and Bacteroides disiens

a. General Considerations

These organisms resemble one another closely and have been isolated from clinical specimens with increasing frequency since their recognition several years ago. They are both found in the normal gingiva and the female urogenital tract. Both species' names refer to their saccharolytic

and proteolytic properties. They are most commonly isolated from infections of the head and neck (also bite wounds), female genital tract, and occasionally intraabdominal sites.

b. Growth Characteristics

B. bivius and *B. disiens* are similar morphologically, They are gram-negative bacilli that can vary in size and shape and often appear in chains. Moderate-size colonies are circular with entire edges.

c. Preliminary Tests

β-Lactamase production (p. 308): Most isolates are positive
Bile tolerance (p. 337): Inhibition of growth
Antibiotic disks (p. 335): Resistant to vancomycin only

d. Definitive Tests

Esculin hydrolysis (p. 340): Negative
Indole production (p. 339): Negative
Nitrate reduction (p. 339): Negative
Carbohydrate fermentation (p. 341): *B. bivius* ferments lactose while *B. disiens* does not; both ferment glucose and maltose.
GLC analysis of PYC fermentation (p. 342): Major acetic and succinic and minor isovaleric peak are produced.

E. Identification of *Fusobacterium*

1. General Considerations

Fusobacterium species are gram-negative bacilli that are most commonly isolated from infections of the head, neck, oral cavity, and upper and lower respiratory tracts. The genus's name implies a fusiform or spindle with pointed ends, but only one species, *F. nucleatum* (Figure 2–1k), has this characteristic morphology. Other species may have rounded ends and exhibit extreme pleomorphism. All *Fusobacterium* species produce major peaks of butyric acid by GLC analysis of PYG fermentation. Some *Bacteroides* species produce butyric acid as well but usually in conjunction with isobutyric and isovaleric acids, which are not produced by *Fusobacterium*.

2. Growth Characteristics

Colonies on blood agar grow to 1 to 3 mm diameter in 48 hr, are convex, and usually produce α-hemolysis. The colonial morphologies of the most

Table 5–10. Indentification of Anaerobic Gram-Negative Bacilli: Genus *Fusobacterium*[a]

Organism	Gram stain morphology	Colonial morphology	β-Lactamase	Bile tolerance[b]
F. gonidiaformans	Slightly pleomorphic bacilli	0.5–1 mm diam., convex, translucent	–	I
F. naviforme	Marked pleomorphism, esp. long bacilli	0.5–2 mm diam., gray translucent	–	I
F. necrophorum	Pleomorphic, contain round bodies	Convex, umbonate with raised opaque center and translucent edge, alpha hemolysis	–	I
F. nucleatum	Long thin bacilli with tapered ends	Convex, internal "irridescent flecking," "bread crumb" colonies	–	I
F. varium	Highly pleomorphic, bizarre forms and round bodies	Convex, opaque center with translucent spreading edge, "fried egg" appearance	–	G
F. mortiferum	Highly pleomorphic, bizarre forms and round bodies	"Fried egg" appearance	–	G

[a] *Fusobacterium symbiosum* now classified as *Clostridium symbiosum.*
[b] G, growth; I, inhibition of growth around a bile disk on a BBA.

commonly isolated species are described in Table 5–10. Excellent photographs of the colonies, as well as Gram-stained smears, are found in the Scope Monograph on Anaerobic Infections.[10]

3. Preliminary Tests (Table 5–10)

Rare isolates of the *Fusobacterium* species produce β-lactamase (p. 308). They are resistant only to vancomycin by the disk antibiogram method (p. 335). The *F. mortiferum/varium* group is not inhibited, while other *Fusobacterium* species are inhibited in the bile disk test (p. 337).

Disk antibiogram[d]	Catalase	Esculin hydrolysis	Indole	Nitrate	Carbohydrate fermented[e]	GLC[f]
R-vanco only	−	−	+	−	glc	a, p, B, l, s thr → P
R-vanco only	−	−	+	−	None	a, p, B, L, s
R-vanco only	−	−	+	−	None	a, p, B, s thr → P L → P
R-vanco only	−	−	+	−	fruc (weak)	a, p, B, l, s thr → P
R-vanco only	−	−	+	−	glc (weak) fruc (weak)	A, B, L, s thr → P
R-vanco only	−	+	−	−	glc fruc (weak) lac (weak) mal (weak) man (weak)	a, p, B, l, s thr → P

[e] glc, glucose; man, mannose; fruc, fructose; lac, lactose; mal, maltose.
[f] GLC, gas liquid chromatographic analysis of glucose (PYG) fermentation end products; A, acetic; p. propionic; ib, isobutyric; b, butyric; iv, isovaleric; l, lactic; s, succinic (capitalized letters for major peaks and small letters for minor peaks).

4. Definitive Tests

All *Fusobacterium* species are catalase (p. 338) and nitrate (p. 339) negative. *F. mortiferum* is the only species that hydrolyzes esculin (p. 340) and does *not* produce indole (p. 339). *Fusobacterium* are generally weakly or nonfermentative (p. 341). GLC analysis of PYG cultures (p. 342) shows that *Fusobacterium* in general produces major peaks of butyric acid and minor peaks of propionic, lactic and succinic. GLC analysis of PRAS cultures containing threonine or lactate may also be analyzed for the production of propionate. *F. naviforme* and *F. russii* are the only species that will *not* convert threonine to propionate, and *F. necrophorum* is the only species which will convert lactate to propionate. The differential

characteristics used to identify *Fusobacterium* species are listed in Table 5–10.

Conversion of Threonine or Lactate to Propionate

Purpose:
To differentiate species of *Fusobacterium.*

Principle:
Some species of *Fusobacterium* when incubated in broth cultures containing either threonine or lactate convert these substrates to propionate which can be detected by GLC analysis (p. 342).

Specimen:
Two- to 5-day culture (showing good growth) of a *Fusobacterium* isolate in PRAS broth containing threonine or lactate.

Procedure:

1. Inject 0.1 ml of a supplemented thioglycollate broth culture into commercially prepared (Carr-Scarborough Microbiologicals) tubes of PRAS peptone-yeast broth containing DL-threonine (0.3%) or lactic acid (0.9%).
2. Incubate in air at 35 to 37°C for 2 to 5 days or until good growth appears, prepare ether extracts (methylate the lactate culture also), and proceed with GLC analysis (p. 342).

Results and Comments:
The chromatographs of both the substrate-containing cultures and PY cultures are examined to determine a relative increase in propionate in the substrate-containing cultures. Chromatography should be performed with inoculated and uninoculated PY-lactate cultures to demonstrate a relative decrease or the disappearance of lactate in the inoculated culture.

F. Identification of *Capnocytophaga*

Capnocytophaga is a new genus that replaces organisms formerly known as *Bacteroides ochraceus* and CDC group DF-1. There will be only a brief discussion here since these organisms are generally considered facultatively anaerobic and capnophilic, although they may appear as obligate

anaerobes on primary isolation. Three species are recognized: *C. gingivalis, C. ochracea,* and *C. sputigena.* They are fusiform-shaped gram-negative bacilli that exhibit a gliding motility and produce a yellow-orange pigment on blood agar. Biochemical reactions include: oxidase, catalase, and indole negative; esculin is usually hydrolyzed, and reduction of nitrates is variable; glucose, sucrose, lactose, and mannose are weakly fermented. Only acetic and succinic acids are detected by GLC analysis of PYG fermentation end products.

G. Identification of the Anaerobic Cocci

1. General Considerations

The gram-positive cocci include the genera *Ruminococcus, Peptococcus,* and *Peptostreptococcus* and the species *Megasphaera elsdenii* and *Sarcina ventriculi*—since organisms belonging to these two species and the genus *Ruminococcus* are so seldom encountered as significant isolates from human specimens, they will not be considered further. The anaerobic gram-negative cocci include *Veillonella parvula* and *Acidominococcus fermentans.* These organisms have rarely been documented as being significant human pathogens and account for a very small minority of the anaerobic cocci isolated from human specimens.

The anaerobic cocci are prominent in the indigenous flora (Table 1–1), especially in the mouth, upper respiratory tract, and large intestine. The gram-positive cocci have been isolated from 31% of anaerobic cultures which yielded growth in our laboratory. The most common isolate, *P. magnus,* has accounted for 32% of anaerobic gram-positive cocci, followed by *P. asaccharolyticus, P. prevotii,* and *P. micros.* The anaerobic cocci have been isolated from many different infections, including abscesses of the breast and other soft tissues, surgical wound infections, osteomyelitis, and infections involving the neck and dentoalveolar tissues, respiratory tract, and female genital tract.[9] At the Mayo Clinic the anaerobic cocci, principally *P. magnus,* have accounted for 6 to 12% of anaerobic bacteremias, which are often polymicrobial. Anaerobic cocci are present alone in approximately 15% of cultures. The recognition of *P. magnus* as a significant cause of septic arthritis, especially when foreign bodies are present, is an important recent finding. *P. indolicus,* previously isolated only from animal sources, has recently been recovered for the first time from a human infection (finger of a sheepherder). Recently, all species of *Peptococcus* (except *P. niger*) have been reclassified as belonging to the genus *Peptostreptococcus.*[8] *P. tetradius* replaces *"Gaffkya anaerobia."*

Table 5–11. Differential Characteristics of Anaerobic Cocci[a]

Organism	Coagulase	Catalase	Indole	Nitrate	Esculin	Gelatin	Carbo- hydrates fermented	GLC[b]
Peptostreptococcus anaerobius	−	−	−	−	−	−	glc	A, p, ib, b, iv, IC
P. micros	−	−	−	−	−	−	None	A, l, s
P. productus	−	−	−	−	+	−	All	A, L, S
P.asaccharolyticus	−	−	+	−	−	−	None	A, p, B, l, s
P. indolicus	+	−	+	+	−	−	None	A, p, B, l, s
P. magnus	−	−	−	−	−	V	None	A, l, s
P. prevotti	−	−	−	−	−	−	None	A, p, b, l, s
P. tetradius	−	−	−	−	−	−	glc, mal, man, suc	a, p, B, L
Veillonella parvula	−	V	−	+	−	−	None	A, P, s

[a] See list of tests in Table 5–7; cel, cellobiose; glc, glucose; lac, lactose; mal, maltose; man, mannose; V, variable reaction.

[b] Abbreviations of volatile fatty acid end products of glucose metabolism as detected by gas liquid chromatography (GLC). Capitalized abbreviations indicate major end products while small letters indicate minor end products; a, acetic; p, propionic; ib, isobutyric; b, butyric; iv, isovaleric; l, lactic; s, succinic; ic, isocaproic.

2. Growth Characteristics

Growth of the anaerobic cocci is usually slower than that of *Bacteroides* or *Clostridium*. The generally small colonies do not become apparent on blood agar plates until after a full 48 hr of incubation. Growth in broth media is also often slow and will produce aggregates or clumps rather than diffuse turbidity. The slow growth rate may necessitate prolonged incubation (3 to 5 days) for biochemical or antibiotic susceptibility testing and results of tests should not be read unless good growth is present. The gram-positive cocci produce small, convex colonies which are grayish-white and opaque, have entire edges, and a stippled or pockmarked surface. Colonies of *Veillonella* are convex, translucent, with an entire edge and exhibit red fluorescence under long wavelength (366 nm) ultraviolet light on blood agar. The gram-positive cocci are usually distinctive in appearance, although coccobacillary forms are occasionally seen. The cells of *P. productus,* in particular, are often elongated and appear in pairs or chains. Generally, the peptococci occur singly or in small groups and are larger than the peptostreptococci which frequently appear in chains; however, there is considerable overlap in cellular arrangements so that this characteristic is not a reliable method for separating these genera. *P. magnus* and *P. micros* are differentiated primarily by the larger size of the former. The observation of unusually large cocci (which do not resemble yeasts), especially when found in groups or "packets," may suggest the presence of *Megasphera, Sarcina,* or *P. tetradius.*

3. Preliminary Tests

P. indolicus is the only anaerobic gram-positive coccus that reliably produces coagulase.

4. Definitive Tests

P. micros and *P. magnus,* which have similar biochemical reactions, may be differentiated on the basis of the latter's larger (1 to 2 μm, diameter) cell size and variable ability to liquefy gelatin (p. 354). The other species may be identified on the basis of the biochemical reactions and volatile fatty acid end products of glucose metabolism listed in Table 5–11.

Gelatin Liquefaction[25]

Purpose:
To speciate anaerobic gram-positive cocci and *Clostridium.*

Principle:
Gelatin is hydrolyzed by a proteolytic enzyme, gelatinase, into its constituent amino acids with loss of its gelling characteristics.

Specimen:
Supplemented thioglycollate broth culture.

Procedure:
a. Conventional
 (1) Inoculate several drops of a 24 hr culture in supplemented thioglycollate broth into the bottom of a tube of Thiogel (BBL Microbiology Systems, cat. no. 11712).
 (2) Incubate under anaerobic conditions at 35°C for 48 hr or until satisfactory growth has occurred.
 (3) Place the inoculated tube and an uninoculated control tube with Thiogel into a refrigerator.
 (4) Examine inoculated tube once control tube has solidified.
b. X-Ray film
 (1) Place a small (0.75 × 0.25 cm) piece of exposed undeveloped X-ray film into a small tube containing a heavy broth suspension of the test organism. The test may be adapted to a well containing 50 μl of the organism suspension in the Minitek plate (BBL Microbiology Systems).
 (2) Incubate at 35°C under anaerobic conditions for 48 hr.

Interpretation:

Gelatin liquefaction

a. Conventional test

Positive: The inoculated medium remains in liquid form when refrigerated. The control medium should become solidified.
Negative: The inoculated and control media become solidified when refrigerated.

b. X-ray film

Positive: The green gelatin emulsion comes off the immersed portion of the film, leaving a transparent, bluish film.
Negative: The green gelatin emulsion remains on the immersed film.

H. Identification of *Clostridium*

1. General Considerations

The *Clostridium* species are anaerobic, spore-forming, gram-positive bacilli; however, the Gram reaction may be variable, especially in young cultures, and some strains appear gram-negative. *C. clostridiiforme* usually is gram-negative. The use of disk antibiogram patterns can help clarify this situation since *Clostridium* (and other gram-positive anaerobes) usually is inhibited by vancomycin but not by colistin. Some *Clostridium* species have even more specific disk antibiogram patterns providing clues to their identification. Spores may also be difficult to demonstrate in Gram or special spore stains. However, with the exception of *C. perfringens, C. clostridiiforme,* and *C. ramosum,* organisms should not be identified as *Clostridium* unless spores can be demonstrated. Special media or conditions to induce spore formation may be necessary. Alternatively, generic designation can be assumed indirectly by demonstrating the organism's ability to survive extreme heat (p. 362). A few *Clostridium* species may be aerotolerant and are able to grow on blood agar incubated in air or CO_2. These species are rarely isolated, but they can be confused with facultative sporeformers (genus *Bacillus*), which also grow both aerobically and anaerobically. *Bacillus* rarely forms spores when grown anaerobically and usually forms catalase, whereas aerotolerant strains of *Clostridium* rarely sporulate when grown aerobically and seldom form catalase. Clostridia may be separated into two general groups based on their ability to produce lecithinase (Fig. 5–3 and Table 5–12), which hydrolyzes lecithin to form a precipitate in egg yolk agar medium (EYA). *C. perfringens* and other *Clostridium* species produce toxins ("alpha toxin") which are lecithinases and can be neutralized by antitoxin; however, the *C. perfringens* type A antitoxin generally used in the United States to neutralize lecithinase activity on EYA ("Naglers' reaction") is not specific for *C. perfringens* so that its usefulness in identifying the species is limited.

Although many different species of *Clostridium* have been described, a relatively small number play a significant role in clinical infections. *C. perfringens* is the most common clostridial isolate and at the Mayo Clinic is responsible for 11% of anaerobic bacteremias (Table 5–5). However, its isolation is infrequently associated with the clinical picture of gas gangrene. *C. ramosum* is also frequently isolated and is especially important because of its resistance to a number of commonly used antimicrobials. *C. septicum* bacteremia appears to occur most frequently in patients who have underlying malignancies. The association seems to be so strong that when *C. septicum* is isolated from blood it has been suggested that the patient be investigated for a malignancy.[9] *C. botulinum* and *C. tetani* cause disease by elaborating a neurotoxin, and a site of

Table 5–12. Identification of Lecithinase-Positive *Clostridium* Species

Organism	Gram-stained morphology	Spores	Colonial morphology	Motility	Lactose fermentation	Gelatin liquefaction	Urease production	Lipase on egg yolk agar	Esculin hydrolysis
C. perfringens	Large "boxcar" shaped (blunt end), spores rarely seen	ST[a] (rarely seen)	Low convex, opaque gray, double zone of hemolysis on a blood agar plate	−	+	+	−	−	−
C. barati	Large bacilli with blunt ends, spores not readily seen	ST	No hemolysis	−	+	−	−	−	+
C. bifermentans	Gram-positive bacilli with oval subterminal spores	ST	Gray, irregular edge with narrow zone of hemolysis	+	−	+	−	−	+
C. sordellii	Gram-positive bacilli with oval subterminal spores	ST	Gray, spreading irregular edge, narrow zone of hemolysis	+	−	+	+	−	−
C. novyi	Gram-positive bacilli with oval subterminal spores	ST	Irregular surface, gray, translucent, hemolytic						
Type A				+	−	+	−	+	−
Type B				+	−	+	−	−	−

[a] ST, subterminal location of spores.

infection likely to contain the organisms is rarely seen. However, the relatively recent recognition of wound botulism and infant botulism has prompted a search for the organism in wounds and feces of infants when the clinical syndrome of botulism is present. Toxin assays are generally not performed by clinical laboratories, and state public health laboratories should be contacted for that purpose. *C. difficile* has been determined to be the etiologic agent of antibiotic-induced pseudomembranous colitis. Although *C. difficile* can be isolated from the feces of such patients on a selective medium (p. 803), a cytotoxicity assay for the enterotoxins is the more specific diagnostic test (p. 364).

2. Growth Characteristics (Tables 5–12 and 5–13)

Most *Clostridium* grow rapidly in broth and will produce significant turbidity after overnight incubation. Growth is slower on agar media, usually taking 48 hr before typical colonies appear. Many species cause hemolysis on blood agar, and *C. perfringens* produces a unique "double zone" of hemolysis consisting of an inner zone of complete or β-hemolysis and an outer zone of partial or α-hemolysis. Many *Clostridium* produce low convex or flattened colonies with irregular edges with rhizoid (rootlike) projections, some being elongated and serpentine in appearance. Gram-variable staining is often seen depending on the age of the culture. However, most species will eventually be consistently gram-positive, except for *C. clostridiiforme,* which almost always stains gram-negatively. Spores may be detected in Gram-stained smears where they appear as nonstaining round or oval, terminal or subterminal vacuoles. Malachite green stains the spores green. Some species will sporulate readily in broth or blood agar; others produce spores only after prolonged incubation (several weeks) on EYA or chopped meat agar (CMA) slants. *C. perfringens, C. clostridiiforme,* and *C. ramosum* rarely produce microscopically detectable spores.

3. Preliminary Tests

a. Detection of Spores

(1) Microscopy

Growth in thioglycollate broth or on blood agar is Gram-stained or stained with malachite green. Though no more sensitive than the Gram stain for detecting spores, malachite green is a specific spore stain. If no spores are seen, subcultures are made onto CMA or EYA, incubated at 35°C for 48 hr, and then stored at room temperature. Stained smears are examined every other day for 4 to 6 days for the appearance of spores. If spores appear, their shape and position should be noted to aid in speciation.

Table 5–13. Identification of Lecithinase-Negative *Clostridium* Species

Organism	Gram-Stained morphology	Spores[a]	Colonial morphology	Disk antibio-gram[b]
C. botulinum	Large bacilli, oval ST spores	ST	Variable, hemolytic	R-colistin only
C. butyricum	Round or blunt ends, large oval ST spores	ST	Nondescript	R-colistin only
C. clostridiiforme	Gram-negative, tapered ends, oval ST spores rarely seen	Rarely seen	Small, convex, translucent, mottled, or mosaic surface	R-colistin and penicillin
C. difficile	Oval T spores	T	Slightly raised, white, glossy	R-colistin only
C. histolyticum	Pleomorphic, oval ST spores	ST	Smooth and rough colonies; rough have flat edges with rhizoids	R-colistin only
C. innocuum	Small bacillus, oval T spores	T	Glossy, white, raised	R-colistin and often to rifampin and erythromycin
C. ramosum	Pleomorphic bacilli in chains with bulges	T	Convex, white, glistening	R-colistin and rifampin
C. septicum	Oval ST spores, long thin cells with some oval shaped	ST	Raised, gray, irregular rhizoid margins, hemolytic	R-colistin only
C. sporogenes	Oval ST spores, filamentous in old cultures	ST	Raised gray-yellow center, rhizoid edge	R-colisin only
C. tertium (aerotolerant)	Large oval T spores	T	Small, low, translucent, glossy	R-colistin only
C. tetani	Slender bacilli, round T spores	T	Translucent, gray, irregular edge, narrow hemolysis	—

[a] T, terminal; ST, subterminal.
[b] R, resistant.
[c] V, variable reaction.
[d] fruc, fructose; glc, glucose; lac, lactose; malt, maltose; mntl, mannitol; man, mannose; raf, raffnose; rham, rhamnose; suc, sucrose; tre, trehalose; xyl, xylose.

Lipase	Gelatin	Motility	Indole	Esculin	Carbohydrates fermented[d]	GLC[e]
+	+	+	−	V[c]	glc, malt (both weak) or nonfermentative	A, p, ib, B, iv, ic, l, s
−	−	+	−	+	arab, fruc, glc, lac, malt, man, starch, suc, tre, xyl	A, B, l, s
−	−	+	−	−	fruc, glc, malt, raf, rham, suc, xyl	A, L, s
−	+	+	−	−	fruc, glc, mntl, man	A, p, ib, B, iv, v, ic, l, s
−	+	+	−	−	None	A, l, s
−	−	−	−	+	fruc, glc, mntl, man, suc, tre	A, B, L, s
−	−	−	−	+	fruc, glc, lac, malt, mntl, man, raf, suc, tre	A, l
−	+	+	−	+	fruc, glc, lac, malt, man	A, B, l, s
+	+	+	−	+	None	A, p, ib, B, iv, v, ic, l, s
−	−	+	−	+	fruc, glc, lac, malt, man, starch, suc	A, B, l, s
−	+	+	+	−	None	A, p, B, l, s

[e] Fatty acids detected by gas liquid chromatography (GLC); capital letter indicates major peak, small letter indicates minor peak: A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; L, lactic; S, succinic; arab, arabinose.

(2) Starch-Heat Test[15]

Indirect evidence of spore formation can be obtained by demonstrating survival of the test organism after heating at 80°C for 10 min. To perform the test, peptone-yeast broth containing 1% glucose and 1% starch is inoculated with growth from solid agar. The broth is heated in a water bath at 80°C for a full 10 min, after which the tube is incubated at 35°C for 24 to 48 hr. Growth is presumptive evidence that spores were present and able to survive the 80°C temperature, which would have killed vegetative cells. Starch tubes with no growth are observed for up to 10 days before being called negative. It is essential for accurate interpretation of this test that appropriate controls be included: an inoculated but unheated starch broth (growth control), a known spore-forming *Clostridium* (positive control), and a nonsporing anaerobe (e.g., *B. fragilis*).

b. Disk Antibiogram (p. 337)[25]

Clostridium will usually be inhibited by all the antibiotic disks except colistin. *C. clostridiiforme* will usually be resistant to penicillin and often to erythromycin. *C. ramosum* is usually resistant to rifampin (as well as to colistin) and is often resistant to kanamycin and erythromycin. *C. innocuum* has an antibiogram pattern similar to that of *C. ramosum.* These unique patterns can give early clues as to the identity of organisms that may be difficult to identify, especially *C. clostridiiforme,* which is usually gram-negative and can rarely be seen to produce spores.

c. Production of Lecithinase and Lipase on EYA

Lecithinase will lyse the egg yolk lecithin in 24 to 48 hr to produce an opaque precipitate in the agar surrounding an area of growth. Lipase breaks down free fats present in egg yolk, liberating insoluble free fatty acids, which appear as an oily, mother of pearl, irridescent film or sheen in the medium just below colonies and on the colony surface. This reaction may be delayed, so that plates should be observed for seven days before being discarded as negative.

A single longitudinal streak with a loopful of an overnight supplemented thioglycollate broth culture of the test organism is sufficient, so that several strains may be tested on the same plate.

4. Definitive Tests

a. Lecithinase-Producing *Clostridium* (Table 5–12)

Those *Clostridium* that form lecithinase on egg yolk agar plates can be differentiated on the basis of the following tests: motility, lactose fermen-

tation (above), gelatin liquefaction (p. 355), urease production, lipase production (above), and hydrolysis of esculin (p. 340). Although spores are rarely seen with *C. perfringens,* this species is usually easily recognized by its large rectangular shape with blunt ends ("boxcars"), lecithinase production, the double zone of hemolysis on blood agar and a positive reverse CAMP test.[13] In this test, an area of synergistic hemolysis is seen when *Streptococcus agalactiae* and *C. perfringens* are cross-streaked on a blood agar plate. A characteristic "arrowhead" of enhanced hemolysis develops near the intersecting streaks. This pattern is unique to *C. perfringens* among the *Clostridium.* The differential characteristics of other species in this group are listed in Table 5–12. Motility can be detected by the hanging drop method (p. 173) with 4 to 6 hour supplemented thioglycollate broth culture or by stabbing a semisolid motility medium (Difco Laboratories, cat. no. 0105–1) with a Pasteur pipette containing a drop of a 24 hr thioglycollate broth culture and looking for growth along the line of inoculation (growth control) or growth diffusing out from line of inoculation indicating motility (p. 174).

The production of urease can be tested in the Minitek System (BBL Microbiology Systems), according to the manufacturer's instructions, or by inoculating several drops from an overnight broth culture to the bottom and middle of a tube of thioglycollate fermentation broth containing 10% (v/v) urea concentrate (Difco Laboratories) and observing for the development of a red color.

b. Non-Lecithinase-Producing *Clostridium*

Clostridium which do not produce lecithinase usually require a full battery of biochemical tests, including GLC analysis of PYG fermentation products, for specific identification (Table 5–13). *C. botulinum* and *C. sporogenes* are the only two members of this group that form lipase; however, they are identical biochemically and can only be differentiated by testing for *C. botulinum* toxin. Since toxin assays are only available through state public health laboratories, the isolation of such organisms should prompt a call to the physician to ask if the patient has any clinical signs suggesting botulism; if not, the most likely identification is *C. sporogenes.* None of these *Clostridium* produce indole except *C. tetani* which in addition is nonfermentative, a characteristic shared by *C. sporogenes* (and often *C. botulinum*) and *C. histolyticum.* The differential characteristics of species in this group are listed in Table 5–13.

5. Special Procedures

a. Isolation of *Clostridium difficile* from Feces

Cultures are performed on a selective medium containing cycloserine, cefoxitin, fructose, and egg yolk agar (Table 3–6). *C. difficile* produces

colonies in 48 hr that are low umbonate, have a filamentous edge, change the color of medium from orange to yellow, and fluoresce yellow when exposed to ultraviolet light.

b. Cytotoxicity Assay for *C. difficile* in Feces

The cytopathic toxin produced by *C. difficile* in feces of patients with antibiotic-associated pseudomembranous colitis is neutralized in vitro by polyvalent gas gangrene or by *C. sordellii* or *C. difficile* antitoxin. Detection of this cytotoxicity is more specific than culture of *C. difficile* in the diagnosis of this gastrointestinal disease.[21] A microtitration method for cytotoxicity is described below.[18]

1. Prepare a 1:4 suspension of feces in phosphate buffered saline (PBS).
2. Mix on mechanical stirrer or shaker.
3. Centrifuge at 3000 × *g* for 30 min or 10,000 × *g* for 10 min.
4. Aspirate supernatant and filter sterilize (0.45 μm membrane filter).
5. Seed a 96-well tissue culture plate with 5000 Chinese Hamster Ovary (CHO) cells/well. Incubate 4–6 hr at 37°C.
6. After *C. difficile* specimens have been filtered, set up toxin and neutralization assays in a 96-well diluting plate.
   a. Eight samples/plate can be tested. This includes a positive and negative control for each plate. Therefore, six unknowns can be tested per plate. Both toxin dilutions and neutralization tests can be done in the same diluting plate. Dilutions of the toxins are made as follows: undiluted, 1:10, 1:100, and 1:1000 with the exception of the positive and negative controls—they are already made at 1/10, so they will start at 1/10 and go to 1/10,000.
   b. *Toxin assay.* 180 μl of PBS is added to all wells *except wells in row A*—stool filtrates, and controls (100 μl) are added to Row A (Figure 5–6).
      i. Add PBS to dilution plate using the Eppendorf repeater pipette and a 2.5 ml Combitip on setting 3. 150 μl of PBS is added to each well. Exchange the 2.5 ml Combitip for a 0.5 ml Combitip and keep setting at 3. Add 30 μl more to each well to bring volume to 180 μl.
      ii. Using a 100 μl Eppendorf pipette, add 100 μl of controls and stool filtrates to Row A wells.
      iii. By using the Titertek Microtitration pipette (it is an 8 channel pipette), one can now make and transfer the dilutions. Set pipette for 20 μl. Starting with Row A, pick up 20 μl from all *eight* wells and dispense to Row B. Continue by picking up 20 μl from Row B and dispense to Row C; then pick up 20 μl from Row C and dispense to Row D.
      1) From Row D dilution plate, transfer 20 μl to Row D of toxin/

		1	2	3	4	5	6	7	8				
UD	A	⊕ 1/10	⊖ 1/10										
1/10	B												
1/100	C												
$1/10^3$	D												
	E												
	F												
	G												
	H												

Figure 5–6. Dilution Plate

neutralization plate; also from Row D dilution plate transfer 20 μl to Row H of toxin/neutralization plate (Figure 5–7).

2) Using the same pipette tips, transfer 20 μl from Row C dilution plate to Row C toxin/neutralization plate. Also from Row C dilution plate, transfer 20 μl to Row G of toxin/neutralization plate. Always remember to go from greatest dilution to least.
3) Do the same for Row B (20 μl)—B and F.
4) Do the same for Row A (20 μl)—A and E.
5) Add 20 μl PBS to all wells Rows A–D of the toxin/neutralization plate. The toxin is now diluted out and is ready for inoculation of CHO cells and neutralization assay.

c. Neutralization assay

i. By the above procedure 20 μl of toxin samples at specific dilutions have already been placed into their assigned wells.

ii. Using the Eppendorf repeater pipette, and a 0.5 ml Combitip, 20 μl (setting #2) of 1:100 of antitoxin is placed in each well E–H of toxin/neutralization plate. The action of the pipette will mix the neutralization mixture; however, after adding the antitoxin the plate should be shaken gently. This allows mixing of toxin and antitoxin. Let the reaction take place at room temperature for 30 min.

iii. During the 30 min incubation period, check the 96-well tissue culture plate containing the CHO cells. Then do the following.
   (a). Mark the plates as shown in Figure 5–8.
   (b). Using the Eppendorf repeater pipette and the 0.5 ml Combitip, add 20 μl (setting 2) of PBS to all the control wells.

iv. After 30 min incubation, using the Titertek microtitration pipette, transfer 20 μl of Row D (10^{-3} dilution) of the toxin/neutralization plate to Row D of CHO cell plate. Transfer Row C of toxin/neutralization plate to Row C of CHO plate. Continue Row B–B CHO plate and Row A–Row A CHO cell plate.

Change pipette tips and transfer 20 μl of specimens in the neutralization half of the plate in the same fashion.

Row H of T/N* plate to Row H of CHO plate
Row G of T/N plate to Row G of CHO plate
Row F of T/N plate to Row F of CHO plate
Row E of T/N plate to Row E of CHO plate

* toxin/neutralization.

			1	2	3	4	5	6	7	8	9	10	11	12
Toxin	UD	A	⊕ 1/10	⊖ 1/10										
	1/10	B												
	1/100	C												
	$1/10^3$	D												
Neut	UD	E	⊕	⊖										
	1/10	F												
	1/100	G												
	$1/10^3$	H												

Figure 5–7 Toxin/Neutralization Plate

											Control Cells			
			*⊕	*⊖	3	4	5	6	7	8	9	10	11	12
	UD	A												
	1/10	B												
Toxin	1/100	C												
	1/1000	D												
	UD	E												
	1/10	F												
Neutralization	1/100	G												
	1/1000	H												

* = ⊕ and ⊖ controls, dilutions begin at 1/10 and go to 1/10,000.

Figure 5–8. 96-Well Tissue Culture Plate

v. Place plates back into CO_2 incubator overnight.
vi. Read plates vertically. A positive toxin result is reported only if a minimum 10^{-1} dilution shows 95–100% cytopathic effect and the first undiluted neutralization well is neutralized.

c. Isolation of *C. botulinum* from Feces[4]

Cultivation of *C. botulinum* from feces may be attempted in suspected cases of infant botulism.

1. Prepare a suspension of feces in saline (approximately equal volumes).
2. Inoculate each of two tubes of peptone-yeast-glucose-starch broth (Gibco Laboratories) with two drops of fecal suspension.
3. Heat one tube at 80°C for 10 min (as in starch-heat test, p. 362).
4. Incubate both tubes anaerobically for 24 hr, subculture to EYA plates, and incubate anaerobically for 48 hr.
5. Examine EYA plates and compare growth subcultured from heated and unheated tubes for suspected sporeforming organisms. Pick suspicious colonies, Gram-stain, and identify as described above.

I. Identification of Anaerobic Non-Spore-Forming Gram-Positive Bacilli

1. General Considerations

The nonsporing gram-positive bacilli are a heterogeneous group comprising the genera *Actinomyces, Bifidobacterium, Eubacterium, Lactobacillus,* and *Propionibacterium.* Some strains of some species are capnophilic or even aerotolerant. Identification within this group can be difficult because of erratic growth and variable test results. In general, these organisms are not pathogenic and rarely cause significant clinical infections, although there are exceptions such as actinomycosis. Identification of only the most frequently encountered organisms is summarized in Table 5–14. It is probably not justifiable for a clinical laboratory to devote excessive time and effort to the identification of organisms other than those listed in the table. In view of the difficulty in achieving a specific identification, many organisms will have to be reported simply as "nonspeciated, nonsporing gram-positive bacillus." For more detailed information on this group of organisms, reference manuals[15,25] should be consulted.

2. Growth Characteristics

Most of these organisms are obligate anaerobes, but some strains of *Actinomyces israelii* and *A. naeslundii* are capnophilic and other strains of

Table 5–14. Identification of Anaerobic Non-Spore-Forming Gram-Positive Bacilli

Organism	Gram stain	Colonial morphology	Aero-tolerance[a]	Catalase	Indole	Nitrate	Gelatin	Esculin
A. israelii	Long thin bacilli; some branching, some club shaped	Rough, "molar tooth"-shaped colonies after 5–7 days	A, C	−	−	+	−	+
A. naeslundii	Long, thin bacilli; many short branches	Smooth or rough, raised, irregular	C, F	−	−	+	−	+
B. adolescentis	Short, thick bacilli with bifurcated ends	White, smooth entire edge	A	−	−	−	−	+
B. eriksonii	Short bacilli with clubbed or bifurcated ends	White, glistening	A	−	−	−	−	+
E. alactolyticum	Pleomorphic bacilli in V-shaped arrangements	Opaque, glistening	A	−	−	−	−	−
E. lentum	Pleomorphic coccobacilli in chains	Opaque, smooth, slightly irregular edge	A	−	−	+	−	−
E. limosum	Pleomorphic bacilli in pairs and short chains	Translucent to white, entire edge	A	−	−	−	−	+
L. catenaforme	Pleomorphic bacilli, often in long chains	Slightly translucent, with entire edge	A	−	−	−	−	+
P. acnes	Pleomorphic bacilli club shapes, pointed	White to pink, shiny opaque, entire edge	A	+	+	+	+	−

[a] A, aerotolerant; C. capnophilic; F, facultative.

the latter may even be facultatively anaerobic. The *Actinomyces* grow best and produce typical "molar tooth" colonies on brain heart infusion agar (Figure 2–3) but may require 7 to 10 days for growth. Other species in this group grow adequately and somewhat faster on blood agar, although 3 to 5 days may be required. Some *A. israelii* typically will be long, thin, and branched, while some *Bifidobacterium* will have short, clubbed bifurcations. However, many of the species look distressingly similar in Gram-stained smears so that microscopic morphology is not a very useful criterion for differentiating them. Typical colonies of *P. acnes* and *A. israelii* (Table 5–14) may be easily recognized; otherwise, the nonsporing bacilli do not have distinctive colonial morphologies.

3. Preliminary Tests

P. acnes and *A. viscosus* are the only members of this group that produce catalase (p. 338); therefore, any catalase positive, nonsporing bacilli with

Carbohydrates fermented													
Arabinose	Fructose	Glucose	Lactose	Maltose	Mannitol	Mannose	Raffinose	Rhamnose	Starch	Sucrose	Trehalose	Xylose	GLC[b]
−	+	+	+	+	+	+	+	−	+	+	+	+	A, L, S
−	+	+	+	+	−	−	+	−	−	+	+	−	A, L, S
+	+	+	+	+	−	+	+	−	+	+	+	+	A, l, s
+	+	+	+	+	+	+	+	−	+	+	+	+	A, l, s
−	+	+	−	−	+	−	−	−	−	−	−	−	A, b, c, s
−	−	−	−	−	−	−	−	−	−	−	−	−	a, l, s
−	+	+	−	−	+	−	−	−	−	−	−	−	A, B, L, s
−	+	+	V	V	−	+	−	−	+	+	−	−	a, L, s
−	+	+	−	−	V	+	−	−	−	−	−	−	A, P, iv, l, s

[b] Fatty acids detected by gas liquid chromatography (GLC); capital letter indicates major peak, small letter indicates minor peak: A, acetic; P, propionic; B, butyric; IV, isovaleric; L, lactic; S, succinic.

typical Gram-stain and colonial morphology can be identified as such. Specification of all other organisms requires a full set of biochemical tests (Table 5–14).

4. Definitive Tests

Actinomyces may be suspected by its morphological and aerotolerant characteristics. This genus is unique in reducing nitrate and hydrolyzing esculin and in producing minor acetic and major lactic and succinic peaks on GLC analysis of PYG fermentation products. *A. israelii* and *A. naeslundii* can be separated on the basis of differential carbohydrate fermentation patterns (Table 5–14).

The differential characteristics of other commonly isolated anaerobic nonsporing gram-positive bacilli are listed in Table 5–14.

J. The ANIDENT™ System for Identification of Anaerobic Bacteria

The ANIDENT™ system (Analytab products, Plainview, N.Y.) is a new, promising method for identification of anaerobic bacteria. The system utilizes a unique set of biochemical substrates designed to detect bacterial enzymes heretofore unexamined by conventional anaerobic bacteriology. The substrates are prepackaged lyophilized in microtubule plates, which require only 4 hr of incubation (35°C) in room air after inoculation. One set of 9 substrates (mostly glycosidic-measuring glycosidases) is detected by the chromogenic reaction of liberated *o*- or *p*-nitrophenol or indoxyl (positive tests). Another set of 9 aminopeptidase substrates (measuring aminopeptidases) is detected by the reaction (chromogenic) of liberated β-naphthylamine after the addition (at the end of incubation) of a cinnamaldehyde reagent. Tests for indole production, arginine utilization and presence of catalase are also included in this system.

We have compared identifications achieved by ANIDENT (provided by phone by the Analytab computer derived database) with those using the conventional methods described on p. 332 for approximately 300 clinical anaerobic bacterial isolates in an ongoing study. Our preliminary results have suggested an approximately 65% to 70% agreement in identifications at Level I (no supplemental tests) and 90% agreement at Level II (Level I identifications plus those requiring supplemental tests). The number of organisms requiring supplemental tests was quite small (~6%) and consisted mostly of *Clostridium* species for which GLC and lipase and lecithinase reactions on egg yolk agar were helpful. Accuracy will probably improve as the system's database expands.

ANIDENT appears to be a promising prepackaged kit system for rapid identification of anaerobic bacteria. Additional critical considerations governing utility of this system will be cost and final determination of need for supplemental tests. ANIDENT has replaced more complex conventional and "kit" systems at the Mayo Clinic.

K. Detection of Anaerobic Bacteremia Using Gas Liquid Chromatography (GLC)[7]

A convenient, rapid, and accurate method has been developed to detect anaerobic bacteremia using GLC.[7] Blood culture broth bottles that become turbid and show organisms in Gram-stained smears but have produced no growth on early aerobic subcultures are tested by GLC. The standard procedure (p. 342) is used except that ether extracts are prepared after centrifugation (300 × *g* for 10 min) of 3 ml samples of broth from blood culture bottles. Isovaleric acid will characteristically be detected from cultures containing *B. fragilis.* Butyric acid will be found in blood

cultures growing *Fusobacterium* or *Clostridium;* these two genera are easily differentiated by Gram stain. This method is used routinely in our laboratory and probably hastens the confirmation of anaerobic bacteremia by approximately 24 hr.

IV. Antimicrobial Susceptibility Testing of Anaerobic Bacteria

A. Indications

Antimicrobial susceptibility testing is not routinely indicated for anaerobes since patients with mixed intraabdominal and wound infections respond well to appropriate surgical measures and nonspecific antimicrobial therapy. On the other hand, patients with anaerobic bacteremia have a poor prognosis and antimicrobial susceptibility testing of their isolates is recommended, as it is for isolates from other serious anaerobic infections, such as brain abscess, septic arthritis, osteomyelitis, and endocarditis. Testing should also be done when specifically requested by a physician.

The antimicrobial susceptibility of many anaerobic bacteria is still predictable; however, β-lactamases, which mediate penicillin resistance and occur in most isolates of *B. fragilis,* are being recognized among many isolates of *B. melaninogenicus, B. disiens, B. bivius,* and other *Bacteroides* species, as well as in certain isolates of *Clostridium clostridiiforme, C. bifermentans, C. butyricum,* and *C. innocuum.* Many anaerobes are resistant to tetracyclines and occasional clindamycin-resistant strains of *B. fragilis* have occurred.[6]

Susceptibility testing may, therefore, be necessary for isolates from serious infections and is probably advisable as a periodic survey of collected batches of organisms to detect significant changes in patterns of susceptibility.

B. Methods

1. General Considerations

An agar dilution reference method has been proposed by the National Committee for Clinical Laboratory Standards (NCCLS)[24]; however, the technique is not practical for testing individual isolates as the need arises. Nonetheless, it serves as a standardized reference procedure with which other methods should be compared.

For practical purposes, the broth–disk elution method, particularly that using aerobically incubated thioglycollate broth,[17] is suggested. Alternatively, a microbroth dilution method can be used to determine minimal

inhibitory concentrations. Both methods have generally been shown to provide results comparable to those of the NCCLS reference method. In a collaborative study comparing these three methods, it was found that significant discrepancies between results of the reference and the broth–disk elution methods (6%) exceeded those encountered between the reference and several microbroth dilution methods (2.0 to 3.7%).[19] Since the majority of discrepancies were attributable to tetracycline, it was concluded that the broth–disk elution method could be used with considerable confidence, although results of testing tetracycline should be interpreted with caution. Resistance to penicillin can be rapidly and simply determined with the chromogenic test for β-lactamase (p. 308).

2. Specific Methods

Broth–Disk Elution[17,26]

Purpose:
To determine the susceptibility or resistance of anaerobic bacteria to a single concentration of antimicrobial.

Principle:
Antimicrobials rapidly elute into broth from commercially available disks. By adding one or more disks of a particular antimicrobial to a specified volume of broth, it is possible to obtain a final antimicrobial concentration that is representative of an expected serum level (Table 5–15).

Specimen:
Overnight broth culture in PRAS chopped meat glucose (CMG) broth.

Table 5–15. Concentration of Antimicrobials Used in the Broth–Disk Method of Susceptibility Testing[a]

Antimicrobial	Antimicrobial content (μg) of each disk	No. of disks per 5 ml of broth	Final concentration (μg/ml)
Penicillin	6	1	1.2
Carbenicillin	100	3	60
Cephalothin	30	1	6
Tetracycline	30	1	6
Clindamycin	2	4	1.6
Chloramphenicol	30	2	12
Erythromycin	15	1	3

[a] Adapted from Wilkins and Thiel.[26]

Reagents:

1. PRAS-CMG broth.
2. PRAS-brain heart infusion (PRAS-BHI) or thioglycollate (BBL Microbiological Systems no. 11720) broth.
3. Antimicrobial disks (Table 5–15).

Procedure:

1. Prepare tubes containing 5 ml each of either PRAS-BHI or thioglycollate broth. Thioglycollate broth should be boiled and cooled prior to use and should, when testing *Bacteroides melaninogenicus,* contain hemin (0.1 μg/ml) and vitamin K_1 (5μg/ml).
2. Add the appropriate number of disks to each tube (Table 5–15). Because thioglycollate contains 0.07% agar, tubes should be stored for two hours at room temperature to allow the antimicrobial to diffuse throughout this medium. *N.B.* All work with the PRAS-BHI technique must be performed under a stream of oxygen-free CO_2 to maintain anaerobiosis.
3. Add one drop of an overnight culture in PRAS-CMG to each tube of PRAS-BHI *or* two drops to each tube of thioglycollate broth. Invert each thioglycollate broth tube twice to ensure mixing of the inoculum. A control tube containing broth without antimicrobial is also inoculated.
4. Tighten the caps on each tube and incubate for 18 to 23 hr at 35°C. Incubation may be extended to 48 hr if growth in the control tube is insufficient by 24 hr.

Interpretation:
The isolate is considered to be resistant when turbidity in the broth containing antimicrobial is 50% or greater than that of the growth control. Susceptibility may be reported as the concentration inhibiting growth of the organism (e.g., "susceptible to chloramphenicol, 12 μg/ml; resistant to penicillin 2 U/ml"). As already noted, the results with tetracycline in the thioglycollate broth–disk elution test are less reliable.[19]

Microbroth Dilution Method[22]

Purpose:
To determine the minimal inhibitory concentration (MIC) of antimicrobial against anaerobic bacteria.

Principle:
A standardized bacterial inoculum is exposed to serially diluted antimicrobials in an enriched broth medium that is suitable for the growth of

anaerobic bacteria. The test has been adapted for use in the Microtiter System (Cooke Laboratory Products). Incubation is under anaerobic conditions. This procedure is used in our laboratory because MICs are routinely reported for aerobic and facultatively anaerobic bacteria and because the system provides flexibility in terms of the antimicrobials and the concentrations tested. Experience with the microdilution method is limited to its use in an anaerobic glove box, obviously a disadvantage in laboratories not possessing a glove box. Prereduction of the trays and Schaedler's broth is required to ensure adequate growth of the organisms; however, prereduction and incubation can be performed in anaerobic jars. Results with the microbroth dilution method have been shown in a collaborative study to be comparable to those obtained with the NCCLS reference method and, in fact, to be more reproducible when a Wilkins-Chalgren broth medium is substituted for Schaedler's broth.[19]

Specimen:
Portions of several morphologically identical colonies on blood agar are transferred to Schaedler's broth which is incubated anaerobically for 24 hr or until turbidity appears. Faintly turbid cultures (corresponding to an O.D. of 0.1 to 0.3 at 650 nm) are diluted 1:10 in Schaedler's broth, while more turbid suspensions are diluted 1:100.

Reagents:

1. Schaedler's broth, prereduced for a least 4 hr prior to use and supplemented with vitamin K_1 (0.1 μg/ml).
2. Microtiter trays and equipment (Cooke Laboratory Products).
3. Antimicrobial standard powders (cefoxitin, chloramphenicol, clindamycin, metronidazole, penicillin, and moxalactam), prepared and frozen in aliquots of 1000 μg/ml as described elsewhere (p. 288).

Procedure:

1. Preparation and distribution of antimicrobial dilutions
   a. Prepare antimicrobial dilutions and add inoculum under anaerobic conditions (e.g., glove box).
   b. Prepare working solutions of all antimicrobials except penicillin and moxalactam by adding 0.64 ml of stock solution to 9.36 ml of Schaedler's broth; for penicillin and moxalactam, add 0.64 ml of stock solution to 4.36 ml of Schaedler's broth.
   c. Pipette 0.05 ml of Schaedler's broth into Microtiter tray wells numbered 2 through 6 (2 through 8 for penicillin and moxalactam) in each horizontal row.
   d. Pipette 0.05 ml of working solution into wells numbered 1 and 2, and serially dilute from well number 2 through well number 6 (number 8 for penicillin and moxalactam), discarding 0.05 ml from

the last well. Care must be taken to mix the contents of each well with the diluter before transferring 0.05 ml to the next well. Each well contains 0.05 ml and double the final antimicrobial concentration.

e. Use the trays immediately or stack them (covering the top one with an empty tray) for freezing at −60°C or below for up to 3 weeks.

2. Susceptibility test
   a. Reduce plates for 4 to 6 hr in an anaerobic environment prior to use.
   b. Add 0.05 ml of inoculum to each well, including a row of six wells with 0.05 ml of broth and without antimicrobial to serve as growth controls. Each well now contains 0.1 ml and the final antimicrobial concentration desired.
   c. Stack the plates (as for freezing, see 1e above).
   d. Incubate anaerobically at 35°C for 48 hr.
   e. Examine on a Microtiter reader. Growth, indicated by a "button" in the bottom of the control wells, should be present.

Interpretation:
The MIC is the lowest concentration of antimicrobial inhibiting at least 80% of growth relative to that observed in the control wells.

References

1. Blazevic, D. J., and Ederer, G. M. *Principles of Biochemical Tests in Diagnostic Microbiology.* New York, Wiley, 1975.
2. Bourgault, A.-M., and Rosenblatt, J. E. Characterization of anaerobic gram-negative bacilli by using rapid slide tests for β-lactamase production. *J. Clin. Microbiol.* 9:654, 1979.
3. Bourgault, A.-M., Rosenblatt, J. E., and Fitzgerald, R. H. *Peptococcus magnus:* A significant human pathogen. *Ann. Int. Med.* 93:244, 1980.
4. Dowell, V. R., McCroskey, L. M., Hatheway, C. L., Lombard, G. L., Hughes, J. M., and Merson, M. A. Coproexamination for botulinal toxin and *Clostridium botulinum. J. Am. Med. Asso.* 238:1829, 1977.
5. Draper, D. L., and Barry, A. L.: Rapid identification of *Bacteroides fragilis* with bile and antibiotic discs. *J. Clin. Microbiol.* 5:439, 1977.
6. Edson, R. S., Rosenblatt, J. E., Lee, D. T., and McVey, E. A. III. Recent experience with antimicrobial susceptibility of anaerobic bacteria. Increasing resistance to penicillin. *Mayo Clin. Proc.* 57:737, 1982.
7. Edson, R. S., Rosenblatt, J. E., Washington, J. A. II, and Stewart, J. B. Gas-liquid chromatography of positive blood cultures for rapid presumptive diagnosis of anaerobic bacteremia. *J. Clin. Microbiol.* 15:1059, 1982.
8. Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S., and Yabuuchi, E. Transfer of *Peptococcus indolicus, Peptococcus asaccharolyticus, Peptococcus prevotii,*

and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal of *Peptostreptococcus tetradius* sp. nov. *Int. J. Syst. Bacteriol.* **33**:683, 1983.
9. Finegold, S. M. *Anaerobic Bacteria in Human Disease.* New York, Academic Press, 1977.
10. Finegold, S. M., Rosenblatt, J. E., Sutter, V. L., and Attebery, H. R. *Scope™ Monograph on Anaerobic Infections.* Kalamazoo, Michigan, The Upjohn Company, 1976.
11. Fitzgerald, R. H., Rosenblatt, J. E., Tenny, J. H., and Bourgault, A.-M. Anaerobic septic arthritis. *Clin. Orthop.* **164**:141, 1982.
12. Hall, B. B., Fitzgerald, R. H., and Rosenblatt, J. E. Anaerobic osteomyelitis. *J. Bone Joint Surg.* **65**:30, 1983.
13. Hansen, M. V. and Elliott, L. R. New presumptive identification test for *Clostridium perfringens:* Reverse CAMP test. *J. Clin. Microbiol.* **12**:617, 1980.
14. Hansen, S. L., and Stewart, B. J.: Slide catalase. A reliable test for differentiation and presumptive identification of certain clinically significant anaerobes. *Am. J. Clin. Pathol.* **69**:36, 1978.
15. Holdeman, L. V., Cato, E. P., and Moore, W. E. C. (eds.). *Anaerobe Laboratory Manual,* 4th ed. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 1977.
16. Holdeman, L. V., Cato, E. P., and Moore, W. E. C. Taxonomy of anaerobes: Present state of the art. *Rev. Infect. Dis.* **6**(Suppl. 1):S3, 1984.
17. Kurzynski, T. A., Yrios, J. W., Helstad, A. G., and Field, C. R. Anaerobically incubated thioglycolate broth disc method for antibiotic susceptibility testing of anaerobes. *Antimicrob. Agents Chemother.* **10**:727, 1976.
18. Lyerly, D. M., Sullivan, N. M. and Wilkins, T. D. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. *J. Clin. Microbiol.* **17**:72, 1983.
19. Rosenblatt, J. E., Murray, P. R., Sonnenwirth, A. C., and Joyce, J. L. Comparison of antibiotic susceptibility results obtained by different methods. *Antimicrob. Agents Chemother.* **15**:351, 1979.
20. Rosenblatt, J. E. Reevaluation of current methods for incubation of anaerobes. *In* Tilton R. C. (ed.) *Rapid Methods and Automation in Microbiology.* Washington, D.C., American Society for Microbiology, 1982, p 271.
21. Rosenblatt, J. E. Laboratory diagnosis of antimicrobial associated diarrhea. *In* Coonrod J. D., Kunz L. J., and Ferraro M. J. (eds.) *The Direct Detection of Microorganisms in Clinical Samples.* New York, Academic Press, 1983.
22. Rotile, C. A., Fass, R. J., Prior, R. B., and Perkins, R. L. Microdilution technique for antimicrobial susceptibility testing of anaerobic bacteria. *Antimicrob. Agents Chemother.* **7**:311, 1975.
23. Smith, L. D. S. *The Pathogenic Anaerobic Bacteria,* 2nd ed. Springfield, Illinois, Charles C Thomas, 1975.
24. Sutter, V. L., Barry, A. L., Wilkins, T. D., and Zabransky, R. J. Collaborative evaluation of a proposed reference dilution method of susceptibility testing of anaerobic bacteria. *Antimicrob. Agents Chemother.* **16**:495, 1979.
25. Sutter, V. L., Citron, D. M., and Finegold, S. M. *Wadsworth Anaerobic Bacteriology Manual,* 3rd ed. St. Louis, C. V. Mosby Co., 1980.
26. Wilkins, T. D., and Thiel, T. Modified broth-disk method for testing the antibiotic susceptibility of anaerobic bacteria. *Antimicrob. Agents Chemother.* **3**:350, 1973.

6

Mycobacteria and Nocardia

Glenn D. Roberts, Ph.D.

I. General Considerations

A. Description of Group

The mycobacteria are members of the order Actinomycetales and family Mycobacteriaceae. Species of the genus *Mycobacterium* show some evidence of branching, are acid-fast, are resistant to treatment with alkali, contain large amounts of lipids in their cell walls, and are aerobic or microaerophilic. Their growth rate is usually slow (2–6 weeks); however, a few species require only ≤ 5 days for growth. Most are resistant to conventional antibacterial and antituberculous antibiotics; however, some exhibit susceptibility to one or the other. Some are nonchromogenic, others may be photochromogenic or scotochromogenic. Current taxonomic trends recommend that members be referred to by their species name rather than by groupings.

B. Source and Clinical Importance

With the exception of *Mycobacterium leprae,* mycobacteria may be recovered from a variety of clinical specimens. Table 6–1 presents common sources from which mycobacteria may be recovered and common infections produced by these organisms. The clinical significance of these organisms in specimens must always be determined in view of the clinical presentation of the patient.

Table 6–1. Mycobacteria Commonly Implicated in Human Infection

Etiological agent	Probable recovery site(s)	Associated infection(s)
Mycobacterium avium-intracellulare complex	Respiratory secretions, thoracentesis fluid, gastric washings, lymph nodes, skin, joints, bone, genitourinary tract, bone marrow, blood, cerebrospinal fluid, small intestine, pericardium, oropharynx	Pulmonary infection, lymphadenitis, cutaneous lesions, arthritis, osteomyelitis, nephritis, disseminated disease, meningitis
Mycobacterium bovis	Respiratory secretions, gastric washings, urine, eyes, lymph nodes	Pulmonary infection, disseminated disease, keratitis, lymphadenitis
Mycobacterium chelonei	Respiratory secretions, gastric washings, lymph nodes, skin, tissue aspirations, joints, bone marrow, blood, eye	Pulmonary infection, lymphadenitis, cutaneous lesions, localized soft tissue abscesses, arthritis, disseminated disease, keratitis
Mycobacterium fortuitum	Respiratory secretions, gastric washings, bone marrow, blood, skin, tissue aspirates, joints, bone, urine, eye, oropharynx, paracentesis fluid	Pulmonary infection, disseminated disease, cutaneous lesions, localized soft tissue abscesses, arthritis, osteomyelitis, nephritis, keratitis
Mycobacterium gastri	Respiratory secretions, gastric washings, joints	Arthritis
Mycobacterium gordonae	Respiratory secretions, gastric washings, urine, vaginal secretions, oropharynx	Not commonly implicated in human infection, prosthetic valve endocarditis, meningitis
Mycobacterium haemophilum	Skin	Cutaneous and subcutaneous lesions causing human infection
Mycobacterium kansasii	Respiratory secretions, bone marrow, joints, lymph nodes, skin, urine, cerebrospinal fluid, thoracentesis fluid	Pulmonary infection, disseminated disease, arthritis, lymphadenitis, cutaneous lesions, nephritis, meningitis
Mycobacterium marinum	Skin, joints	Cutaneous lesions, arthritis
Mycobacterium scrofulaceum	Lymph nodes, respiratory secretions	Lymphadenitis, pulmonary disease, disseminated disease
Mycobacterium simiae	Respiratory secretions	Pulmonary infection
Mycobacterium smegmatis	Urine	Not commonly implicated in causing human infection

Table 6–1 (*continued*)

Etiological agent	Probable recovery site(s)	Associated infection(s)
Mycobacterium szulgai	Respiratory secretions, gastric washings, lymph nodes, skin, bursa	Pulmonary infection, lymphadenitis, cutaneous lesions, bursitis
Mycobacterium terrae	Gastric washings, respiratory secretions	Not commonly implicated in causing human infection
Mycobacterium tuberculosis	Respiratory secretions, gastric washings, bone marrow, blood, cerebrospinal fluid, urine, lymph nodes, skin, joints, eye, bone, intestine, uterus, sinuses, thoracentesis fluid	Pulmonary infection, disseminated disease, meningitis, nephritis, lymphadenitis, pericarditis, cutaneous lesions, arthritis, keratitis, osteomyelitis, intestinal infection, endometritis
Mycobacterium xenopi	Respiratory secretions, gastric washings, urine, oropharynx	Pulmonary infection, epididymitis

II. Identification

A. General Considerations

The tentative identification of clinically important mycobacteria has traditionally been based on a number of criteria including acid-fastness, growth rate, pigment production, colonial morphology, and homogeneity of a suspension of the organisms in a liquid medium. Table 6–2 presents characteristic features of each of the common species of mycobacteria that are useful for making a *presumptive* identification after an acid-fast stain has been examined. However, the *definitive* identification of each species has been based on characteristic biochemical reactions derived from the following tests: nitrate reduction, hydrolysis of Tween-80, arylsulfatase production, catalase production and inactivation, sodium chloride tolerance, urease production, tellurite reduction, niacin production, and susceptibility to thiophene-2-carboxylic acid hydrazide.

It is not necessary to perform an entire battery of tests for the definitive identification of each organism recovered from clinical specimens. Based on the presumptive identification of an organism, one can select appropriate conventional biochemical tests as shown in Table 6–3. Figures 6–1 to 6–4 present a step-by-step approach to the identification of clinically important mycobacteria while Table 6–4 provides a complete summary of tests and results characteristic for most clinically important mycobacteria.

Table 6–2. Features Useful for Making a Presumptive Identification of Mycobacteria

Organism	Growth rate (days)	Pigment production in		Colonial morphology on Middlebrook 7H10 agar	Features of suspension in Middlebrook 7H9 broth
		light	dark		
Mycobacterium avium-intracellulare complex	10–21	Buff to yellow	Buff to yellow	Colonies are thin, transparent, glistening or matte, smooth, entire and rounded; some colonies rough and wrinkled	Uniformly homogeneous suspension
Mycobacterium bovis	25–90	Colorless to buff	Colorless to buff	Colonies are small, thin, often nonpigmented, raised, rough, later wrinkled and dry; some colonies inhibited on this medium	Heterogeneous; fine granular suspension
Mycobacterium chelonei	3–7	Buff	Buff	Colonies are rounded, smooth, matte, periphery entire or scalloped, no branching filaments; some colonies are rough and wrinkled	Heterogeneous; coarsely granular suspension
Mycobacterium fortuitum	3–7	Buff	Buff	Colonies are circular, convex, wrinkled or matte; branching filaments on periphery are obvious	Heterogeneous; coarsely granular suspension
Mycobacterium gastri	10–21	Colorless to buff	Colorless to buff	Colonies are round, smooth, convex and glistening; often resembles *M. avium-intracellulare* complex	Uniformly homogeneous suspension
Mycobacterium gordonae	10–25	Yellow to orange	Yellow to orange	Colonies are round, smooth, convex, yellow to orange and glistening	Uniformly homogeneous yellow suspension
Mycobacterium haemophilum	14–28	Buff to gray	Buff to gray	Colonies are grayish-white, smooth to rough	Usually homogeneous suspension

Mycobacterium kansasii	10–21	Yellow	Buff	Colonies are raised and smooth; some are rough and wrinkled; carotene crystals numerous after exposure to light	Usually heterogeneous, finely granular suspension; some isolates give a uniformly homogeneous suspension
Mycobacterium marinum	5–14	Yellow	Buff	Colonies are round, smooth; some may be wrinkled	Uniformly homogeneous suspension
Mycobacterium scrofulaceum	10–14	Yellow	Yellow	Colonies are smooth, moist, yellow and round	Uniformly yellow and homogeneous
Mycobacterium simiae	7–14	Yellow	Buff	Colonies are smooth, domed and slightly pigmented	Heterogeneous; coarsely granular suspension
Mycobacterium smegmatis	3–7	Buff to yellow	Buff to yellow	Colonies are raised, rough, wrinkled and have scalloped edges	Heterogeneous; finely granular suspension
Mycobacterium szulgai	14–28	Yellow to orange	Buff at 25°C; yellow at 37°C	Colonies are smooth to rough; periphery somewhat irregular	Heterogeneous; finely granular suspension
Mycobacterium terrae	10–21	Buff	Buff	Colonies are round, smooth, glistening and sometimes colorless	Uniformly homogeneous suspension
Mycobacterium tuberculosis	12–28	Buff	Buff	Colonies are flat, rough, spreading with irregular periphery	Uniformly heterogeneous; coarsely granular suspension
Mycobacterium xenopi	28–42	Yellow	Yellow	Colonies are small, domed, yellow, smooth or rough; at 45°C, resemble a miniature "birds nest"	Uniformly homogeneous suspension

Table 6–3. Selection of Tests for the Identification of Commonly Isolated Mycobacteria

Rapidly Growing Organism	Photochromogen	Scotochromogen	Nonchromogen
Arylsulfatase Nitrate reduction NaCl tolerance	Nitrate reduction Growth at 30° and 35°C Tween-80 hydrolysis	Nitrate reduction Tween-80 hydrolysis Urease production NaCl tolerance	Niacin Nitrate reduction Tween-80 hydrolysis Tellurite reduction NaCl tolerance Catalase Thiophene-2-carboxylic acid hydrazide tolerance[a]

[a] When *M. bovis* or bacille Calmette-Guerin suspected.

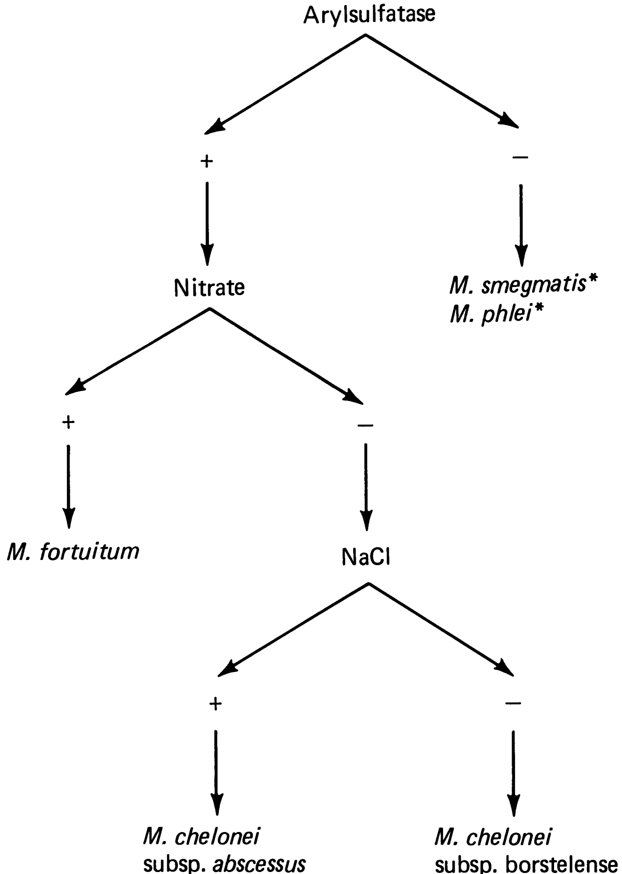
Since 1979, our laboratory has utilized gas-liquid chromatography (GLC) for the identification of mycobacteria. The processing of a specimen (1 colony) requires approximately 1 hr from start to finish. It gives a result days to weeks faster than conventional biochemical testing. GLC identification is used along with selected biochemical tests and directs their selection. For example, a GLC identification of *Mycobacterium fortuitum* complex, which is consistent with colonial morphology and growth rate would require only a positive arylsulfatase test and nitrate test for species identification. GLC is an easy and rapid method for the identification of clinically important mycobacteria and is used on a routine basis in our laboratory.

A radiometric approach to the rapid detection has been under evaluation in our laboratory during recent months.[10,12] Currently, its role to replace or supplement conventional methods is being assessed. From data available, it appears that this method may soom have a definite place in the routine clinical microbiology laboratory.

B. Additional Characteristics of Clinically Important Mycobacteria

1. Mycobacterium avium-intracellulare Complex

Colonies appear in 2 or more weeks on primary isolation and 1 or more weeks on subculture; growth may be matte or wrinkled on Lowenstein-Jensen (L-J) medium but the surface is usually moist. Optimal temperature is variable with some strains growing best at 42°C.



* Require additional biochemical testing for differentiation.

Figure 6–1. Identification of rapidly growing mycobacteria of clinical significance.

2. Mycobacterium bovis

Colonies, usually smaller than those of *M. tuberculosis,* appear after 3 weeks when recovered on L-J medium. Some strains grow only on glycerol-free L-J on primary isolation. On subculture, scanty white or buff-colored growth appears in 2 weeks or more. The niacin test is negative.

3. Mycobacterium chelonei

Growth appears within 7 days on L-J medium as rounded smooth or matte buff colonies on primary isolation. On subculture, luxuriant growth appears within 3 days and growth is smooth, matte, or wrinkled and rough. Colonies grow well at 24° to 37°C but not at 45°C. Growth occurs on MacConkey agar within 5 days.

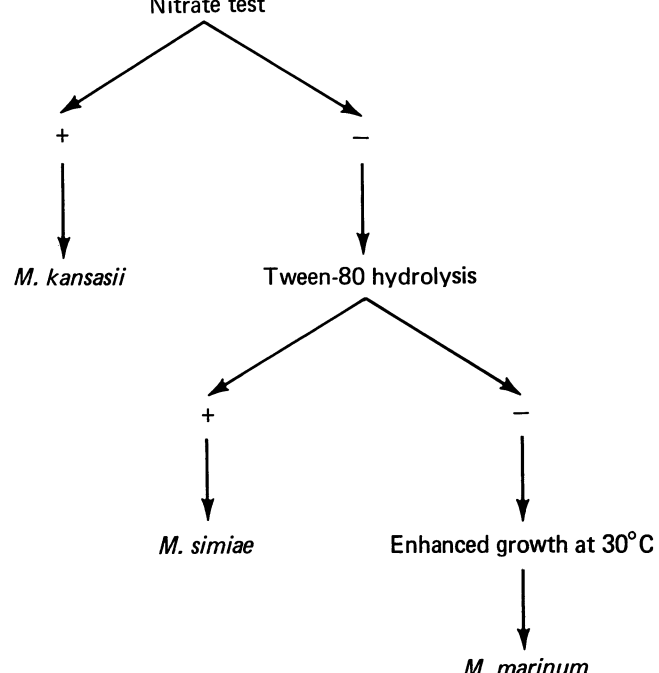


Figure 6–2. Identification of common photochromogenic mycobacteria.

4. Mycobacterium fortuitum

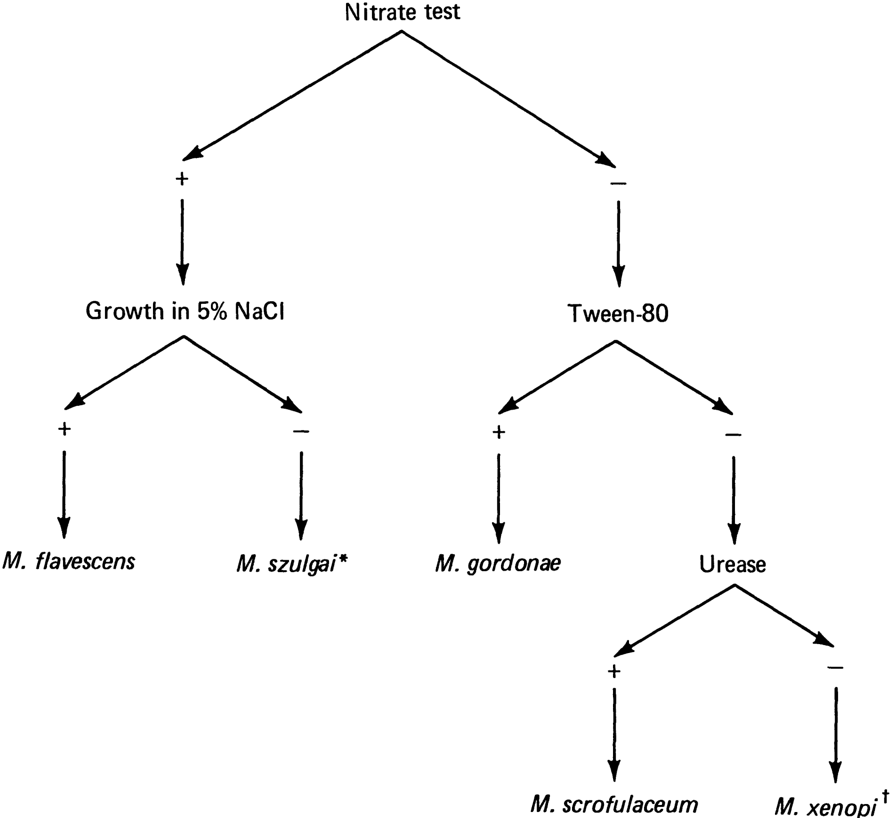
Colonies are wrinkled or matte and appear within 7 days of L-J medium. On subculture, luxuriant growth is evident within 3 days. Colonies grow well at 24° to 37°C but not at 45°C. Growth occurs on MacConkey agar within 5 days.

5. Mycobacterium gastri

Colonies grow slowly on L-J medium and are smooth to rough and nonchromogenic in 2 weeks or longer. On subculture colorless to buff colonies form in 1 week or longer.

6. Mycobacterium gordonae

Colonies appear in 3 or more weeks on L-J medium, usually as single or a few isolated colonies. On subculture colonies appear within 2 weeks as smooth yellow growth in the presence or absence of light. Tween-80 is hydrolyzed within 5 days.



* *M. szulgai* may be photochromogenic at 25°C and scotochromogenic at 35°C; arylsulfatase test is sometimes positive.

† *M. xenopi* has the ability to grow at 42°C; arylsulfatase test is sometimes positive.

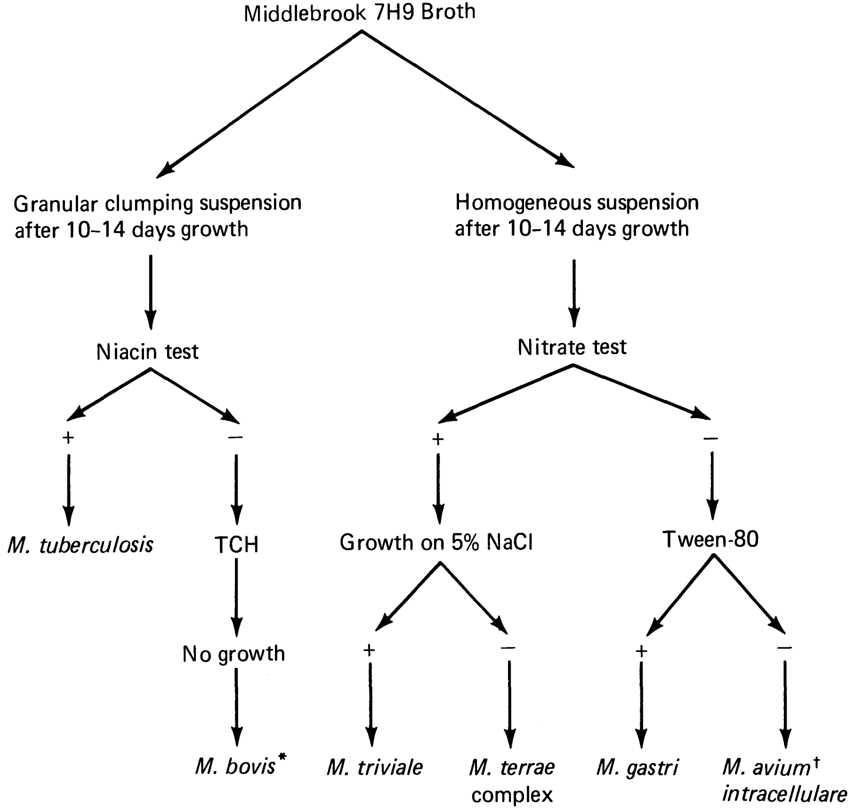
Figure 6–3. Identification of common scotochromogenic mycobacteria.

7. Mycobacterium haemophilum

Colonies appear within 2–4 weeks on L-J or Middlebrook 7H10 medium enriched with hemin or ferric ammonium citrate, 15 mg/ml, incubated at 30°C. Colonies are smooth to rough and nonchromogenic.

8. Mycobacterium kansasii

Colonies appear in 2 or more weeks on L-J medium on primary isolation and in 1 week or longer on subculture. Colonies are photochromogenic. Nitrate is reduced and niacin is occasionally formed.



* *M. bovis* is sometimes positive for niacin.
† Tellurite is often positive with *M. avium-intracellulare* complex.

Figure 6–4. Identification of common nonchromogenic mycobacteria.

9. Mycobacterium marinum

Colonies appear in 2 or more weeks at 24° to 30°C on L-J medium; growth is photochromogenic. Growth occurs poorly or not at all at 37°C. Specimens thought to contain this organism should be incubated at temperatures lower than 37°C.

10. Mycobacterium scrofulaceum

Colonies appear in 2 or more weeks on L-J medium and are rounded, smooth, glistening, and scotochromogenic. On subculture smooth, moist yellow colonies appear after 1 week. Tween-80 is not hydrolyzed within 2 weeks.

Table 6–4. Identification Characteristics of Mycobacteria[a,b]

Organism	Niacin test	Nitrate reduction	Tween-80 hydrolysis 5 or 10 days	Arylsulfatase 3 days	Growth on 5% NaCl	Catalase: Semi-quantitative[c]	Catalase: pH 7.0 68°C	Urease	Tellurite reduction 3 days	Resistance to T_2H 1 μg/ml
M. tuberculosis	+	3–5+	∓	−	−	<40[d]	−	+	−	+
M. africanum	+	V	−	−	−	<20	−		−	+
M. bovis	−	−	−	−	−	<20	−	+	−	−
M. ulcerans	−	−	−	−		>50	+			+
M. kansasii	−	1–5+	+[e]	−	−	>50	+	+	−	+
M. marinum	∓	−	+	−	−	<40	∓		−	+
M. simiae	+	−	−			>50	+	+		+
M. szulgai	−	+	∓	±	−	>50	+	+	−	+
M. scrofulaceum	−	−	−	−	−	>50	+	+	−	+
M. gordonae	−	−	+	−	−	>50	+	−	−	+
M. flavescens	−	+	+	−	+	>50	+	+	∓	+
M. xenopi	−	−	−	±	−	<40	+	−	−	+
M. intracellulare-avium complex	−	−	−	−	−	<40	+	−	±	+
M. gastri	−	−	+	−	−	<40	−	+	−	+
M. haemophilum	−	−	−	−	−	<40	−	−	−	+
M. terrae complex	−	+	+	−	−	>50	+	−	−	+
M. triviale	−	+	+	∓	+	>50	+		−	+
M. fortuitum	−	+	±	+	+	>50	+	+	±	+
M. chelonei	V	−	−	+	−	>50	+	+	∓	+
subsp. *abscessus*	V	−	−	+	+	>50	+	+	∓	+
M. smegmatis	−	+	+	−	+	>50	±		+	+

[a] Adapted from Kubica, C. P.: Identification of mycobacteria. *Am. Rev. Resp. Dis.* **107,** 1973; *Diagnostic Standards,* 1974 edition, American Thoracic Society, 1740 Broadway, New York, NY 10019.

[b] +, 84% of strains +; ±, 50–84%; ∓, 16–49%; −, 16% of strains +; V, variable; blank spaces, little or no data.

[c] Numbers indicate millimeters of bubbles.

[d] INH resistant strains may be negative.

[e] Positive (most) in 24–48 hr.

11. Mycobacterium simiae

Colonies appear within 2 weeks or longer on L-J medium and are smooth, moist and photochromogenic. Growth is poor at 25°C, good at 37°C, and absent at 45°C. Niacin is produced, nitrate is not reduced, and catalase activity is high.

12. Mycobacterium smegmatis

Colonies appear after 2 to 4 days on L-J medium, are finely wrinkled to coarsely folded, and are creamy white in color. Smooth, glistening colonies are commonly seen and pigmented colonies are rare. Colonies of this species may be distinguished from those of *Mycobacterium phlei* by the lack of pigment production.

13. Mycobacterium szulgai

Smooth or rough colonies appear within 2 to 4 weeks on L-J medium. Colonies are scotochromogenic at 37°C and photochromogenic at 25°C after 18 hr of exposure to light. Growth is slower at 25°C than at 37°C and absent at 42°C. Nitrate is reduced. Tween-80 is hydrolyzed slowly and weakly, and arylsulfatase is strong.

14. Mycobacterium tuberculosis

Colonies appear in 2 weeks or longer on primary isolation and after 1 week or longer on subcultures. Growth on L-J medium is raised, rough or matte and becomes wrinkled, dry, and buff-colored. Catalase is inactivated at 68°C.

15. Mycobacterium xenopi

Colonies grow slowly on L-J medium and are small and yellow after 4 or more weeks; colonies are scotochromogenic. The optimal temperature range for growth is 42° to 45°C. Young colonies examined microscopically appear spiderlike.

C. Preliminary Tests

Acid-Fast Stains (Kinyoun and Ziehl-Neelsen)

Purpose:
To detect members of the Actinomycetales that retain basic fuchsin staining after treatment with strong mineral acids.

Principle:
Mycobacterial cells are difficult to stain with common aniline dyes; however, they will stain with basic fuchsin. Once stained, they retain the dye despite treatment with strong mineral acids, i.e., HCl. The mechanism responsible for the retention of basic dyes is not clearly understood. Previously it was thought that high concentrations of mycolic acids present within cells were permanently stained with basic dyes; however, it has been proposed that acid-fastness is due to the retention of basic fuchsin–mycolic acid complexes within the mycobacterial cell by a barrier created by the cell membrane. More recently, it has been postulated that acid fastness is due to the absorption of dye by the mycolic acid residues that are linked to the arabinogalactan-peptidoglycan layer of the cell wall skeleton.[1]

Specimen:
Isolated colonies from Middlebrook or L-J media.

1. Kinyoun Stain

Reagents:

Carbol Fuchsin Stain

Basic fuchsin (J. T. Baker Chemical Co.)	4.0. g
Phenol (melted) (Mallinckrodt Co.)	8.0 ml
Ethanol (95%)	20.0 ml
Distilled water	to 100.0 ml

Acid Alcohol Decolorizing Agent

Hydrochloric acid (concentrated) (J. T. Baker Chemical Co.)	12.0 ml
Ethanol (95%)	388.0 ml

Add acid to alcohol and mix well.

Methylene Blue Counterstain

Methylene blue (Fisher Scientific)	4.0 g
Distilled water	400.0 ml

Dissolve by shaking.

Procedure:

1. Cover slide with a strip of filter paper and flood with carbol fuchsin for 5 min at room temperature.
2. Remove filter paper and rinse with water. Decolorize slide with acid-alcohol until no further stain can be rinsed off.
3. Counterstain with methylene blue for 1 min.

4. Rinse, drain, and dry by blotting.
5. Examine using bright field microscopy and an oil-immersion objective.

Interpretation:

Positive test: Organisms retain carbol fuchsin and stain red.
Negative test: Organisms stain blue with the methylene blue counterstain.

2. Ziehl-Neelsen Stain

Reagents:

Carbol Fuchsin Stain

Basic fuchsin (J. T. Baker Chemical Co.)	0.3 g
Phenol (melted) (Mallinckrodt Co.)	5.0 ml
Ethanol (95%)	10.0 ml
Distilled water	95.0 ml

Dissolve basic fuchsin in ethanol and phenol in water. Mix both solutions and allow to stand for 2 to 3 days before using.

Acid Alcohol Decolorizing Agent

Hydrochloric acid (J. T. Baker Chemical Co.)	3.0 ml
Ethanol (95%)	97.0 ml

Methylene Blue Counterstain

Methylene blue (Fisher Scientific)	0.3 g
Distilled water	100.0 ml

Dissolve by shaking.

Procedure:

1. Flood slides with carbol fuchsin and heat slowly to steaming; maintain for 4 to 5 min.
2. Wash with water and decolorize with acid-alcohol until no further stain can be rinsed off.
3. Counterstain with methylene blue for 20 to 30 sec.
4. Rinse, drain, and dry by blotting.
5. Examine using bright field microscopy and an oil immersion objective.

Interpretation:

Positive test: Organisms retain carbol fuchsin and stain red.
Negative test: Organisms stain blue with the methylene blue counterstain.

Controls:

Positive: *M. tuberculosis*
Negative: *Corynebacterium* species

Pigment Production by Mycobacteria[22]

Purpose:
To determine photochromogenic or scotochromogenic nature of mycobacterial cultures.

Principle:
Some species of mycobacteria possess carotenoid pigments in the presence or absence of light and othes are dramatically induced to form yellow-orange β-carotene crystals only by photoactivation. Those producing pigment either in the presence or absence of light are described as scotochromogenic and those whose pigment is induced only by photoactivation are described as photochromogenic. Some species of mycobacteria lack β-carotene and are nonchromogenic.

Reagents:
Isolated colonies on primary recovery media. L-J medium is preferable; however, Middlebrook media are satisfactory. Subcultures of the organism onto L-J slants may be used after adequate growth appears.

Procedure:

1. Two tubes of media containing isolated colonies or those recently inoculated are used and one is covered entirely with aluminum foil or black paper.
2. Cultures presumed to be photochromogenic should be incubated at 30° and 37°C and those thought to be scotochromogenic should be incubated at room temperature and 37°C.
3. After adequate growth occurs, expose the uncovered tube to bright tungsten light for 60 to 120 min while the caps are left loosened.
4. Reincubate both tubes for an additional 12 to 24 hr. Unwrap the covered tube and compare both for pigmentation.

Interpretation:

1. If growth remains a buff color when compared to the uncovered (yellow) culture, the organism is considered to be photochromogenic.
2. If both tubes exhibit pigmentation, the organism is considered to be scotochromogenic.

3. If both tubes remain nonpigmented, the organism is considered to be nonchromogenic.

Controls:

Photochromogenic control: *M. kansasii*
Scotochromogenic control: *M. gordonae*
Negative control: *M. avium-intracellulare* complex

D. Definitive Tests

Nitrate Reduction (Virtanen Method[20,22])

Purpose:
To detect the presence of the enzyme nitrate reductase produced by some species of mycobacteria.

Principle:
Many bacteria, including mycobacteria, produce nitrate reductase which reduces inorganic nitrate to nitrite (p. 221).

Specimen:
Isolated colonies from a freshly growing culture.

Reagents:

Hydrochloric Acid

Hydrochloric acid (concentrated) (J. T. Baker Chemical Co).	10.0 ml
Distilled water	10.0 ml

Add acid to water to prevent excessive heat generation.

Sodium Nitrate Phosphate Buffer

$NaNO_3$ (J. T. Baker Chemical Co.)	0.085 g
$Na_2HPO_4 \cdot 12\ H_2O$	0.485 g
KH_2PO_4 (J. T. Baker Chemical Co.)	0.117 g
Distilled water	100.0 ml

pH to 7.0.

Sulfanilic Acid

Sulfanilamide (J. T. Baker Chemical Co.)	0.2 g
Distilled water	100.0 ml

N-Naphthylethylenediamine Dihydrochloride

N-Naphthylethylenediamine dihydrochloride (ICN Pharmaceuticals, Inc.)	0.1 g
Distilled water	100.0 ml

Procedure:

1. Place three to five drops of sterile distilled water in a sterile screw-cap test tube.
2. Emulsify one loopful of the culture in the distilled water.
3. Add 2 ml of the $NaNO_3$ buffered solution to the test suspension, shake, and incubate for 2 hr at 37°C.
4. Add one drop of the hydrochloric acid solution to the test.
5. Add two drops of the sulfanilic acid solution to the test.
6. Add two drops of the *N*-naphthylethylenediamine dihydrochloride solution to the test.
7. Observe for the presence of a red color.

Interpretation:

1. If a red color develops, the test is positive and the organism produces nitrate reductase.
2. If no color change develops, the test must be confirmed by adding a small amount of zinc dust to the strip or test suspension. The development of a red color then indicates that the test result is negative. If no color change develops, the test result is positive due to the complete reduction of nitrite to ammonia.

Controls:

Positive: *M. kansasii*
Negative: *M. avium-intracellulare* complex

Tween-80 Hydrolysis

Purpose:
To detect the ability of certain mycobacteria to produce esterases that will hydrolyze polysorbate-80 (Tween-80).

Principle:
Certain, usually nonpathogenic species, of mycobacteria produce an esterase that hydrolyzes polysorbate-80 (polyoxyethylene sorbitan monooleate) into oleic acid and polyoxyethylated sorbitol. The released oleic acid changes the optical characteristics of the substrate so that the neutral red indicator changes from an original amber color to pink.

Specimen:
Isolated colonies from an actively growing culture.

1. Commercial Test

Reagents:

TB hydrolysis reagent (Difco Laboratories)

Procedure:

1. Add 1.0 ml of distilled water to a sterile 13 × 75 mm screw-capped tube.
2. Add two drops of Bacto-TB hydrolysis reagent to each tube.
3. Add one loopful of actively growing (3 to 4 weeks old) culture.
4. Tighten caps on tubes and incubate at 37°C for 10 days.
5. Observe for a color change.

2. Kilburn Method[6,24]

Reagents:

Phosphate buffer, pH 7.0, M/15	100.0 ml
Na_2HPO_4 (J. T. Baker Chemical Co.)	
9.47 g/liter of distilled water	(61.1 ml)
KH_2PO_4 (J. T. Baker Chemical Co.)	
9.09 g/liter of distilled water	(38.9 ml)
Polysorbate-80 (Emulsion Engineers Inc.)	0.5 ml
0.1% neutral red solution, prepared by correcting for dye content	2.0 ml

Combine reagents and dispense 2.0 ml amounts into 16 × 125 mm screw-capped tubes.
Autoclave tubes for 10 min at 121°C. (Substrate is stable for 2 to 4 weeks only.)

Procedure:

1. Inoculate the polysorbate-80 substrate with a 3-mm loopful of an actively growing culture.
2. Incubate for 10 days at 35° to 37°C.
3. Observe for a color change.

Interpretation:

1. A color change from amber to pink or red within 5 days is representative of a positive test while a color change produced between 5 and 10 days is considered to be equivocal.
2. No color change is regarded as a negative test.

Controls:

Positive: *M. kansasii*
Negative: *M. avium-intracellulare* complex

Arylsulfatase Production[21]

Purpose:
To detect the enzyme, arylsulfatase, that is produced by certain, usually rapidly growing, species of mycobacteria.

Principle:
The enzyme, arylsulfatase, which is produced primarily by the rapidly growing mycobacteria, is detected by its degradation of the sulfate molecules of a tripotassium phenolphthalein disulfate salt into free phenolphthalein and the remaining salts. The addition of a base, sodium carbonate, reacts with the free phenolphthalein, and produces a red diazo reaction that is easily visible.

Specimen:
Isolated colonies of an actively growing culture.

Reagents:

Substrate

Middlebrook 7H9 broth with 2% glycerol	100.0 ml
Phenolphthalein disulfate tripotassium salt (Eastman Chemical Co.)	0.2 g

Sodium Carbonate Solution

Sodium carbonate (J. T. Baker Chemical Co.)	10.6 g
Distilled water	100.0 ml

Procedure:

1. Dispense 3 ml amounts of sterile Middlebrook 7H9 broth and glycerol into 16 × 125 mm screw-capped tubes.
2. Inoculate a 3-mm loopful of the test culture into a tube of the arylsulfatase substrate.
3. Incubate for 3 days at 35° to 37°C.
4. Add four to five drops of Na_2CO_3 to each tube and observe for the presence of a pink to purple color.

Interpretation:

1. A color change to pink, red or purple is representative of a positive test.
2. No color change is indicative of a negative test.

Controls:

Positive: *M. fortuitum*
Negative: *M. avium-intracellulare* complex

Alternative Methods:
Dubos broth may be substituted for the Middlebrook 7H9 broth. In addition, the method described by Wayne[21] uses an unenriched agar base medium (Wayne's arylsulfatase agar, BBL Microbiology Systems).

Catalase Tests

Purpose:
To detect catalase production by mycobacteria.

Principle:
The semiquantitative catalase test detects differences among certain mycobacteria in their production of catalase by measuring the height of the column of bubbles produced after the addition of H_2O_2. In addition, certain mycobacteria produce a catalase that is heat-labile and can be detected by heating the culture to 68°C before adding H_2O_2. The principle of the catalase test has been described elsewhere (p. 134).

Reagents:

Hydrogen Peroxide Solution

Hydrogen peroxide (30%)	
Polysorbate-80 (Emulsion Engineers Inc.)	10.0 ml
Distilled water	90.0 ml

Mix equal parts of the Tween-80 solution and 30% hydrogen peroxide shortly before performing catalase tests.

M/15 Phosphate Buffer, pH 7.0

Na_2PO_4 (J. T. Baker Chemical Co.)	
9.47 g/liter of distilled water	(63.9 ml)
KH_2PO_4 (J. T. Baker Chemical Co.)	
9.09 g/liter of distilled water	(36.1 ml)

The combination of the two salt solutions should give a final pH of 7.0.

Lowenstein-Jensen Medium
Lowenstein-Jensen medium is prepared by adding 5 ml into 20 × 150 mm screw-capped tubes to make deep butts. This medium is available commercially as Lowenstein medium, Jensen Deeps from Difco Laboratories.

Procedure:

Semiquantitative Test[8]

1. Prepare a dense suspension of the test organism in 1.0 ml of distilled water.
2. Inoculate the entire surface of a Lowenstein medium, Jensen Deep tube.
3. Incubate cultures in an upright position with caps loose for 2 weeks at 35° to 37°C.
4. After incubation, add 1.0 ml of the Tween-80 hydrogen peroxide solution to each tube.
5. Allow reaction to proceed for 5 min at room temperature and measure the height of the column of bubbles produced and report as ≤50 mm or >50 mm.

Catalase Inactivation at 68° C[9]

1. Suspend a 3-mm loopful of growth from an actively growing culture into a 16 × 125 mm screw-capped tube containing 0.5 ml of M/15 phosphate buffer, pH 7.0.
2. Place the tube in a 68°C water bath for 20 min.
3. Allow the suspension to cool to room temperature.
4. Add 0.5 ml of the Tween-80-hydrogen peroxide solution to each tube.
5. Observe for the presence of bubble formation.
6. Tubes should be kept for 20 min before being reported as negative.

Interpretation:

1. The semiquantitative method of detecting catalase production is useful for separating species that produce large and small amounts of catalase. Most isolates of isoniazid resistant *M. tuberculosis* will produce little or no detectable catalase.
2. *M. tuberculosis, M. bovis,* and *M. gastri* have heat-labile catalase, which is inactivated at 68°C, whereas the catalase produced by other species of mycobacteria is heat stable.

Controls:

Positive: *M. fortuitum*
Negative: *M. tuberculosis* H37Rv

Sodium Chloride Tolerance[7]

Purpose:
To detect those species of rapidly growing mycobacteria that are able to grow in the presence of 5% sodium chloride.

Principle:
The differentiation of *M. fortuitum* from other slower growing mycobacteria is accomplished by its ability to grow in a medium containing 5% sodium chloride. In addition, *M. fortuitum* may be distinguished from *M. chelonei,* another rapidly growing species, by its ability to grow in the presence of 5% salt.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:

Lowenstein-Jensen Medium Containing 5% NaCl
Lowenstein-Jensen medium slants containing 5% sodium chloride are prepared. Bacto Lowenstein medium, Jensen with 5% NaCl and Bacto ATS medium with 5% NaCl are commercially available from Difco Laboratories.

Procedure:

1. Inoculate the surface of a slant of Lowenstein medium, Jensen with 5% NaCl with 0.1 ml of a slightly turbid suspension of the test organism.
2. Observe the slants for the presence of growth after 2 to 3 weeks of incubation at 35° to 37°C.

Interpretation:
Growth on the medium containing 5% NaCl is indicative of a positive test while growth only on a NaCl-free medium is considered to be a negative test.

Controls:

Positive: *M. fortuitum*
Negative: *M. avium-intracellulare* complex

Urease Production

Purpose:
To detect urease production by certain species of mycobacteria.

Principle:
Refer to page 194.

Specimen:
Isolated colonies from an actively growing culture.

Methods:

1. Disk Method[11]

Reagents:
Bacto-Differentiation Disks Urea (Difco Laboratories).

Procedure:

1. Emulsify several colonies of the test organism in 0.5 ml distilled water.
2. Add a single urea disk.
3. Incubate at 37°C for 4 hr.
4. Examine for the presence of a cerise (cherry red) color after 10 or more min.

2. Broth Method[17]

Reagents:

Bacto-urea agar base concentrate (Difco Laboratories	10.0 ml
Distilled water	90.0 ml

Dispense in 4 ml amounts in 16 × 125 mm screw-capped tubes.

Procedure:

1. Inoculate the urea substrate with a 3-mm loopful of the test culture.
2. Incubate for 3 days at 35° to 37°C.
3. Observe for a color change of pink or red.

Interpretation:

1. A positive test with a color change to red or purple is evidence of the production of urease by the test organism.
2. No color change is indicative of a negative test.

Controls:

Positive control: *M. kansasii*
Negative control: *M. avium-intracellulare* complex

Alternative Methods:
A simplified broth method was developed by Steadham[14] and appears to be reliable as an alternate method.

Tellurite Reduction[5]

Purpose:
To distinguish *M. avium-intracellulare* complex from other nonchromogenic, slower growing mycobacterial species.

Principle:
Mycobacterium avium-intracellulare complex produces an enzyme, tellurite reductase, that reduces a tellurite salt to the metal tellurium. Potassium tellurite acts as an artificial electron acceptor and is reduced to metallic tellurium.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:

Tellurite Test Medium

Middlebrook 7H9 broth *without* glycerol	100.0 ml
Polysorbate-80 (Emulsion Engineers, Inc.)	5.0 ml

Prepare Middlebrook 7H9 according to manufacturer's instructions with the addition of Polysorbate-80. Dispense 5 ml amounts into sterile 16 × 125 mm screw-capped tubes.

Tellurite Test Reagent

Potassium tellurite (Difco Laboratories)	0.1 g
Distilled water	50.0 ml

Dissolve tellurite and autoclave for 15 min at 15 pounds pressure (121 °C). Store in refrigerator and dispense in 2 ml amounts just prior to use.

Procedure:

1. Emulsify a small amount of growth in the tellurite test medium to obtain a *slightly* turbid suspension.

2. Allow culture to incubate for 7 days at 35° to 37°C in the absence of 5 to 10% CO_2.
3. Add two drops of the potassium tellurite test reagent to the 7-day-old culture and incubate for an additional 3 days.
4. Examine the sediment in the tube daily for the presence of a black color.

Interpretation:

1. A positive reaction may begin as a light brown or gray color, but the presence of a black color should be present to indicate the presence of tellurite reductase.
2. No color change is indicative of a negative test.

Niacin Production (Konno-Runyon Method[6,13])

Purpose:
To distinguish *M. tuberculosis* from other slowly growing nonchromogenic mycobacteria.

Principle:
Niacin is a precursor in the biosynthesis of the coenzymes, nicotinic acid adenine dinucleotide and nicotinic acid adenine dinucleotide phosphate. A blocked enzymatic pathway prevents cells of *M. tuberculosis* from utilizing niacin to produce coenzymes and leads to its accumulation.

Niacin is detected when it reacts with cyanogen bromide in the presence of an aromatic amine, i.e., aniline to produce a colored schiff base (yellow).

Specimen:

1. A 3- to 4-week-old culture actively growing on an egg base medium, i.e., Lowenstein-Jensen medium. Middlebrook agar base media are *not* satisfactory unless supplemented with 0.1 to 0.5% asparagine. A large amount of growth is necessary to perform the test. Cultures may be autoclaved for 15 min prior to testing if desired.
2. When growth is insufficient in primary cultures, it should be subcultured into 5.0 ml of Middlebrook 7H9 broth. Testing may proceed when growth attains visible turbidity.

Reagents:

Aniline Reagent

Ethanol (95%)
Aniline (J. T. Baker Chemical Co.) 4.0 ml

Store refrigerated in a brown bottle. Discard solution when a yellow color develops.

Cyanogen Bromide

Cyanogen bromide (Eastman Chemical Co.)	5.0 g
Distilled water	50.0 ml

Prepare only in a well-ventilated safety cabinet. Solution should be stored in a tightly capped brown bottle under refrigeration. If a precipitate forms during refrigeration, warm to room temperature to dissolve. Prepare in small quantities to prevent volatilization and loss of potency.

Procedure:

1. If adequate growth is present on egg base media, add 1.0 ml of sterile water or saline to the slant. Scrape colonies free from the surface of the medium to allow the liquid to be in direct contact with the medium's surface. Allow the liquid to incubate for 15 to 30 min.
2. Remove 0.5 ml of the liquid from the slant or from the 7H9 broth subculture.
3. Add 0.5 ml of alcoholic aniline solution.
4. Add 0.5 ml of cyanogen bromide.
5. Observe for the presence of a yellow color within 1 to 2 min.

Interpretation:
The presence of a yellow color indicates the presence of free niacin. A false negative test may occur if an insufficient amount of growth is present prior to testing. False negative tests may also occur if growth is not removed from the slant surface when an egg base medium is used. A colorless test indicates a lack of niacin and is interpreted as being negative.

Controls:

Positive: *M. tuberculosis*
Negative: *M. avium-intracellulare* complex

Thiophene-2-Carboxylic Acid Hydrazide (TCH) Susceptibility[3,19]

Purpose:
To distinguish *M. bovis* from *M. tuberculosis.*

Principle:
TCH selectively inhibits the growth of *M. bovis;* however, other species of mycobacteria are unaffected.

Specimen:
Isolated colonies from an actively growing culture.

Procedure:
The proportion susceptibility method described on page 408 is employed by testing 1.0 and 5.0 μg/ml of TCH.

Interpretation:
A lack of growth on the medium containing either 1 or 5 μg/ml of TCH is considered to be a positive test and is characteristic of *M. bovis.* Occasionally, an isoniazid-resistant culture of *M. tuberculosis* will fail to grow on the antimicrobial-containing medium.

Controls:

Positive: *M. bovis*
Negative: *M. tuberculosis,* H37Rv

Gas-Liquid Chromatographic Identification[15,16]

Purpose:
To rapidly identify clinically important mycobacteria recovered from specimens.

Principle:
Organisms are saponified in methanolic NaOH, and the mixture is treated with boron trifluoride in methanol and extracted with a hexane-chloroform mixture. Fatty acids found in mycobacterial cells are identified by their retention times, and peak heights are considered when interpreting a profile characteristic for certain species of mycobacteria.

Specimen:
Isolated colony from a freshly growing culture.

Reagents:

Reagent	Concentration
Sodium hydroxide in methanol (Mallinckrodt)	0.5 mol/liter
Boron trifluoride–methanol (Applied Science Laboratories)	14% w/v
Sodium chloride (J. T. Baker Chemical Co.)	Saturated solution
Chloroform-hexane (Burdick and Jackson Laboratories, Inc.; M. C. B. Reagents)	1:4 v/v
Chloroform (Burdick and Jackson Laboratories, Inc.)	

Procedure:

1. Suspend 1 loopful (colony) of mycobacteria in 0.05 ml of sodium hydroxide in methanol using a 6 × 50 test tube. A 200 μl polypropylene centrifuge (Bio-Rad Laboratories) is used as a cork.
2. Incubate at 70°C for 30 min.
3. After cooling, add 0.1 ml of boron trifluoride.
4. Incubate at 70°C for 5 min.
5. After cooling, add 0.05 ml of saturated NaCl.
6. Add 0.2 ml of chloroform-hexane.
7. Agitate for 5 min.
8. Centrifuge at 1000 × g for 5 min.

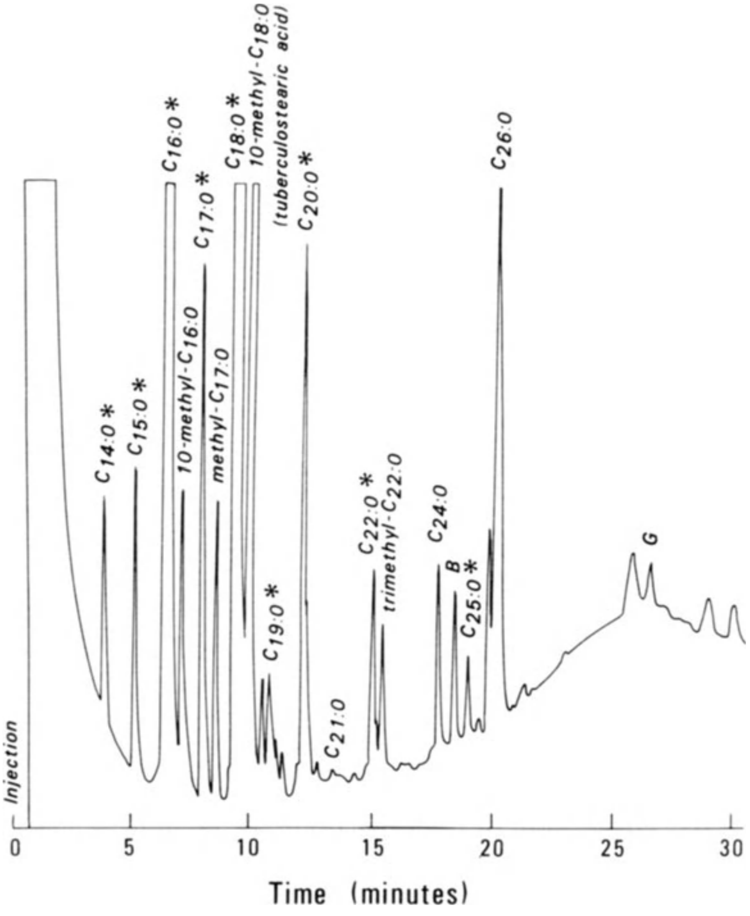


Figure 6–5. Typical chromatogram of fresh isolate of *M. tuberculosis*.

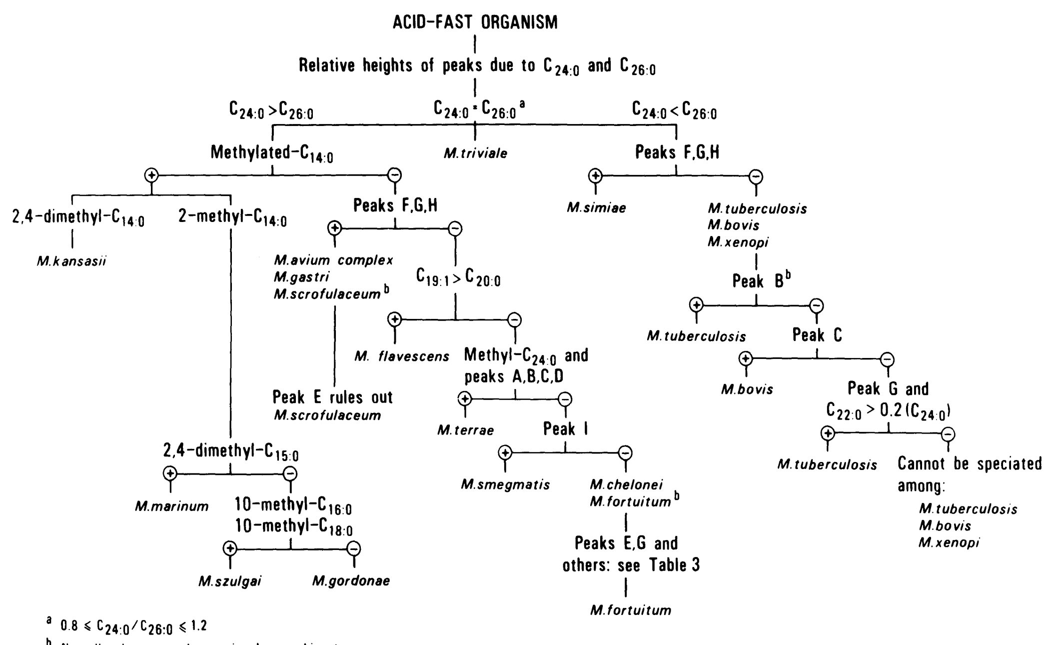


Figure 6–6. Identification scheme of mycobacteria by chromatographic pattern.

9. Transfer upper phase to a 6 × 50 test tube.
10. Evaporate to dryness in a water bath at 50–60°C under a stream of air.
11. Reconstitute with 20 μl chlorofom.
12. Inject 2 ml into a gas liquid chromatograph with a flame-ionization detector.

GLC Conditions:

Column:	OV-17 (Supelco, Inc.), glass, 6 ft in length
Injector port temperature:	275°C
Detector temperature:	315°C
Oven temperature:	170°C for 2 min after injection then raised 6°C/min to 300°C for 15 min.

Interpretation:

Figure 6–5 presents a chromatogram typical of *Mycobacterium tuberculosis.* All chromatograms are examined and organisms are characterized according to the decision tree presented in Figure 6–6.

Supplementary biochemical tests are selected, as shown below, for certain groups of organisms:

M. tuberculosis, M. bovis	Niacin, TCH
M. xenopi	Yellow pigmentation
M. avium-intracellulare complex	Tellurite reduction
M. scrofulaceum	Urease production
M. gastri	Catalase inactivation at 68°C
M. marinum	Nitrate reduction
M. szulgai	
M. smegmatis	Arylsulfatase
M. fortuitum	
M. chelonei	Nitrate reduction

Controls:
Standards of fatty acids C_{14}—C_{22}, C_{24}, C_{12-14}, C_{14-16}, C_{16-18}, myristyl laurate, myristyl palmitate, palmityl palmitate, and stearyl stearate (Nu-Chek-Prep, Inc., Elysian, MN) are included with each series of sample testing.

III. Antimicrobial Susceptibility Testing

A. General Considerations

It is well documented that resistant and susceptible populations of mycobacteria coexist within the same culture. If the culture is exposed to only one antimicrobial, the resistant population survives and predominates. Hence, the typical antimycobacterial regimen includes three appropriate antimicrobials that theoretically reduce the resistant population to an insignificant level and allow normal host defense mechanisms to eliminate the remaining organisms.

Proportional Antimicrobial Susceptibility Testing Method[18]

Purpose:
To determine the minimum inhibitory concentration of mycobacteria to antimicrobial agents.

Principle:
If greater than 1% of a population of mycobacteria exhibits resistance in vitro to an antimicrobial, the infection will not respond favorably to that agent. The proportional antimicrobial susceptibility test method is designed to determine the proportion of mycobacteria exhibiting growth in the presence and absence of antimicrobials.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:

1. Antimicrobial solutions
   Obtain the specific activity (μg/mg) of antimicrobial from the manufacturer and prepare the solutions as described in Table 6–5.
2. Test medium
   Prepare the susceptibility test medium by adding the appropriate concentration of antimicrobial solution to the desired amount of Middlebrook 7H11 which has been cooled to 50° to 52°C. Three quadrants of each quadrant plate should be filled with 5 ml amounts of the medium containing appropriate concentrations of antimicrobials, while one quadrant is used as a control and is filled with antimicrobial-free Middlebrook 7H11 agar.

Table 6–5. Antimicrobial Agents Used in Determining *In Vitro* Susceptibility of Mycobacteria

Antimicrobial	Solvent	Diluent	Final concentration(s) (μg/ml) incorporated into Middlebrook 7H11 agar used for testing
Primary			
Ethambutol	Distilled water	Distilled water	4, 8
Isoniazid	Distilled water	Distilled water	1, 4
Rifampin	Dimethyl sulfoxide	Distilled water	1, 4
Streptomycin	Distilled water	Distilled water	8, 16
Secondary			
Cycloserine	Distilled water	Distilled water	16, 32
Ethionamide	Distilled water	Distilled water	4, 8
Kanamycin	Distilled water	Distilled water	8, 16
p-Aminosalicylic acid	Distilled water	Distilled water	2
Capreomycin	Distilled water	Distilled water	8, 16
Thiophene-2-carboxylic acid hydrazide[a]	Distilled water	Distilled water	1.0, 5.0

[a] Used only to distinguish *M. bovis* from *M. tuberculosis*.

Procedure:

1. Remove a 1-mm loopful of the test organism and inoculate a tube containing 10 ml of Middlebrook 7H9 broth with five (1–2 mm) glass beads.
2. Mix the cells for 10 to 15 sec on a Vortex mixer.
3. Allow the organisms to incubate for 5 to 7 days at 35°C, until growth is equivalent to the turbidity of half of a MacFarland No. 1 barium sulfate standard.
4. Mix cells on a Vortex mixer for 10 to 15 sec and allow larger clumps to settle for 5 to 10 min.
5. Prepare 10^{-2} and 10^{-4} dilutions of the test organism in sterile Middlebrook 7H9 broth or saline.
6. With a capillary pipette, inoculate three drops of the suspension onto the surface of each antimicrobial-containing quadrant. Repeat the procedure with the other dilution of the suspension.
7. Tilt the plates until the surface of each quadrant has been evenly covered with the suspension.
8. Incubate the inoculated plates in sealed polyethylene bags in an atmosphere of 5 to 10% CO_2 at 35°C and read when colonies can be counted.

Interpretation:

1. Record the amount of growth on the control and antimicrobial-containing quadrants as follows:

   4+ = confluent growth (>500 colonies)
   3+ = 200 to 500 colonies
   2+ = 100 to 200 colonies
   1+ = 50 to 100 colonies
   actual number – ≤50 colonies

2. Calculate the percent resistance as follows:

$$\frac{\text{no. of colonies on antimicrobial-containing quadrant}}{\text{no. of colonies on control quadrant}} \times 100$$

3. Count colonies in the dilution giving 100 to 200 colonies on the control quadrant. Additional dilutions may be necessary if the colony count exceeds 200 colonies.
4. Report the lowest concentration (μg/ml) of antimicrobial to which the test organism exhibits <1% resistance as the minimum inhibitory concentration.

Controls:
M. tuberculosis isolates, TMC 102, TMC 306, TMC 330, and TMC 331, NJH 4292 and 8328, obtained from National Jewish Hospital and Research

Table 6–6. Antimicrobial Susceptibility of Quality Control Organisms

Antimicrobial	TMC 102	TMC 306	TMC 330	TMC 331
		No Growth at		
Isoniazid	1.0 μg/ml	4.0 μg/ml[a]	1.0 μg/ml	1.0 μg/ml
Rifampin	1.0 μg/ml	1.0 μg/ml	1.0 μg/ml	4.0 μg/ml*
Streptomycin	8.0 μg/ml	16.0 μg/ml[a]	8.0 μg/ml	8.0 μg/ml
Ethambutol	4.0 μg/ml	4.0 μg/ml	8.0 μg/ml*	4.0 μg/ml
Kanamycin	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml
Ethionamide	4.0 μg/ml	4.0 μg/ml	4.0 μg/ml	4.0 μg/ml
Cycloserine	16.0 μg/ml	16.0 μg/ml	16.0 μg/ml	16.0 μg/ml
p-Aminosalicylic acid	2.0 μg/ml	2.0 μg/ml	2.0 μg/ml	2.0 μg/ml
Capreomycin	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml
Pyrazinamide	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml	8.0 μg/ml

[a] Growth at concentration given.

Center, Denver, CO. Table 6–6 presents the susceptibility patterns for the four quality-control organisms.

IV. Common Aerobic Actinomycetes

A. Description of Group

Common aerobic actinomycetes, which include species of *Nocardia, Streptomyces,* and *Actinomadura,* are branching bacteria and members of the order Actinomycetales. Replication is by the production of spores or fragmentation of filaments 1 μm or less in diameter into coccoid or bacillary elements. Their growth rate is usually rapid (3 to 7 days) at temperatures of 30° to 37°C. All are gram-positive; however, none is acid-fast except for *Nocardia,* which exhibits partial acid-fastness. Many are susceptible to antibacterial antibiotics. All contain diaminopimelic acid (DAP) in their cell walls; the streptomyces contain the levo isomer while the actinomadurae and nocardiae contain the meso isomer.

B. Source and Clinical Importance

Members of the *Streptomyces* group are commonly recovered from clinical specimens (i.e., skin and respiratory secretions) but are rarely associated with clinical disease. Perhaps the greatest challenge for the microbiology laboratory is to distinguish between the genera *Streptomyces* and

Nocardia. *Nocardia asteroides* is commonly recovered from clinical specimens and is most frequently associated with pulmonary infection and disseminated disease in the compromised patient. In addition, it has been reported to cause subcutaneous abscesses, brain abscess, osteomyelitis, endophthalmitis, and ocular keratitis. It is important to note that *N. asteroides* may also be recovered from patients without clinical evidence of infection.[2]

Nocardia brasiliensis, uncommon to the clinical laboratory, has been reported to cause disseminated, lymphocutaneous, and subcutaneous infection in the United States. It is most often seen in Central and South America as the etiologic agent of mycetoma.

Other genera and species of Actinomycetes, which are known to be common etiological agents of mycetoma in other countries, are not presented here because of their rarity in the United States.

C. Identification

1. General Considerations

Aerobic actinomycetes are tentatively identified by their rapid growth rate (3 to 7 days) and characteristic small, heaped and wrinkled, moist to chalky colonies. The microscopic morphologic features and acid-fast staining properties of the Nocardiae serve to distinguish them from the other genera. Table 6–7 presents features useful for making a tentative identification of the common aerobic actinomycetes.

The definitive identification of the aerobic actinomyctes is based on the following: detection of the levo or meso isomers of diaminopimelic acid (DAP) in the cell wall; detection of hydrolytic enzymes for substrates such as xanthine, tyrosine, and casein; production of urease; and the detection of characteristic carbohydrates in the cell wall. The information provided by these criteria will identify most species of aerobic actinomycetes that are pathogenic for man. Table 6–7 includes characteristic features for several uncommon organisms that might possibly be encountered.

2. Preliminary Tests

Acid-Fast Stain for *Nocardia* (Modified Kinyoun's)

Purpose:
To detect members of the genus *Nocardia* and separate them from other members of the aerobic actinomycetes.

Table 6–7. Features Useful for the Identification of Common Aerobic Actinomycetes

	Tentative			Hydrolysis of			
Organism	Acid-fastness	Microscopic morphology	Colonial morphology	Casein	Xanthine	Tyrosine	Urease production
Streptomyces sp.	−	Filaments thick with prolific branching. Some isolates produce small chains of spores	Young colonies moist becoming chalky or powdery, slightly raised or flat	±	+	+	±
Nocardia asteroides	+	Filaments delicate, breaking into coccobacillary forms	Heaped, folded, chalky, orange to pink to white, mealy or leathery or dry	−	−	−	+
N. brasiliensis	+	Filaments delicate, breaking into coccobacillary forms	Heaped, folded, chalky, orange to pink to white, mealy or leathery or dry	+	−	+	+
N. caviae	+	Filaments delicate, breaking into coccobacillary forms	Heaped, folded, crusty, buff to pink	−	+	−	+
Actinomadura madura	−	Filaments delicate, nonfragmenting, some isolates produce arthroconidia on branches	Waxy, heaped, moist, white to pink to orange or red-orange	+	−	+	−
A. pelletieri	−	Filaments delicate, nonfragmenting	Heaped, waxy, dark red, resembles a mulberry	+	−	+	−

Principle:
Refer to page 391. Members of the genus *Nocardia* exhibit partial acid-fastness. Some filaments are stained well with the carbol fuchsin while others are only stained with the counterstain.

Specimen:
Isolated colonies from suitable culture media.

Reagents:

Carbol Fuchsin Stain
Refer to page 391.

Decolorizing Agent

Sulfuric acid (concentrated) (J. T. Baker Chemical Co.)	0.5 ml
Distilled water	99.5 ml

Add acid to water and mix well.

Methylene Blue Counterstain
Refer to page 391.

Procedure:

1. Flood smear with Kinyoun's carbol fuchsin for 5 min.
2. Rinse with distilled water.
3. Flood smear with 50% ethanol and pour off until excess carbol fuchsin is removed.
4. Rinse with distilled water.
5. Decolorize with 0.5% aqueous H_2SO_4 for 3 min.
6. Rinse with distilled water.
7. Counterstain with 1% methylene blue for 1 min.
8. Rinse with distilled water.
9. Rinse, drain and dry by blotting.
10. Examine using bright field microscopy and an oil immersion objective.

Interpretation:

Positive test: Some portions of the organisms retain carbol fuchsin and stain red.
Negative test: Organisms stain only blue with the methylene blue counterstain.

Controls:

Positive: *N. asteroides*
Negative: *Streptomyces* sp.

3. Definitive Tests

Substrate Hydrolysis (Casein, Xanthine, and Tyrosine)

Purpose:
To detect the presence of hydrolytic enzymes that degrade casein, xanthine, and tyrosine.

Principle:
Certain species of aerobic actinomycetes produce hydrolytic enzymes (currently unclassified) that degrade proteins, purines, and amino acids. Casein, xanthine and tyrosine are common substrates used for the identification of aerobic actinomycetes and are incorporated into an agar medium for testing. Hydrolysis of the substrate is detected by observing a clearing of the medium around the area of inoculum.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:

Casein Agar

Skim milk (dehydrated or instant nonfat)	50.0 g
Agar (Difco Laboratories)	10.0 g
Distilled water	1000.0 ml

Dissolve milk and agar in 500 ml distilled water each and autoclave for 15 min at 15 psi. Combine ingredients and pour into petri dishes.

Xanthine Agar

Nutrient agar (Difco Laboratories)	23.0 g
Xanthine (National Biochemical Corporation)	4.0 g
Distilled water	1000.0 ml

Dissolve xanthine in 100 ml distilled water, add to nutrient agar solution and pH to 7.0. Autoclave for 15 min at 15 psi, swirl medium as it is poured into petri dishes.

Tyrosine Agar
Prepare as described for xanthine agar (above) but use 5.0 gm of tyrosine (Eastman Chemical Co.).

Procedure:

1. Inoculate each medium by placing a small amount of growth in the center of the petri dish.
2. Incubate for 2 weeks at 30°C.
3. Observe periodically for hydrolysis (clearing) around the colonies.

Interpretation:
Clearing of the medium around and below colonies is indicative of hydrolysis of the substrate. Table 6–7 presents characteristic reactions for common species.

Controls:

Positive control: *Streptomyces* sp.
Negative control: *N. asteroides*

Urease Production

Purpose:
To distinguish the nocardiae from other aerobic actinomycetes by their ability to produce urease.

Principle:
Refer to page 194.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:
Bacto-Christensen's urea agar slants, (Difco Laboratories).

Procedure:

1. Inoculate each slant with growth from an actively growing culture.
2. Incubate for 2 weeks at 30°C and observe for a color change from yellow to pink or purple.

Interpretation:

1. A positive test with a color change to pink or purple is evidence of the production of urease by the test organism.

2. No color change is indicative of a negative test.
3. Common species of *Nocardia* and approximately 50% of *Streptomyces* sp. produce urease while common species of *Actinomadura* do not.

Controls:

Positive: *N. asteroides*
Negative: Non-urease-producing strain of *Streptomyces* sp.

References

1. Barksdale, L., and Kim, K. Mycobacterium. *Bacteriol. Rev.* **41**:217, 1977.
2. Frazier, A. R., Rosenow, E. C., III, and Roberts, G. D. Nocardiosis: a review of twenty-five cases occurring during 24 months. *Mayo Clinic Proc.* **50**:657, 1975.
3. Harrington, R., and Karlson, A. G. Differentiation between *M. tuberculosis* and *M. bovis* by in vitro procedures. *Am. J. Vet. Res.* **27**:1193, 1967.
4. Kilburn, J. O., O'Donnell, K. F., Silcox, V. A., and David, H. L. Preparation of a stable mycobacterial Tween hydrolysis test substrate. *Appl. Microbiol.* **26**:826, 1973.
5. Kilburn, J. O., Silcox, V. A., and Kubica, G. P. Differential identification of mycobacteria. V. The tellurite reduction test. *Am. Rev. Resp. Dis.* **99**:94, 1969.
6. Konno, K. New chemical method to differentiate human-type tubercule bacilli from other mycobacteria. *Science* **124**:985, 1956.
7. Kubica, G. P. Differential identification of mycobacteria. VII. Key features for identification of clinically significant mycobacteria. *Am. Rev. Resp. Dis.* **107**:9, 1973.
8. Kubica, G. P., Jones, W. D., Jr., Abbott, V. D., Beam, R. E., Kilburn, J. O., and Cater, J. C., Jr. Differential identification of mycobacteria. I. Tests on catalase activity. *Am. Rev. Resp. Dis.* **94**:400, 1966.
9. Kubica, G. P., and Pool, G. L. Studies on the catalase activity of acid-fast bacilli. I. An attempt to subgroup these organisms on the basis of their catalase activities at different temperatures and pH. *Am. Rev. Resp. Dis.* **81**:387, 1960.
10. Morgan, M. A., Horstmeier, C. D., DeYoung, D. R., and Roberts, G. D. Comparison of a radiometric method (BACTEC) and conventional media for recovery of mycobacteria from smear negative specimens. *J. Clin. Microbiol.* **18**:384, 1983.
11. Murphy, D. B., and Hawkins, J. E. Use of urease disks in the identification of mycobacteria. *J. Clin. Microbiol.* **1**:465, 1975.
12. Roberts, G. D., Goodman, N. L., Heifets, L., Larsh, H. W., Lindner, T. H., McClatchy, J. K., McGinnis, M. R., Siddiqi, S. H., and Wright, P. Evaluation of BACTEC radiometric method for recovery of Mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast positive specimens. *J. Clin. Microbiol.* **18**:689, 1983.
13. Runyon, E. H., Selin, M. J., and Harris, H. W. Distinguishing mycobacteria by the niacin test. *Am. Rev. Tuberc.* **79**:663, 1959.

14. Steadham, J. E. Reliable urease test for identification of mycobacteria. *J. Clin. Microbiol.* **10**:134, 1979.
15. Tisdall, P. A., Roberts, G. D., and Anhalt, J. P. Identification of clinical isolates of mycobacteria with gas-liquid chromatography alone. *J. Clin. Microbiol.* **10**:506, 1979.
16. Tisdall, P. A., DeYoung, D. R., Roberts, G. D., and Anhalt, J. P. Identification of clinical isolates of Mycobacteria with gas-liquid chromatography: a 10-month follow-up study. *J. Clin. Microbiol.* **16**:400, 1982.
17. Toda, T. Y., Hagihara, Y., and Takeya, K. A simple urease test for the classification of mycobacteria. *Am. Rev. Resp. Dis.* **83**:757, 1960.
18. Vestal, A. L. Procedures for the isolation and identification of mycobacteria. U.S. Department of Health, Education and Welfare, Public Health Service Publication (CDC) 75–8230, 1975.
19. Vestal, A. L., and Kubica, G. P. Differential identification of mycobacteria. III. Use of thiacetazone, thiophene-2-carboxylic acid hydrazide and triphenyltetrazolium chloride. *Scand. J. Resp. Dis.* **48**:142, 1967.
20. Virtanen, S. A study of nitrate reduction by mycobacteria. *Acta Tuberc. Scand.* (*Suppl.*) **48**:119, 1960.
21. Wayne, L. G. Recognition of *Mycobacterium fortuitum* by means of a three day phenolphthalein sulfatase test. *Am. J. Clin. Pathol.* **36**:185, 1961.
22. Wayne, L. G., and Doubek, J. R. The role of air in the photochromogenic behavior of *Mycobacterium kansasii. Am. J. Clin. Pathol.* **42**:431, 1964.
23. Wayne, L. G., and Doubek, J. R. Classification and identification of mycobacteria. II. Tests employing nitrate and nitrite as substrates. *Am. Rev. Resp. Dis.* **91**:738, 1965.
24. Wayne, L. G., Doubek, J. R., and Russell, R. L. Classification and identification of mycobacteria. I. Tests employing Tween-80 as a substrate. *Am. Rev. Resp. Dis.* **90**:588, 1964.

7

Fungi

Glenn D. Roberts, Ph.D.

I. General Considerations

A. Description of the Group

The fungi are a group of organisms that lack chlorophyll, reproduce by the production of spores (sexually and/or asexually), and contain chitin or cellulose in their cell walls. In contrast to bacteria, they are eukaryotic and have cell membranes that are rich in sterols, i.e., ergosterol. They are not susceptible to usual antibacterial antimicrobials but are to polyene antimicrobials that have a marked affinity for sterols.

The fungi of importance to the clinical microbiology laboratory are the yeasts and filamentous fungi, both monomorphic and dimorphic. Yeasts are unicellular and usually reproduce by budding, while the filamentous fungi are composed of hyphae and reproduce by spores. The dimorphic fungi produce hyphae at room temperature and a yeast form in vitro at 37°C or in the infected host; however, *Coccidioides immitis* lacks a yeast form and produces spherules and endospores.

B. Source and Clinical Importance

The significance of fungi recovered from most clinical specimens is, at best, difficult to assess. Most exist ubiquitously in the environment as saprophyte and are commonly found as transient flora in "normal" persons and have little clinical significance. However, many of these fungi have been associated with severe and often fatal infections of compromised patients. The clinical laboratory cannot determine the clinical significance

of fungi and is obligated to identify and report all organisms recovered so that the clinician may consider them when making a diagnosis.

The monomorphic filamentous fungi commonly recovered from clinical specimens and their association with reported cases of human infection are presented in Table 7–1. Studies correlating their clinical significance in clinical specimens are lacking.

The dimorphic filamentous fungi, including *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*, must always be regarded as clinically significant when recovered from clinical specimens.

Yeasts are more commonly recovered from clinical specimens than are other fungi which makes an assessment of their importance challenging. Table 7–2 gives the frequencies and distribution of yeasts recovered

Table 7–1. Common Filamentous Fungi and Their Association with Human Mycotic Infections

Etiological agent	Common recovery sites	Clinical association
Acremonium species	Skin, nails, respiratory secretions, cornea, vagina, gastric washings	Skin and nail infections, mycotic keratitis
Alternaria species	Skin, nails, conjunctiva, and respiratory secretions	Skin and nail infections, conjunctivitis, hypersensitivity pneumonitis, palatitis, osteomyelitis
Aspergillus flavus	Skin, respiratory secretions, gastric washings, nasal sinuses	Skin infections, allergic bronchopulmonary infection, sinusitis, myocarditis, disseminated infection, renal infection, subcutaneous mycetoma
Aspergillus fumigatus	Respiratory secretions, skin, ear, cornea, gastric washings, nasal sinuses, stool, bone	Allergic bronchopulmonary infection, fungus ball, invasive pulmonary infection, skin and nail infections, osteomyelitis, external otomycosis, mycotic keratitis, sinusitis, myocarditis, renal infection and brain abscess. Normal flora in 16% of population.[42]
Aspergillus niger	Respiratory secretions, gastric washings, ear, skin	Fungus ball, pulmonary infection, external otomycosis, mycotic keratitis
Blastomyces dermatitidis[a]	Respiratory secretions, skin, oropharyneal ulcers, bone, prostate	Pulmonary infection, skin infection, oropharyngeal ulceration, osteomyelitis, prostatitis, arthritis, CNS infection

Table 7–1. (*continued*)

Etiological agent	Common recovery sites	Clinical association
Cladosporium species	Respiratory secretions, skin, nails, nose, cornea	Skin and nail infections, mycotic keratitis. Chromoblastomycosis, brain abscess, and tinea nigra palmaris caused by *Cladosporium carrionii, Exophiala werneckii,* and *C. trichoides,* respectively
Coccidioides immitis[a]	Respiratory secretions, skin, bone, cerebrospinal fluid, synovial fluid, urine, gastric washings	Pulmonary infection, skin infection, osteomyelitis, meningitis, arthritis, disseminated infection
Drechslera species	Respiratory secretions, skin	Pulmonary infection (rare), osteomyelitis
Epidermophyton floccosum	Skin, nails	Tinea cruris, tinea pedis, tinea corporis, onychomycosis
Fusarium species	Skin, respiratory secretions, cornea	Mycotic keratitis, skin infection (in burn patients), disseminated infection
Geotrichum species	Respiratory secretions, urine, skin, stool, vagina, conjunctiva, gastric washings, throat	Bronchitis, skin infection, colitis, conjunctivitis, thrush, wound infection
Histoplasma capsulatum[a]	Respiratory secretions, bone marrow, blood, heart, urine, adrenals, skin, cerebrospinal fluid, eye, pleural fluid, liver, spleen, oropharyngeal lesions, vagina, gastric washings, larynx	Pulmonary infection, oropharyngeal lesions, CNS infection, skin infection (rare), uveitis, peritonitis, endocarditis
Microsporum audouinii	Hair	Tinea capitis
Microsporum canis	Hair, skin	Tinea corporis, tinea capitis, tinea barbae, tinea manuum
Microsporum gypseum	Hair, skin	Tinea capitis, tinea corporis
Mucor species	Respiratory secretions, skin, nose, brain, stool, orbit, cornea, vitreous humor, gastric washings, wounds, ear	Rhinocerebral infection, pulmonary infection, gastrointestinal infection, mycotic keratitis, intraocular infection, external otomycosis, orbital cellulitis
Nocardia asteroides[b]	Respiratory secretions, skin, urine, blood, brain, conjunctiva, bone, cornea, gastric washings	Pulmonary infection, mycetoma, brain abscess, conjunctivitis, osteomyelitis, mycotic keratitis, disseminated infection

Table 7–1. (*continued*)

Etiological agent	Common recovery sites	Clinical association
Penicillium species	Respiratory secretions, gastric washings, skin, urine, ear, cornea	Pulmonary infection, skin infection, external otomycosis, mycotic keratitis, endocarditis, cutaneous ulceration
Phialophora species (including *Wangiella dermatitidis* and *Exophiala jeanselmei*)	Respiratory secretions, gastric washings, skin, cornea, conjunctiva	Some species produce chromoblastomycosis or mycetoma; mycotic keratitis, conjunctivitis, intraocular infection
Pseudallescheria (*Petriellidium*) *boydii*	Respiratory secretions, gastric washings, skin, cornea	Pulmonary fungus ball, mycetoma, mycotic keratitis, brain abcess
Rhizopus species	Respiratory secretions, skin, nose, brain, stool, orbit, cornea, vitreous humor, gastric washings, wounds, ear	Rhinocerebral infection, pulmonary infection, mycotic keratitis, intraocular infection, orbital cellulitis, deep wound infection, external otomycosis, dermatitis
Scopulariopsis species	Respiratory secretions, gastric washings, nails, skin, vitreous humor, ear	Pulmonary infection, nail infection, skin infection, intraocular infection, external otomycosis
Sporothrix schenckii[a]	Respiratory secretions, skin, subcutaneous tissue, maxillary sinuses, synovial fluid, bone marrow, bone, cerebrospinal fluid, ear, conjunctiva	Pulmonary infection, lymphocutaneous infection, sinusitis, arthritis, osteomyelitis, meningitis, external otomycosis, conjunctivitis, disseminated infection, fungemia
Trichophyton mentagrophytes	Hair, skin, nails	Tinea barbae, tinea capitis, tinea corporis, tinea cruris, tinea pedis, onychomycosis
Trichophyton rubrum	Hair, skin, nails	Tinea pedis, onychomycosis, tinea corporis, tinea cruris, mycetoma
Trichophyton tonsurans	Hair, skin, nails	Tinea capitis, tinea corporis, onychomycosis, tinea pedis
Trichophyton verrucosum	Hair, skin, nails	Tinea capitis, tinea corporis, tinea barbae

[a] Clinically significant regardless of the site from which they are recovered.
[b] Although *N. asteroides* is a bacterium, it is commonly recovered on fungal culture media due to its slow growth rate.

Table 7–2. Frequencies of Yeasts Recovered from Various Body Sites[24]

Type of patient	No. of patients	Specimen	Yeasts recovered (%)		*Candida albicans* recovered (%)	
			Mean	Range	Mean	Range
"Normal"	457	Skin	0.6	0–2.0	0.2	0–0.5
Hospitalized	12,786	Skin	10.5	4.7–16.5	5.8	0.4–12.4
"Normal"	1,820	Mouth	14.3	2–37	10.3	2–23
Hospitalized	8,085	Mouth	45.2	13–76	42.9	11–70
"Normal"	1,215	Anorectal tract	23.7	19–40	14.6	12–23
Hospitalized	3,162	Anorectal tract	25.2	6–62	22.0	1–48
"Normal"	1,692	Vagina	9.5	4.3–13	7.8	0–11.3
Hospitalized	8,900	Vagina	20.7	8–68	14.9	7–52
Patients with vaginitis	8,720	Vagina	29.1	26–76	25.7	12–45
Hospitalized	706	Urine	8.9	8.4–9.3	3.0	1.6–4.4
Outpatients and	9,797 specimens	Sputum	53.3	—	82.5	—
hospitalized[22]	2,902 specimens	Induced sputum	56.6	—	73.9	—
	3,135 specimens	Bronchial washings	33.6	—	52.4	—

Table 7–3. Common Yeasts and Their Association with Human Infection[a]

Organism	Common Recovery Sites	Clinical Association
Candida albicans	Respiratory secretions, vagina, urine, skin, oropharynx, gastric washings, blood, stool, transtracheal aspiration, cornea, nails, cerebrospinal fluid, bone, peritoneal fluid, bile	Pulmonary infection, vaginitis, urinary tract infection, dermatitis, fungemia, mycotic keratitis, onychomycosis, meningitis, osteomyelitis, peritonitis, myocarditis, endocarditis, endophthalmitis, disseminated infection, thrush, arthritis, common duct stone formation
Candida glabrata	Respiratory secretions, urine, vagina, gastric washings, blood, skin, oropharynx, transtracheal aspiration, stool, bone marrow, skin (rare)	Pulmonary infection, urinary tract infection, vaginitis, fungemia, disseminated infection, endocarditis
Candida tropicalis	Respiratory secretions, urine, gastric washings, vagina, blood, skin, oropharynx, transtracheal aspiration, stool, pleural fluid, peritoneal fluid, cornea, bone	Pulmonary infection, vaginitis, thrush, endophthalmitis, endocarditis, arthritis, peritonitis, mycotic keratitis, fungemia, osteomyelitis, meningitis
Candida parapsilosis	Respiratory secretions, urine, gastric washings, blood, vagina, oropharynx, skin, transtracheal aspiration, stool, pleural fluid, ear, nails	Endophthalmitis, endocarditis, vaginitis, mycotic keratitis, external otomycosis, paronychia, fungemia, onychomycosis
Saccharomyces species	Respiratory secretions, urine, gastric washings, vagina, skin, oropharynx, transtracheal aspiration, stool	Pulmonary infection (rare), endocarditis, fungemia (rare)
Candida krusei	Respiratory secretions, urine, gastric washings, vagina, skin, oropharynx, blood, transtracheal aspiration, stool, cornea	Endocarditis, vaginitis, urinary tract infection, fungemia, mycotic keratitis
Candida guilliermondii	Respiratory secretions, gastric washings, vagina, skin, nails, oropharynx, blood, cornea, bone, urine	Endocarditis, fungemia, dermatitis, onychomycosis, mycotic keratitis, osteomyelitis, urinary tract infection
Rhodotorula species	Respiratory secretions, urine, gastric washings, blood, vagina, skin, oropharynx, stool, cerebrospinal fluid, cornea	Fungemia, endocarditis, mycotic keratitis
Trichosporon species	Respiratory secretions, skin, oropharynx, stool	Pulmonary infection, brain abscess, disseminated infection, piedra

Table 7–3. (*continued*)

Organism	Common Recovery Sites	Clinical Association
Cryptococcus neoformans	Respiratory secretions, cerebrospinal fluid, bone, blood, bone marrow, urine, skin, pleural fluid, gastric washings, transtracheal aspiration, cornea, orbit, vitreous humor	Pulmonary infection, meningitis, osteomyelitis, fungemia, disseminated infection, endocarditis, skin infection, mycotic keratitis, orbital cellulitis, endophthalmic infection, chorioretinitis
Candida pseudotropicalis	Respiratory secretions, vagina, urine, gastric washings, oropharynx, blood	Vaginitis, urinary tract infection, fungemia
Cryptococcus albidus/ albidus	Respiratory secretions, skin, gastric washings, urine, cornea	Meningitis, pulmonary infection (rare)
Cryptococcus luteolus	Respiratory secretions, skin, nose	Not commonly implicated in human infection
Cryptococcus laurentii	Respiratory secretions, cerebrospinal fluid, skin, oropharynx, stool	Not commonly implicated in human infection
Cryptococcus albidus/ diffluens	Respiratory secretions, urine, cerebrospinal fluid, gastric washings, skin	Not commonly implicated in human infection
Cryptococcus terreus	Respiratory secretions, skin, nose	Not commonly implicated in human infection

[a] Arranged in order of occurrence (most frequent to least frequent) in the clinical laboratory.

from various clinical specimens of "normal" and hospitalized patients. The information presented suggests that yeasts are more often recovered from hospitalized patients than from "normal" persons. Furthermore, it appears that yeasts are not common flora of certain body sites, such as the skin and urinary tract. No definite association between colony counts and urinary tract infections with yeasts has been established, but it has been suggested that ≥10,000 colonies/ml is significant.[10]

The presence of yeasts in other normally sterile body fluids, i.e., blood and cerebrospinal fluid is always considered clinically significant. A listing of commonly encountered yeasts, their most common sources, and their association with disease is shown in Table 7–3.

The importance of yeasts in vaginal secretions is uncertain; however, currently it is thought that their presence is abnormal and indicates increased vaginal morbidity.

In contrast, the presence of yeasts other than *Cryptococcus neoformans* in respiratory secretions is common and represents indigenous flora in

most persons.[22] Our laboratory routinely screens all yeasts recovered from respiratory secretions and gastric washings only for urease production. Results for nonurease producing yeasts are reported as "yeast present; not *Cryptococcus.*" The routine identification of other yeasts in respiratory secretions is of little or no value to the clinician, and the time and expense of their identification is not warranted except in specimens from compromised patients or those suspected of having primary pulmonary yeast infections.

II. Identification of Yeasts

A. General Considerations

Whether yeasts recovered from all clinical specimens should be identified is questionable. However, the following criteria are recommended for all laboratories, regardless of their experience or size:

1. Yeasts recovered from blood, cerebrospinal fluid, urine, and other normally sterile body fluids should be identified to species.
2. Yeasts recovered from several consecutive specimens, except respiratory secretions, from the same patient should be identified to species. Respiratory secretions should be screened for the presence of *Cryptococcus neoformans.*
3. Yeasts recovered in large numbers from any clinical specimen, except respiratory secretions, should be identified to species. Respiratory secretions should be screened for the presence of *Cryptococcus neoformans.*
4. Yeasts recovered from compromised patients or those in whom a mycotic infection is suspected should be identified to species.

The extent to which yeasts should be identified in situations other than those previously listed must be determined by each individual laboratory. Optimally, each laboratory should identify yeasts from all specimens so that the clinical significance of each can be definitely established but this is much too impractical except in large clinical laboratories having this capability.

The screening of yeasts for urease and germ tube formation should be minimum requirements for all laboratories. If urease production is observed, the organism should be identified to species to ensure the detection of *Cryptococcus neoformans.* Those yeasts exhibiting germ tube formation need no further characterization and may be identified as *Candida albicans.*

Approaches and methods that are practical for the clinical laboratory are numerous and not well standardized. Commercial products, including

the API 20C System (Analytab Products, Inc.) and Uni-Yeast-Tek System (Flow Laboratories, Inc.), offer some degree of standardization and allow laboratories with little expertise to provide an accurate identification of most yeasts. Laboratories that prefer using commercial systems for any of a number of reasons will find them reliable and helpful.

Some clinical laboratories prefer to use microscopic morphological features and conventional biochemical tests for the identification of yeasts. It is not within the scope of this chapter to include all methods that have been described to have practical value. However, methods that our laboratory has found to be useful will be described and suitable alternatives for some will be included.

Figure 7–1 presents a conventional schema that is helpful for the identification of commonly encountered species of yeasts seen in the clinical laboratory. It uses pigmentation (red or pink) as a method detecting *Rhodotorula* and *Sporobolomyces*. *Sporobolomyces* is often detected by the presence of satellite colonies that appear around the original inoculum as the result of the release of ballistoconidia.

The identification of yeasts is best accomplished using a combination of colonial, microscopic, and biochemical features. The colonial morphological features are extremely variable and often are not helpful; however, Table 7–4 provides a brief description of common species for reference purposes.

The microscopic morphological features of the yeasts on cornmeal Tween-80 agar has proved helpful in making a presumptive identification of common organisms based on the characteristics presented in Table 7–5 and Figure 7–2. Commercial yeast identification systems require the use of microscopic morphologic features. Biochemical features including carbohydrate utilization profiles and, in some instances, carbohydrate fermentation are necessary for the definitive identification of all yeasts except *Candida albicans,* which may be identified by germ tube production. The cryptococci may be detected by urease production; however, a rapid presumptive identification of *Cryptococcus neoformans* can be made using a combination of the rapid nitrate reduction, selective urease, and levodopa–ferric citrate tests. The definitive identification of *C. neoformans* must be based on pigment production on niger seed agar in addition to the previously mentioned tests. Characteristic biochemical features for yeasts commonly encountered in the clinical laboratory are found in Table 7–6. It is important to note that variations in biochemical profiles exist among the yeasts, and sometimes it is necessary to make an identification on a "best fit" basis.

There are numerous schemata available for the identification of clinically important yeasts, and the methods described here have proved useful for our laboratory; however, some may not be appropriate in another. Laboratories are encouraged to use methods that are most suitable to their own situation.

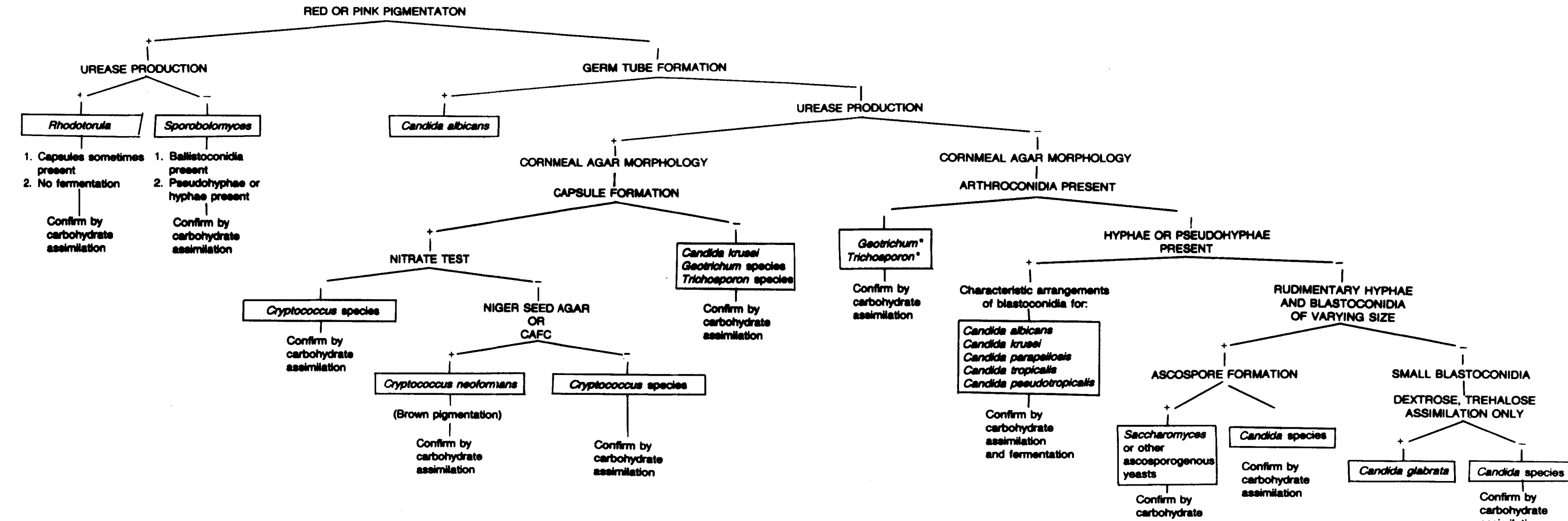


Figure 7–1. Conventional schema for the identification of yeasts commonly encountered in the clinical laboratory. (Note: Some species of *Geotrichum* and *Trichosporon* produce urease.)

B. Preliminary Tests

Urease Production[31]

Purpose:
To detect urease production by certain species of yeasts including *Cryptococcus, Candida krusei,* and some members of the genus *Trichosporon.*

Principle:
Refer to page 194.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:
Bacto Urea R Broth (Difco Laboratories).

Procedure:

1. Reconstitute each vial of Difco Urea R broth with 3 ml of sterile distilled water on the day that it is to be used.
2. Dispense 3 to 4 drops into each well to be used in a microtiter plate.
3. Transfer a heavy inoculum of each yeast colony to a well containing urea broth. A subculture or reincubation of the isolation plate may be necessary if there is insufficient growth of the colony. Isolation of the yeast will be necessary if the culture is contaminated with bacteria. Colonies to be tested should be no older than 7 days.
4. Seal the wells with clear plastic tape and incubate for 4 hr at 37°C.

Interpretation:

1. A positive test with a color change to red or purple is evidence of the production of urease by the test organism.
2. No color change is indicative of a negative test.

Controls:

Positive control: *Cryptococcus neoformans*
Negative control: *Candida albicans*

Alternative Methods:
The rapid selective urease test developed by Zimmer and Roberts[48] has proved to be very helpful in making a presumptive identification of *Cryptococcus neoformans* cultured on Sabouraud's dextrose agar. However,

Table 7–4. Morphology of Common Yeasts on Inhibitory Mold Agar

Species	Gross colonial morphology
Candida albicans	Colonies are white, smooth and domed, shiny and moist; variants may be dry, waxy, and wrinkled. Some isolates produce pseudohyphae fringes at the periphery of colonies.
Candida glabrata	Colonies are small or large and flat, shiny, smooth and creamy.
Candida krusei	Colonies are flat, dry, spreading and have a ground glass appearance. Variants are often dull and nonspreading; sometimes wrinkled at the periphery.
Candida parapsilosis	Colonies are shiny, cream to yellow, moist and smooth to wrinkled. Colonies become rust colored at the periphery with age.
Candida pseudotropicalis	Colonies are white and shiny, later becoming dull and wrinkled.
Candida tropicalis	Colonies are dull, dry, heaped, white to cream, have pseudohyphal fringes and are wrinkled or smooth. Some isolates become rust colored at the periphery with age.
Cryptococcus species	Colonies are mucoid to dry and smooth, domed, shiny to dull and cream to golden with age.
Rhodotorula species	Colonies are pink to orange or salmon color, mucoid and smooth to dry and wrinkled.
Saccharomyces species	The colonial morphological features are highly variable but many colonies are heaped, wrinkled, shiny and white to gray. Most colonies become rust colored at the periphery with age.
Trichosporon species	Colonies range from white to cream colored, smooth and mucoid to pasty, heaped and very wrinkled to those that are white and powdery.

the test has not been evaluated using organisms cultured on other media. Another reasonable alternative for small laboratories is to heavily inoculate the tip of a slant of Christensen's urea agar and incubate it at 37°C for at least 48 hr. Most cultures of *C. neoformans* will produce detectable urease within a few hours.

Rapid Nitrate Reduction Test[12]

Purpose:
To detect the enzyme, nitrate reductase, produced by some species of yeasts.

Table 7–5. Microscopic Morphological Features of Common Yeasts and Yeastlike Organisms on Cornmeal Tween-80 Agar

Organism	Microscopic morphological features[a]
Candida	Numerous blastoconidia and/or pseudohyphae and occasionally true hyphae are produced.
Candida albicans	Irregular or *spherical clusters of blastoconidia occur at septa. Chlamydospores are borne singly* and may be very numerous. Chlamydospores do not develop well at 37°C.
Candida glabrata	*Small, spherical, tightly compacted blastoconidia* with no hyphae present are produced.
Candida krusei	Elongated cells form a branched mycelium that is easily disintegrated. *Clusters of elongated blastoconidia occur at the septa.*
Candida parapsilosis	Fine and coarse mycelium (*giant hyphae*) are commonly seen. Blastoconidia occur singly or in short chains at the septa or distal ends of cells. *The sagebrush appearance of hyphae is characteristic.*
Candida pseudotropicalis	Very elongated *blastoconidia* are produced which readily fall apart and *lie parallel to give a "logs in stream" arrangement.*
Candida tropicalis	*Blastoconidia are produced randomly* or in regular clusters along the hyphae. Chlamydospores are very rare but hyphae and blastoconidia are commonly produced.
Cryptococcus	*A wide variation in size of the round to oval blastoconidia exists* and the *cells are separated by spaces* (*capsule*). Hyphae are not usually produced.
Saccharomyces	*Large to small, primarily spherical blastoconidia* with some rudimentary hyphae are commonly produced.
Trichosporon	*Numerous arthroconidia are produced. Blastoconidia may be present* but are few in number. *Geotrichum produces only arthroconidia* and may resemble *Trichosporon.*

[a] Characteristic features are in *italics*.

Principle:

Some yeasts, including some of the cryptococci, produce nitrate reductase which reduces inorganic nitrate to nitrite. Benzalkonium chloride, 1% is used to disrupt the cell wall structure of yeasts and rapidly liberate the endogenous enzyme present. In addition, an excess of substrate is present to prevent a false negative reaction due to the complete reduction of nitrate to ammonia.

Table 7-6. Characteristics of Yeasts Commonly Recovered from Clinical Specimens[a]

Organism	Assimilations											Fermentations						Other reactions					
	Dextrose	Maltose	Sucrose	Lactose	Galactose	Erythritol	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dextrose	Maltose	Sucrose	Lactose	Galactose	Trehalose	Urease	KNO_3	Pseudohyphae	Germ tubes	Ascospore formation	Pigment on niger seed
Candida albicans	+	+	V	–	+	V	V	–	+	–	+	F	F	V	–	V	V	–	–	+	+		–
C. famata	+	+	+	V	+	V	+	–	+	+	+	V	V	V	–	V	–	–	–	–	–		–
C. glabrata	+	–	–	–	–	–	–	–	–	–	+	F	–	–	–	–	F	–	–	–	–		–
C. guillermondii	+	+	+	–	+	–	+	–	+	+	+	F	V	F	–	V	V	–	–	+	–		–
C. krusei	+	–	–	–	–	–	–	–	–	–	–	F	–	–	–	–	–	V	–	+	–		–
C. lipolytica[b]	+	V	–	–	V	+	V	–	–	–	–	–	–	–	–	–	–	–	–	+	–		–
C. parapsilosis	+	+	+	–	+	–	–	–	V	–	+	F	V	V	–	V	–	–	–	+	–		–
C. pseudotropicalis	+	–	+	+	+	–	+	–	V	+	–	F	–	F	F	F	–	–	–	+	–		–
C. rugosa	+	–	–	–	+	–	–	–	V	–	–	–	–	–	–	–	–	–	–	+	–		–
C. tropicalis	+	+	V	–	+	V	V	–	V	–	+	F	F	V	–	F	F	–	–	+	–		–
Cryptococcus albidus	+	+	+	V	V	V	+	+	+	+	+	–	–	–	–	–	–	+	+	V	–		–
Cr. gastricus	+	+	V	V	+	–	+	+	+	–	+	–	–	–	–	–	–	+	–	–	–		–
Cr. laurentii	+	+	+	+	+	+	+	+	+	V	V	–	–	–	–	–	–	+	–	+	–		–
Cr. luteolus	+	+	+	V	+	V	+	+	+	V	+	–	–	–	–	–	–	+	–	–	–		–
Cr. neoformans	+	+	+	–	+	–	+	V	+	V	+	–	–	–	–	–	–	+	–	R	–		+
Cr. terreus	+	V	V	–	V	–	+	+	+	–	V	–	–	–	–	–	–	+	+	–	–		–
Cr. uniguttulatus	+	+	+	–	V	–	V	+	+	V	V	–	–	–	–	–	–	+	–	–	–		–
Geotrichum candidum	+	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–		–

G. capitatum	+	−	−	−	V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−		−
G. penicillatum	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−		−
Saccharomyces cerevisiae	+	V	V	−	V	−	−	−	−	+	V	F	V	V	−	V	V	−	−	+	−	+	−
Trichosporon cutaneum	+	V	V	+	V	V	V	V	+	V	V	−	−	−	−	−	−	V	−	+	−		−
T. pullulans	+	+	+	V	+	+	+	V	V	V	+	−	−	−	−	−	−	+	+	+	−		−

[a] V, strain variation; +, assimilation; F, fermentation as detected by gas production; —, negative; R, rare.
[b] *C. lipolytica* distinguished from *C. krusei* by presence of true hyphae and pseudohyphae.

Specimen:
Several isolated colonies of an actively growing culture.

Reagents:

Medium

KNO_3 (J. T. Baker Chemical Co.)	5 g
Na_2HPO_4 (J. T. Baker Chemical Co.)	1.14 g
NaH_2PO_4 (J. T. Baker Chemical Co.)	11.7 g
Benzalkonium chloride (17%) (Winthrop Laboratories)	1.2 ml
Distilled water	200 ml

Standard, medium-sized cotton-tipped applicators are saturated in the test medium. Impregnanted applicators may be lyophilized or air dried, autoclaved, and stored in sterile containers.

Developing Reagents
Refer to p. 221 for a description of Reagents A and B.

Procedure:

1. Sweep the tip of an impregnated applicator across two or three colonies of the yeast being tested.
2. Swirl the inoculated applicator against the bottom of an empty test tube to embed the yeast well into the cotton fibers.
3. Incubate the tube and applicator for 10 min at 45°C.
4. Remove the applicator and add two drops each of Reagents A and B to the tube and replace the applicator.
5. Observe for the presence or absence of a red color.

Interpretation:
The presence of a red color is indicative of the presence of nitrate reductase.

Controls:

Positive control: *Candida albicans*
Negative control: *Cryptococcus neoformans*

Alternative Methods:
The method described by Rhodes and Roberts[29] is also an acceptable alternative; however, it requires 72 hr and cannot be used as a rapid test.

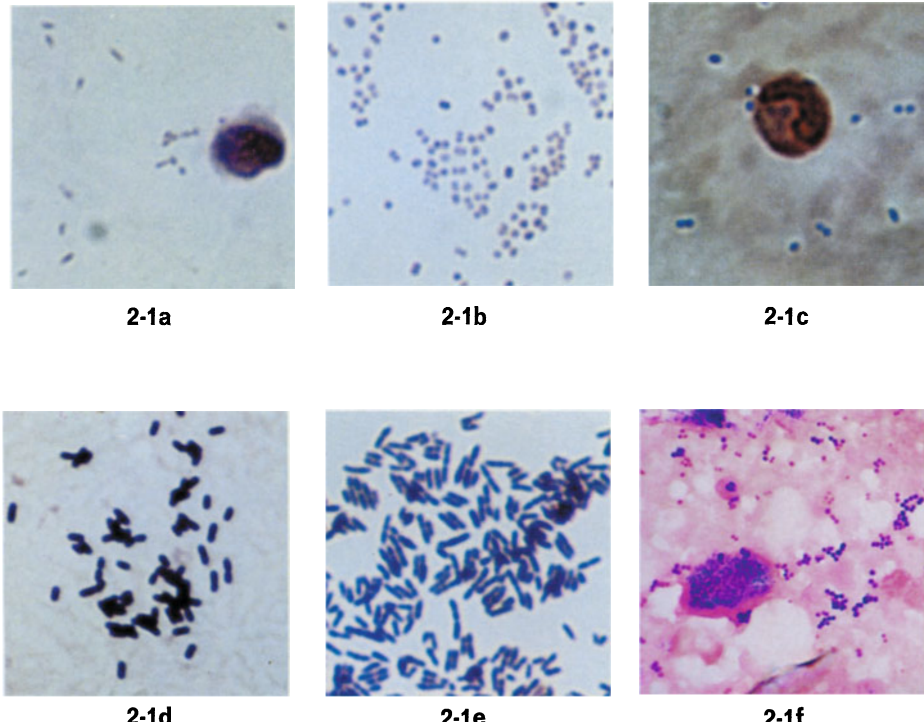
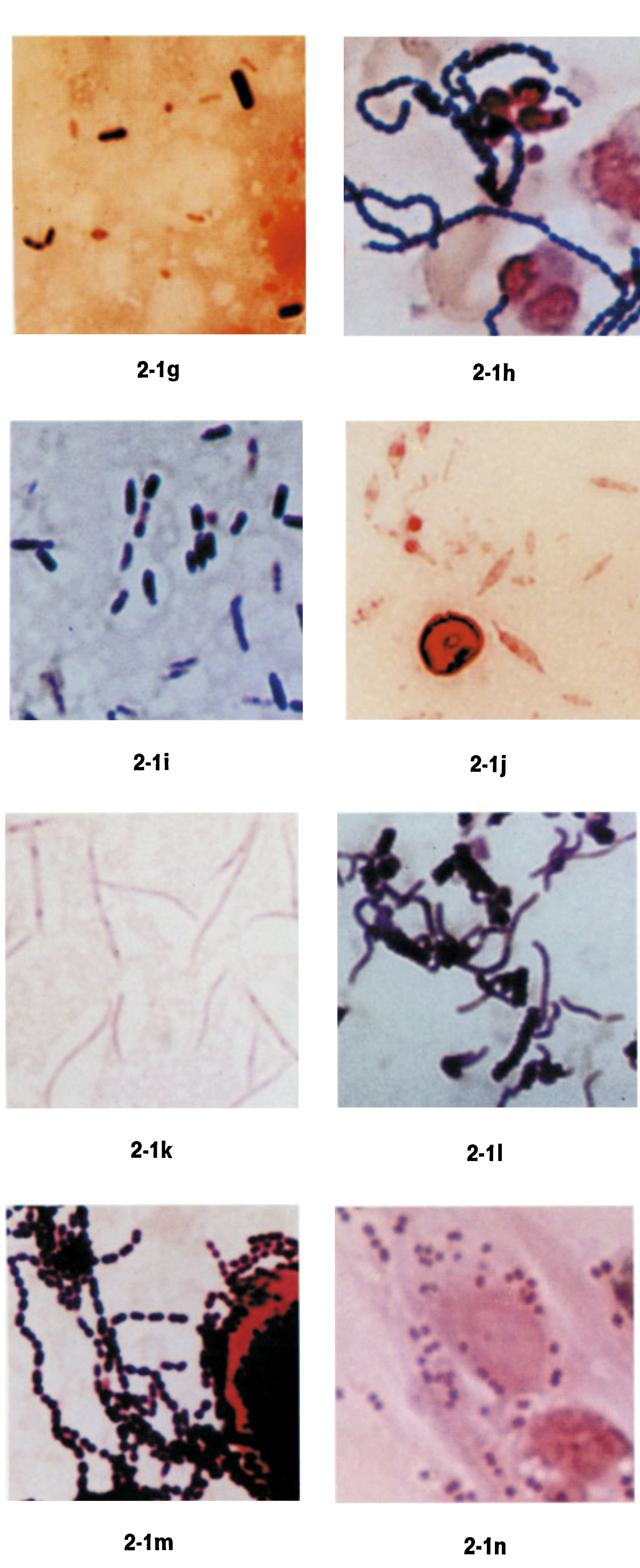


Figure 2-1. Gram stained smears (original magnification, 1000×). Cerebrospinal fluid demonstrating (a) faintly staining, pleomorphic gram-negative coccobacilli and bacilli, typical of *Haemophilus influenzae*; (b) gram-negative diplococci typical of *Neisseria meningitidis*; (c) encapsulated gram-positive diplococci typical of *Streptococcus pneumoniae.* Cultures of (d) *Listeria monocytogenes* and (e) *Corynebacterium* demonstrating angular and palisading arrangement of nonsporulating gram-positive bacilli. Exudate or pus demonstrating (f) round gram-positive cocci in clusters typical of *Staphylococcus aureus*; (g) gram-positive bacilli, gram-negative bacilli, and cocci suggesting mixed anaerobic bacterial infection; (h) gram-positive cocci in chains typical of group A streptococci; and (i) large, nonsporulating gram-positive bacilli resembling *Clostridium* or *Bacillus.* Blood cultures demonstrating (j) highly pleomorphic gram-negative bacilli suggesting bacteremia due to *Bacteroides fragilis*, (k) slender gram-negative bacilli with tapered ends typical of *Fusobacterium nucleatum*, and (l) nonsporulating, nonbranching bacilli demonstrating extreme pleomorphism and gram-variability from a patient with lactobacillemia. Pharyngeal exudate demonstrating (m) gram-positive cocci typical of streptococci in association with a polymorphonuclear leukocyte demonstrating loss of cytoplasmic and cellular integrity, suggesting group A streptococcal pharyngitis. Expectorated sputum from patients with (n) *Branhamella catarrhalis* and (o) *Pasteurella multocida* pneumonia. Male urethral exudate demonstrating intracellular, gram-negative diplococci diagnostic of gonorrhea (p). (q) Yeasts with branching hyphae from left ureter.



2-1g
2-1h
2-1i
2-1j
2-1k
2-1l
2-1m
2-1n

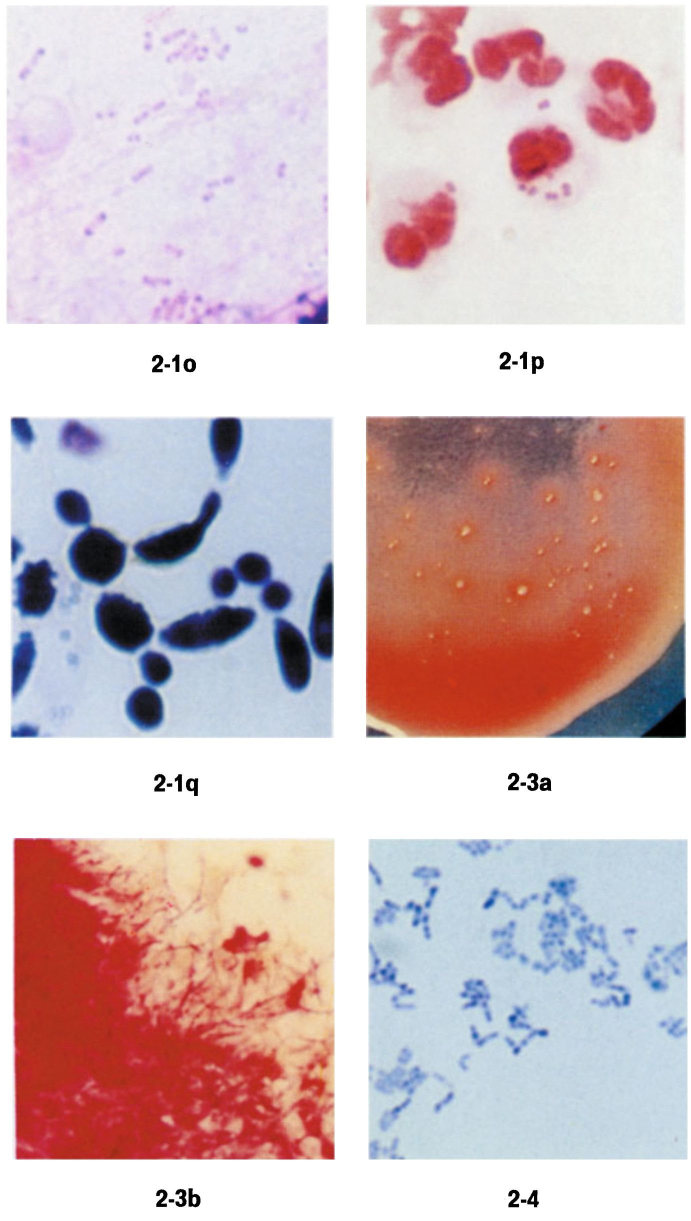


Figure 2-3. Sinus tract exudate with sulfur granules (a) comprising masses of nonsporulating gram-positive bacilli (b) identified as *Actinomyces israelii.*

Figure 2-4. Loeffler's methylene blue stain of *Corynebacterium diphtheriae* demonstrating metachromatic granules ($\times$1000).

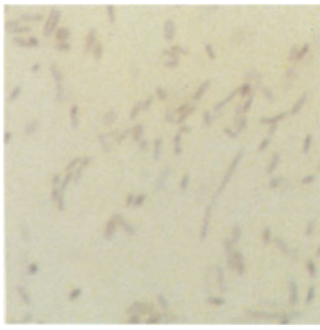


Figure 2–8. Gram stained smear of *Legionella pneumophila.*

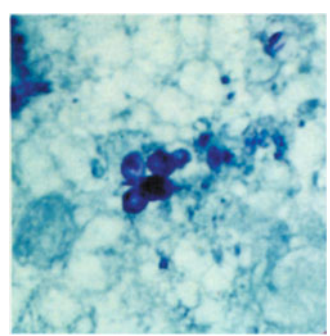
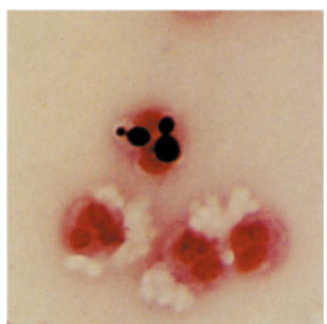


Figure 2–9. Toluidine blue stained impression smear of open lung biopsy demonstrating *Pneumocystis carinii* cysts [×640].



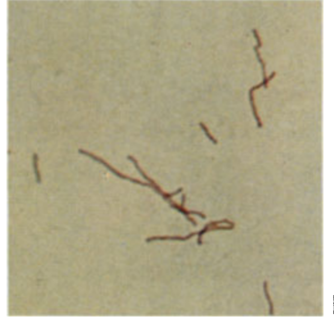


Figure 2–10. Gram-stained smears of drops of well-mixed urine specimens: **a.** yeasts, pus cells; **b.** gram-negative bacilli, no pus cells [×1000].

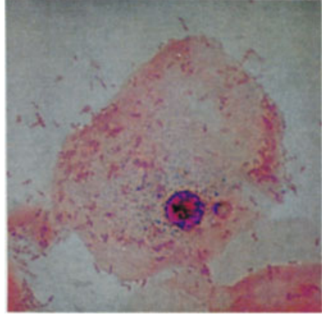


Figure 2–12. Gram-stained smear demonstrating clue cell in *Gardnerella vaginalis* associated nonspecific vaginosis [×1000].

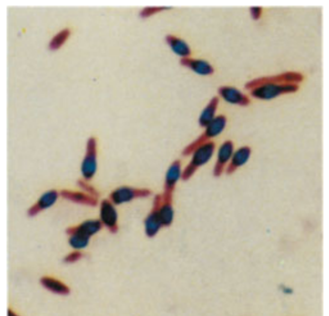


Figure 2–13. Spore stain of *Clostridium sporogenes.*

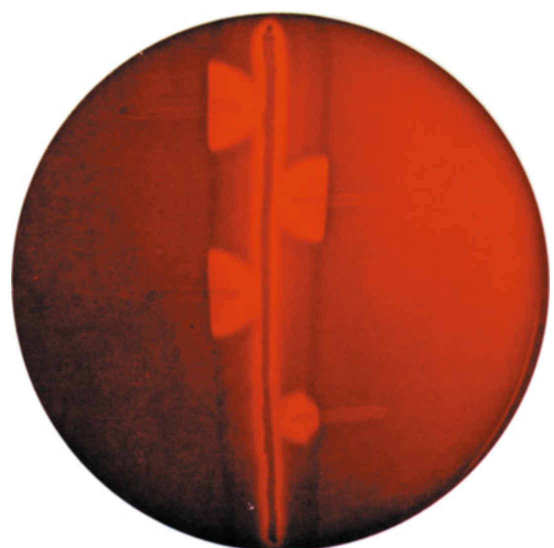


Figure 4.2-2. CAMP reaction consisting of arrowhead-shaped area of *β*-hemolysis at juncture of lines of growth on blood agar of *Staphylococcus aureus* and group B streptococci.

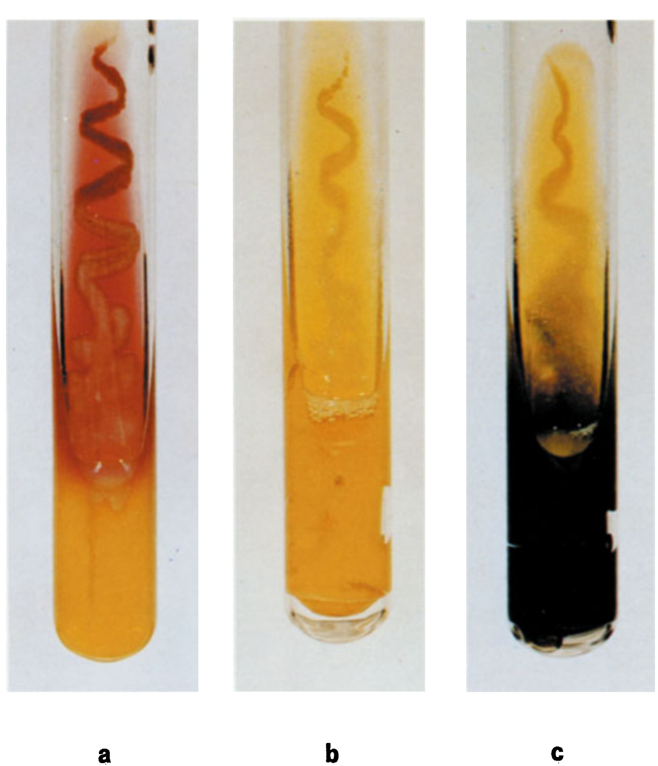


Figure 4.2-3. Reactions in triple sugar iron agar (TSIA) demonstrating: (a) alkaline slant/acid butt (K/A) reaction without gas or H_2S, (b) A/A reaction with gas and no H_2S, (c) A/A reaction with gas and H_2S.

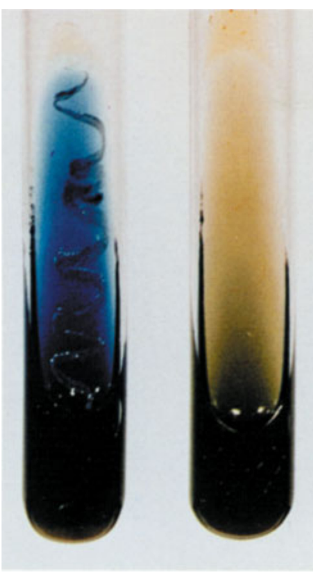


Figure 4.2-4. Positive (left) and negative (right) reactions on Simmons' citrate agar.

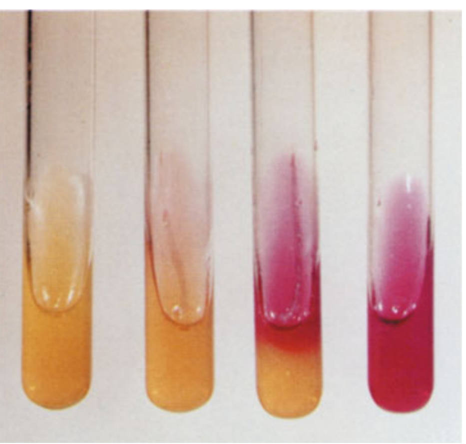


Figure 4.2-5. Christensen's urea agar demonstrating negative reaction in the first tube on the left and increasing gradations of positive reactions in the next three tubes.

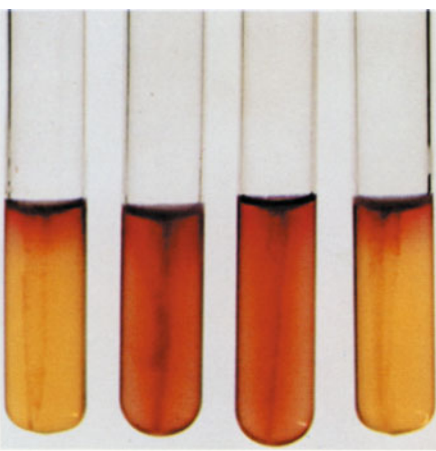


Figure 4.2-6. Decarboxylation of arginine and ornithine (center two tubes) but not of lysine (left) or in the control (right) semisolid Moeller's medium. Diffuse growth extending away from stab line of inoculation reflects the organism's motility.

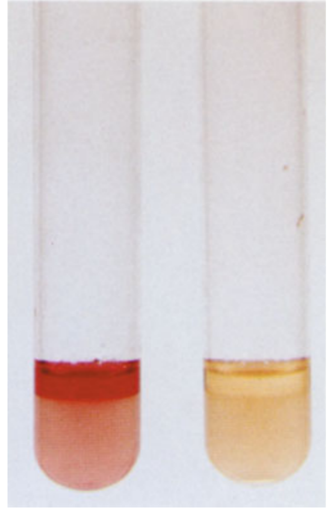


Figure 4.2-7. Positive (left) and negative (right) indole tests.

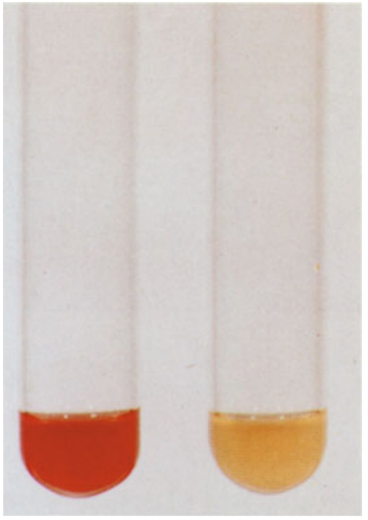


Figure 4.2-8. Positive (left) and negative (right) methyl red tests.

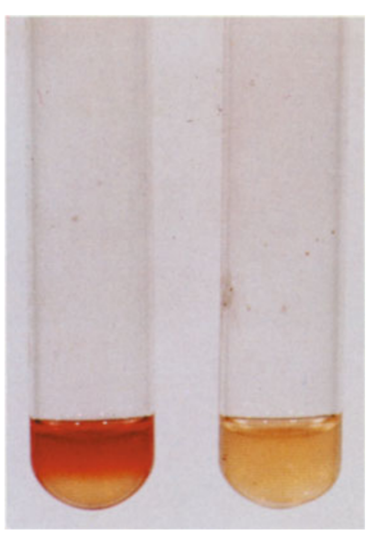


Figure 4.2-9. Positive (left) and negative (right) Voges–Proskauer tests.

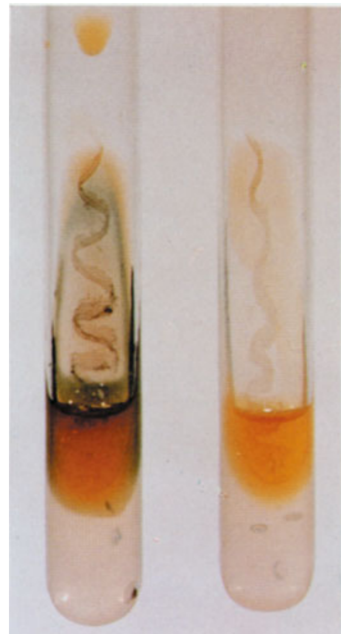


Figure 4.2-10. Positive (left) and negative (right) phenylalanine deaminase tests.

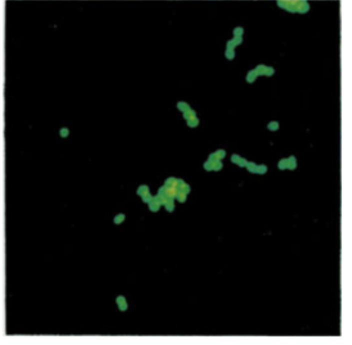


Figure 4.3-1. Examples of a positive (4+) and negative (≤1+) FA stain using fluorescein-labeled antibody against group A *Streptococcus:* (a) group A *Streptococcus;* (b) non-group A *Streptococcus.*

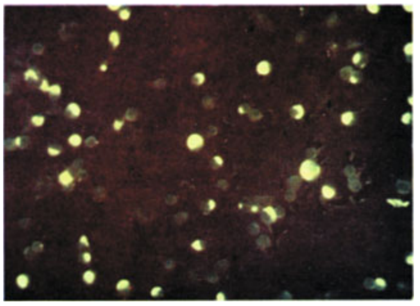


Figure 8-3. Fluorescence of intracytoplasmic inclusion bodies resulting from reaction of antibodies to *C. trachomatis* with LGV-1-infected McCoy's cells in the indirect test (×250).

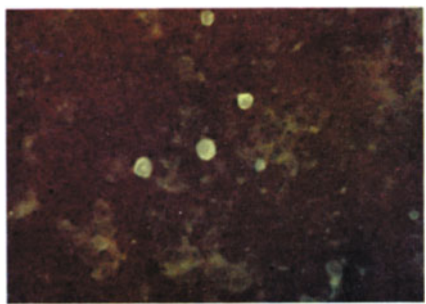


Figure 10-21. Immunofluorescence of MRC-5 cells infected with herpes simplex virus (×250).

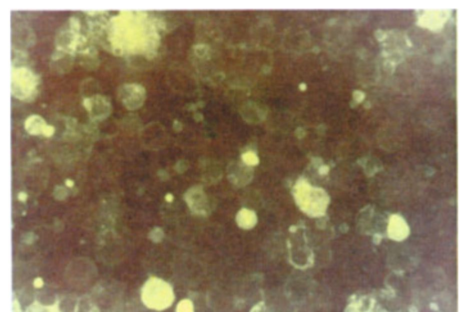


Figure 10-24. Immunofluorescence of Epstein–Barr virus-infected HR1K cells after reaction with serum containing IgG antibodies to the viral capsid antigen (×250).

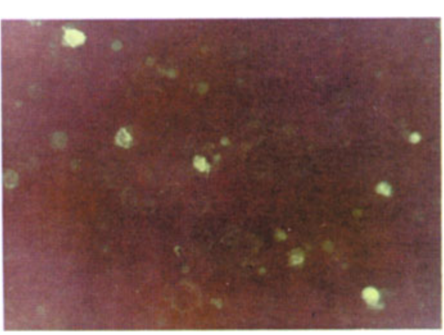


Figure 10-25. Immunofluorescence of Epstein–Barr virus-infected HR1K cells after reaction with serum containing IgM antibodies to the viral capsid antigen (×250).

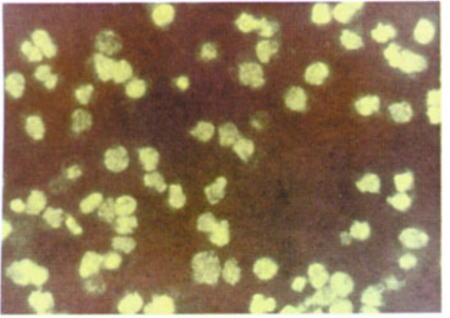


Figure 10-26. Immunofluorescence of Epstein–Barr virus-infected Raji cells after reaction with serum containing antibodies to EBV nuclear antigen (EBNA) (×400).

Levodopa–Ferric Citrate Test[13]

Purpose:
To detect the enzyme, phenoloxidase, produced by *Crytococcus neoformans*.

Principle:
Cryptococcus neoformans is the only member of the genus known to produce the enzyme, 3,4-dihydroxyphenylalanine-phenoloxidase. When reacted with L-β-3,4-dihydroxyphenylalanine in the presence of an iron-containing compound, *C. neoformans* oxidizes the *o*-diphenol to melanin, which is characteristically brown to black in color.

Specimen:
Several isolated colonies from an actively growing culture.

Reagents:

Buffer

A.	KH_2PO_4 (J. T. Baker Chemical Co.)	0.912 g
	Distilled water	100 ml
B.	Na_2HPO_4 (J. T. Baker Chemical Co.)	0.951 g
	Distilled water	100 ml

Mix equal volumes of A and B; adjust pH to 6.8 and filter sterilize. Store at room temperature.

Levodopa (*L-β-3,4-dihydroxyphenylalanine*) *Solution*

Levodopa—in three drops on dimethyl sulfoxide	3.0 mg
Distilled water-sigma	1 ml

Ferric Citrate Solution (*1.0 mg/ml*)

Ferric citrate (Fisher Scientific)	1.0 mg
Distilled water—apply gentle heating	1 ml

Levodopa–Ferric Citrate Solution

Levodopa solution	1 ml
Ferric citrate solution	0.5 ml
Phosphate buffer	3.5 ml

The final mixture should be light blue to purple in color. This reagent should be stored in a dark bottle to prevent deterioration.

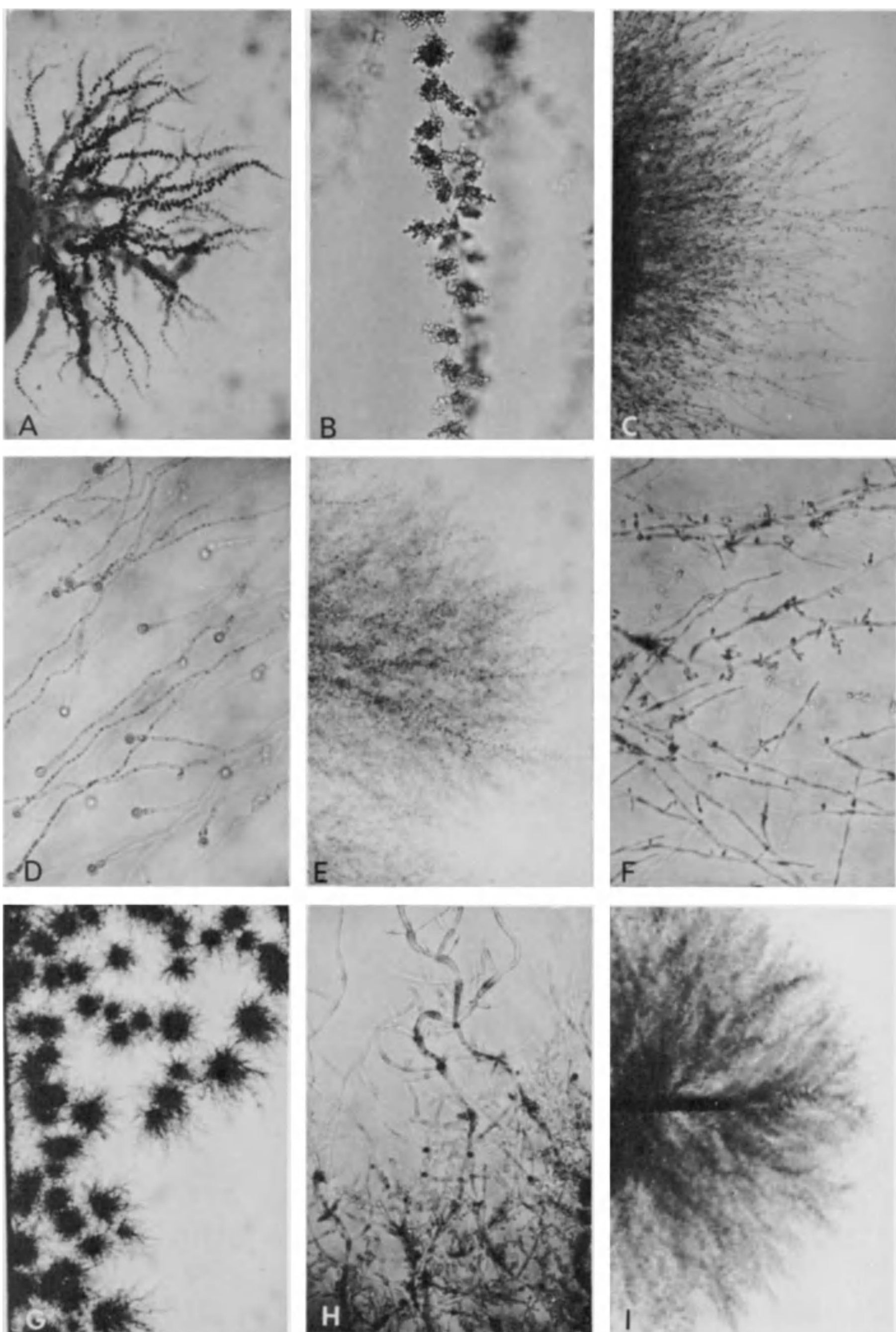
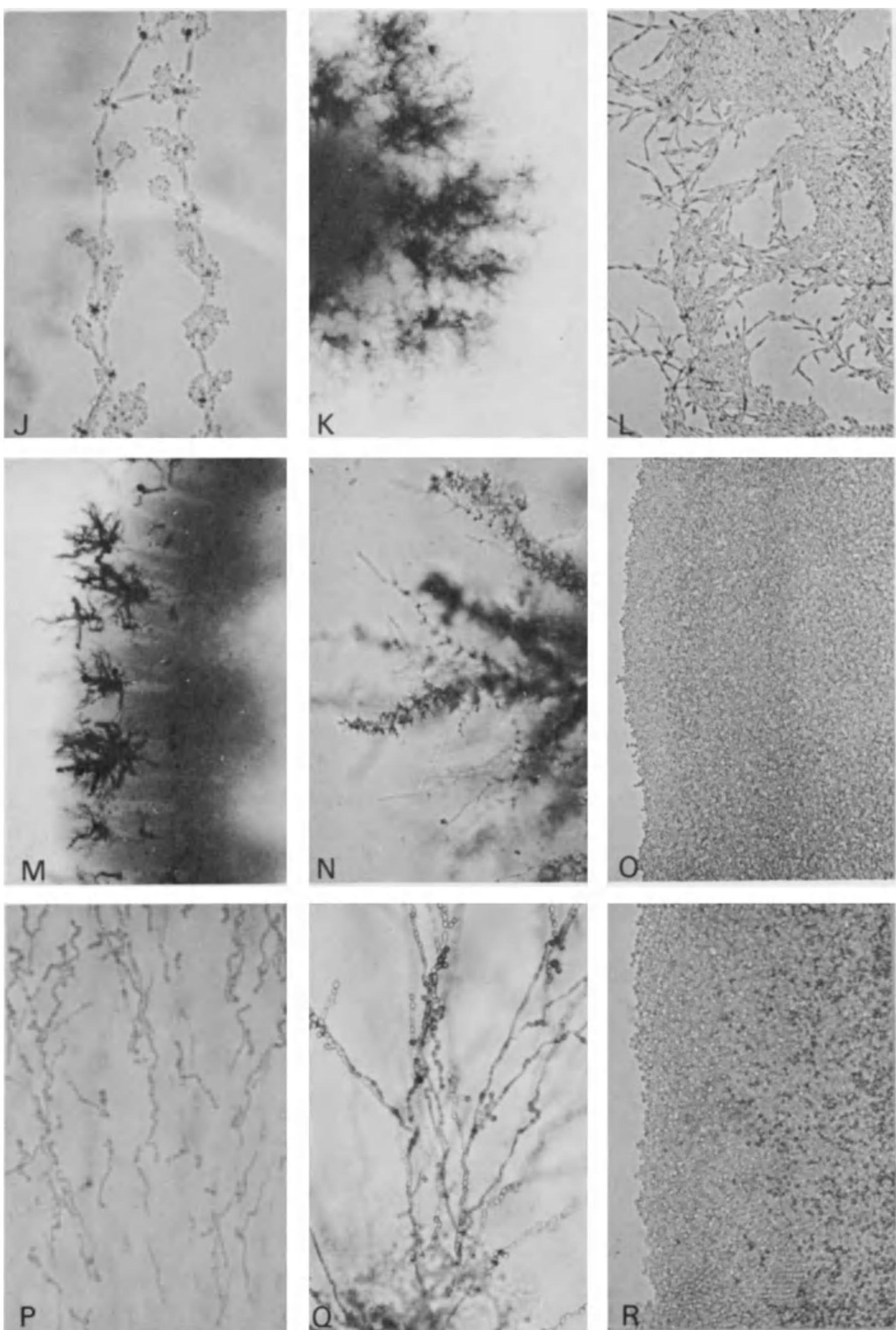


Figure 7–2. Microscopic morphological features of common yeastlike organisms on cornmeal Tween-80 agar. A, B, C, and D, *Candida albicans,* illustrating spherical clusters of blastoconidia in A and B and chlamydospores in C and D (A, ×125; B, ×800; C, ×125; D, ×800). E and F, *Candida tropicalis,* with blastoconidia irregularly arranged along hyphae and, rarely, chlamydospores (E, ×125; F, ×800). G and H, *Candida parapsilosis,* illustrating "giant" hyphae with blastoconidia singly or at end of hyphae (G, ×125; H, ×800). I, *Candida krusei* (×125). J. *Candida krusei,* illustrating elongated blastoconidia in the cross-matchsticks arrangement (×800). K and L,



Candida pseudotropicalis, illustrating elongated cells that break apart and lie in parallel (K, ×125; L, ×800). M and N, *Candida guilliermondii,* illustrating very small hyphae with clusters of small blastoconidia (M, ×125; N, ×800). O, *Candida glabrata,* illustrating small (2 to 4 μm) oval cells (×800). P, *Geotrichum candidum,* showing rectangular arthroconidia (×800). Q, *Trichosporon* species, showing rectangular arthroconidia (×800). R, *Cryptococcus neoformans,* illustrating encapsulated blastoconidia, 7 to 10 μm (×800). (From Dolan, C. T., *Am. J. Clin. Pathol.* **55:**580, 1971. By permission of *American Journal of Clinical Pathology.*)

Impregnation of Disks
Sterile blank paper disks are each *saturated* with the Levodopa–ferric citrate solution. Disks may be dried in petri dishes with lids partially ajar at 37°C overnight. Disks should be stored in a dark bottle at −20°C for up to 6 months.

Procedure:

1. Moisten one plain disk with buffer and one Levodopa–ferric citrate disk with two to three drops of distilled water prior to testing.
2. Place disks into a petri dish and smear a liberal amount of the test organism onto the surface of both disks.
3. Seal the petri dish with tape and incubate at 37°C for no longer than 3–6 hr. Examine at 30-min intervals.
4. Tests should be read for the presence of a dark pigment on the test disk. If no pigment is observed, incubate the disks for the full 3 hr and reexamine.

Interpretation:
Cryptococcus neoformans produces a dark diffusible pigment on the test disk and not on the buffer disks. A positive test indicates the production of phenoloxidase. Occasionally other species of cryptococci produce a dark diffusible pigment on both disks and the test should be regarded as negative. False positive and negative tests occur, and it is necessary to use the results of this test in conjunction with those of other biochemical tests.

Controls:

Positive control: *Cryptococcus neoformans*
Negative control: *Candida albidus*

Alternative Methods:
The Tween-80 oxgall caffeic acid (TOC) medium developed by Fleming et al.[8] is a suitable alternative method.

Microscopic Morphological Features on Cornmeal Tween-80 Agar

Purpose:
To determine the microscopic morphological features of yeasts commonly recovered from clinical specimens.

Principle:
The incorporation of Polysorbate 80 (Tween-80) into cornmeal agar reduces the surface tension of the medium to promote the germination and sporulation of yeasts. Trypan blue is present to provide a contrasting background on which to observe the characteristic microscopic morphological features of yeasts.

Specimen:
An isolated colony from an actively growing culture.

Reagents:

Cornmeal Agar

Cornmeal (yellow)	62.5 g
Distilled water	1500 ml
Agar (Difco Laboratories)	19 g

Heat cornmeal and agar for 1 hr at 52°C. Filter through a filter paper and readjust the volume to 1500 ml with distilled water.

Trypan Blue Solution

Trypan blue (Harleco)	0.1 g
Distilled water	10 ml

Polysorbate 80 (Tween-80)

Polysorbate-80 (Emulsion Engineers, Inc.)	200 ml
Distilled water	800 ml

Add 1.5 ml of Trypan blue solution and 15 ml of the Tween-80 solution to the cornmeal agar after boiling. Autoclave for 15 min and dispense 15 ml amounts into culture dishes.

Procedure:

1. Inoculate a plate of cornmeal agar containing Tween-80 and Trypan blue by making three parallel cuts ½ in. apart at a 45° angle into the agar medium. Cover one cut with a sterile coverslip.
2. Incubate cultures for 48 hr at 30°C.
3. Observe the areas where the agar cuts were made using the low and high power objectives of a bright field microscope for the presence of hyphae, pseudohyphae, blastoconidia, arthroconidia, or chlamydospores.

Interpretation:
Commonly encountered yeasts and dermatophytes may be tentatively identified using the characteristics presented in Tables 7–5 and Figures 7–2 and 7–3. The definitive identification must be based on a combination of morphological and biochemical features.

Controls:
The production of chlamydospores by *Candida albicans* is used as a control.

Alternative Methods:
Suitable alternatives include Wolin–Bevis medium[46] and a number of rise extract preparations.

III. Definitive Tests

Germ Tube Production

Purpose:
To detect germ tube formation by strains of *Candida albicans*.

Principle:
When placed in a liquid nutrient environment, strains of *C. albicans* produce hyphal shoots from blastoconidia after incubation for no longer than 3 hr at 35°C.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:
Sheep serum (Granite Diagnostics, Burlington, North Carolina).

Procedure:

1. Suspend a *very small* inoculum of yeast cells obtained from an isolated colony in a small tube containing 0.5 ml of sheep serum.
2. Incubate the inoculated tubes at 37°C for no longer than 3 hr.
3. Remove a portion of the sediment, place on a slide, and cover with an 18 by 18 mm no. 2 coverslip.
4. Examine microscopically for the presence of germ tubes. A germ tube is defined as an appendage one half the width of and three to four times the length of the cell from which it arises. A germ tube contains no indentation at its origin from the blastoconidium from which it

arises. In contrast, a pseudo-germ tube produced by *Candida tropicalis* is constricted at this point of origin and contains at least one septum.

Interpretation:
The presence of a true germ tube is characteristic of *Candida albicans*. Caution must be exercised in distinguishing a true germ tube from a pseudo-germ tube of *Candida tropicalis*.

Controls:

Positive control: *Candida albicans*
Negative control: *Candida tropicalis*

Alternative Methods:
Numerous substrates have been described for use in the germ tube test; however, most of the investigators recommend use of normal human serum, sheep serum, or fetal calf serum. If other substrates are used, they must be evaluated prior to use by using a known strain of *Candida albicans*.

Carbohydrate Utilization[30]

Purpose:
To determine the characteristic carbohydrate utilization patterns for clinically important yeasts.

Principle:
Yeasts contain specific enzyme systems that determine their ability to utilize a carbohydrate as a sole source of carbon in a chemically defined medium. Growth with the production of acid by-products in the presence of a single carbohydrate substrate reflects utilization of that compound as the sole carbon source. In the method described below, carbohydrate utilization is determined by the presence of growth and a color change around carbohydrate-impregnated disks placed on the surface of a basal medium containing an indicator. In some instances, growth may occur around the carbohydrate-containing disks without a corresponding color change, reflecting carbohydrate utilization with diminished acid production.

Reagents:

Bromcresol Purple Indicator Solution

Bromcresol purple (Allied Chemical and Dye Corp.)	0.04 g
Distilled water	100 ml

Add a small amount of 1 *N* NaOH to make the solution alkaline. Allow to stand overnight and after the dye is in solution, add 1 *N* HCl until neutrality is approached.

Yeast Nitrogen Base Agar

Yeast nitrogen base (Difco Laboratories)	6.7 g
Distilled water	100 ml

Adjust the pH to 6.2 to 6.4, and sterilize by filtration using a 0.45 μ filter.

Carbohydrate Utilization Medium
Add 88 ml of sterile yeast nitrogen base and 100 ml of filter-sterilized bromcresol purple indicator to 1000 ml of sterile 2% agar. Pour into sterile plastic petri dishes (20 ml/plate).

Carbohydrate-Impregnated Disks
Bacto differentiation disks (Difco Laboratories) impregnated with specific carbohydrates are used for detecting carbohydrate utilization. The disks used for hyphal-producing species, as detected on cornmeal agar, include dextrose, maltose, sucrose, lactose, and raffinose. Disks used for non-hyphal-producing yeasts include dextrose, maltose, sucrose, lactose, trehalose, inositol, galactose, melibiose, and raffinose.

Procedure:

1. Flood the surface of the carbohydrate utilization medium with a suspension of yeast in saline with turbidity equivalent to that of a McFarland standard no. 4 and aspirate the excess.
2. Place the carbohydrate disks onto the surface of the agar and press down firmly with a flamed forceps. Disks are placed on the four quadrants and the center of the plates containing the carbohydrate utilization medium.
3. Incubate the cultures for 24 to 48 hr at 30°C. Observe for presence of growth and/or a color change in the medium surrounding the carbohydrate-containing disks.

Interpretation:
Growth around the carbohydrate-containing disks and a color change in medium reflects utilization of specific carbohydrates. Results obtained using carbohydrate utilization tests may be compared with the characteristic reactions of common species presented in Table 7–6.

Controls:
Cryptococcus laurentii and *Candida pseudotropicalis* should be used as controls when determing the activity of the carbohydrate impregnated disks.

Alternative Methods:
Numerous alternative methods are available for determining carbohydrate utilization patterns. Some of the more practical methods include those of Land et al.,[17] Segal and Ajello,[34] Adams and Cooper,[1] and Mickelsen et al.[21] In addition, the API 20C and Uni-Yeast-Tek commercial systems are certainly adequate for the identification of yeasts in a clinical laboratory setting.

Carbohydrate Fermentation

Purpose:
To determine the ability of yeasts to utilize specific carbohydrates fermentatively.

Principle:
Yeasts contain specific enzyme systems that allow for the anaerobic degradation of specific carbohydrates with the production of CO_2 as the end by-product. Carbohydrate utilization may also occur with the production of acid by-products; however, this is not an indication of fermentation.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:
Purple broth (Gibco Diagnostics) containing: dextrose, maltose, sucrose, lactose, and galactose.

Procedure:

1. Add 0.2 ml of a yeast suspension in saline equivalent to a McFarland standard no. 4.
2. Incubate at 37°C and observe tubes for the presence of gas bubbles in the inverted Durham tubes.
3. Incubate tubes for at least 6 days before reporting the tests as negative.

Interpretation:
The production of gas is the only criterion for detecting the fermentation of specific carbohydrates. The observed reactions may be compared to those given in Table 7–6.

Controls:

Positive controls: *Candida albicans* and *Candida pseudotropicalis*
Negative control: *Cryptococcus neoformans*

Pigment Production on Niger Seed Agar[25]

Purpose:
To detect the enzyme, phenoloxidase, produced by *Cryptococcus neoformans.*

Principle:
Refer to p. 437.

Specimen:
An isolated colony from an actively growing culture.

Reagents:

Thistle seed (*Guizotia abyssinica*)	50 g
Agar (Difco Laboratories)	15 g

Add the thistle seed to 100 ml of distilled water and grind in a blender. Add distilled water to give a final volume of 1 liter and boil for ½ hr. Strain the mixture through a cloth and adjust the volume to 1 liter with distilled water. Add the agar and adjust the pH to 5.5. Autoclave for 15 min and dispense into petri dishes (35 ml/dish).

Procedure:

1. Inoculate a plate of niger seed agar by streaking a heavy inoculum across the surface.
2. Incubate at 25°C and observe daily for the presence of a dark brown or black pigment produced by the colonies of the test organism.
3. Incubate for at least 7 days before reporting a test as negative.

Interpretation:
The presence of a dark brown or black pigment indicates the presence of phenoloxidase, which is produced only by *Cryptococcus neoformans.*

Controls:

Positive control: *Cryptococcus neoformans*
Negative control: *Cryptococcus albidus*

Ascospore Production

Purpose:
To detect the presence of ascospores produced by ascosporogenous yeasts, including *Saccharomyces cerevisiae.*

Principle:
Members of the ascomycetes, under certain environmental conditions, reproduce sexually and produce ascospores within an ascus. Ascospores are easily visible when stained by the Kinyoun acid-fast stain.

Specimen:
Isolated colonies from an actively growing culture.

Reagents:

Ascospore Medium

Potassium acetate (J. T. Baker Chemical Co.)	10 g
Yeast extract (Gibco Diagnostics)	2.5 g
Dextrose (Difco Laboratories)	1 g
Agar (Difco Laboratories)	30 g
Distilled water	1000 ml

Prepare and autoclave the medium for 15 min and dispense 15 ml amounts into sterile petri dishes.

Kinyoun's acid-fast stain
Refer to p. 390.

Procedure:

1. Inoculate the surface of the ascospore medium and incubate at 30°C.
2. Prepare smears daily and stain with Kinyoun's acid-fast stain and observe for the presence of ascospores.
3. Incubate cultures for at least 10 days before reporting as negative for ascospore production.

Interpretation:
The presence of acid-fast structures within an ascus is evidence of ascospore production. *Saccharomyces cerevisiae* is the most commonly encountered yeast that exhibits ascospore production.

Controls:

Positive control: *Saccharomyces cerevisiae*
Negative control: *Candida albicans*

Alternate Methods:
Other media, including V-8 juice agar, malt extract agar and other acetate media, may be used to induce ascospore formation.

IV. Identification of Filamentous Fungi

A. General Considerations

The filamentous fungi represent one of the largest and most diverse groups of microorganisms found in nature. They are commonly encountered in clinical specimens and often pose problems to laboratories unfamiliar with their characteristic features. Fortunately, many are common to most areas of the United States and are easily recognized by many laboratories.

Many criteria including the growth rate, colonial morphology, and microscopic morphology are used to identify the filamentous fungi. However, experience is the most important tool used for identification of these fungi.

The remainder of this section will be devoted to a discussion of the general characteristics of common fungi with the presentation of a practical classification schema (Table 7–7) and a number of photographs and descriptions of common organisms. The classification schema (Table 7–7) generally categorizes common organisms into groups based on growth rate and the presence or absence of pigment or septa within the hyphae.

1. Growth Rate

The growth rate of the filamentous fungi varies greatly depending on the group of organisms being considered, the number of organisms present in the specimen, and the type of medium and incubation conditions used. Growth rates may be helpful diagnostically but are variable.

Zygomycetes with nonseptate hyphae usually require from 1 to 3 days for growth to appear. The growth rate of the remainder of the monomorphic hyaline molds usually varies between 3 and 7 days, with some dermatophytes requiring as long as 10 to 14 days.

The slow growth rate of the dimorphic hyaline molds has long been regarded as a useful criterion for their identification; however, this rate is not absolute and wide variations occur. In general, the recovery time for *Histoplasma capsulatum* and *Blastomyces dermatitidis* is from 10 to 14 days, but some isolates, when present in large numbers, may be recovered within 4 to 5 days. In contrast, single colonies of these organisms often require as long as 21 to 45 days to appear. *Coccidioides immitis* typically requires 4 to 5 days of incubation for growth; however, growth may occur within 48 hr when many organisms are present, or in 21 to 28 days when few organisms are present. Large numbers of *Sporothrix schenckii* may be detected within 48 hr, but they usually require 5 days of incubation. *Paracoccidioides brasiliensis* may grow in 5 to 25 days but usually require an incubation period of 10 to 15 days.

Table 7–7. Filamentous Fungi Commonly Encountered in a Clinical Microbiology Laboratory

Hyaline monomorphic molds			
Aseptate hyphae (zygomycetes)	Septate hyphae	Dimorphic hyaline molds	Dematiaceous molds
Absidia species	*Acremonium* species	*Blastomyces dermatitidis*	*Alternaria* species
Cunninghamella species	*Aspergillus flavus*	*Coccidioides immitis*	*Aureobasidium pullulans*[a]
Mucor species	*Aspergillus fumigatus*	*Histoplasma capsulatum*	*Cladosporium* species
Rhizopus species	*Aspergillus niger*	*Paracoccidioides brasiliensis*	*Curvularia* species
Syncephalastrum species	*Chrysosporium* species	*Sporothrix schenckii*	*Drechslera* species
	Fusarium species		*Epicoccum* species
	Geotrichum candidum		*Exophiala jeanselmei*
	Gliocladium species		*Nigrospora* species[a]
	Paecilomyces species		*Phialophora verrucosa*
	Penicillium species		*Wangiella dermatitidis*
	Pseudallescheria boydii		
	Scopulariopsis species		
	Sepedonium species		
	Trichoderma species		
	Epidermophyton floccosum		
	Microsporum audouinii		
	Microsporum canis		
	Microsporum gypseum		
	Trichophyton mentagrophytes		
	Trichophyton rubrum		
	Trichophyton tonsurans		
	Trichophyton verrucosum		

[a] Some hyphae appear hyaline while others are pigmented.

2. Colonial Morphology

Colonial morphology is of limited value in identifying the filamentous fungi since wide variations in colonial morphological features, including pigmentation, texture, and size, may exist among isolates of the same species. In addition, variation is often medium-related. For example, *Histoplasma capsulatum,* which is typically described as being fluffy white to tan on Sabouraud's dextrose agar, may appear as a heaped, wrinkled, glabrous colony, neutral in color and yeastlike in appearance on a blood-enriched medium. Most glabrous colonies exhibit tufts of adherent hyphae that appear on the surface of heaped colonies; this feature is also seen with *Blastomyces dermatitidis* and *Coccidioides immitis.* It is important to recognize that natural variation in the colonial morphological features commonly occur and that textbook descriptions of organisms are often misleading.

3. Microscopic Morphology

The microscopic morphological features of the filamentous fungi provide the only reliable criterion for their identification. The absence of hyphal septa easily distinguishes the Zygomycetes from the remainder of the hyaline molds. The size of the hyphae is also often helpful in grouping the filamentous fungi, since most of the rapidly growing monomorphic hyaline molds have hyphae with a diameter of 3 to 5 μm while those of the Zygomycetes and dimorphic fungi are 10 to 15 μm and 1 to 2 μm, respectively.

The definitive identification of the filamentous fungi, however, is based on the characteristic shape, size and arrangement of spores on the hyphae. Most have characteristic and usually distinctive microscopic features. In many instances the filamentous fungi, particularly the dimorphic molds, fail to sporulate on primary isolation media. It is then necessary to use appropriate subculture medium to induce sporulation. Table 7–8 provides

Table 7–8. Recommended Subculture Media for the Filamentous Fungi

Group	Medium
Zygomycetes	Cornmeal agar; Sabouraud's dextrose (2%) agar; inhibitory mold agar
Monomorphic hyaline molds (septate)	Sabouraud's dextrose (2%) agar; inhibitory mold agar; cornmeal agar
Aspergillus	Czapek Dox agar
Dermatophytes	Cornmeal agar; potato dextrose agar
Dimorphic hyaline molds	Inhibitory mold agar; yeast extract–phosphate agar[37]; Sabouraud's dextrose (2%) agar
Dematiaceous molds	Cornmeal agar

a list of subculture media which are useful for inducing sporulation of the various groups of fungi.

Variation among the microscopic morphological features occurs most commonly with the dimorphic molds. *Histoplasma capsulatum,* for example, has been shown to exhibit a wide variation in the size and shape of conidia produced. Textbooks commonly describe the presence of tuberculate macroconidia as being characteristic, but is not uncommon to find smooth-walled spherical macroconidia that fail to become tuberculate despite numerous attempts at subculture. Some isolates fail to produce macroconidia and only produce small smooth-walled microconidia. It is important to recognize that variation in the microscopic morphological features commonly occurs.

B. Preparation of Cultures for Microscopic Examination

Since the definitive identification of the filamentous fungi is based on the recognition of characteristic microscopic features, the selection of an appropriate mounting preparation for microscopic examination is of importance. Current methods use lactophenol cotton blue or lactophenol aniline blue as the mounting medium to ensure that the microscopic features are easily visualized.

1. Mounting Media

a. Lactophenol Aniline Blue with Polyvinyl Alcohol

Reagents:

Polyvinyl alcohol (Gelvatol, Monsanto Chemical Co.)	15 g
Lactic acid (Mallinckrodt, Inc.)	39 ml
Phenol, melted (Mallinckrodt, Inc.)	39 ml
Aniline blue or cotton blue (Porrier's blue) (National Aniline Division)	0.1 g
Distilled water	100 ml

Add the polyvinyl alcohol to distilled water, place in an 80°C water bath, and stir until smooth and the solution clears. Add the lactic acid and then the phenol. Finally, dissolve the aniline or cotton blue in the solution.

This mounting medium is useful only for Scotch tape preparations and interferes with the visualization of fungi when wet mounts containing a small portion of the agar medium are made.

b. Lactophenol Aniline Blue Mounting Medium without Polyvinyl Alcohol

Reagents:

Lactic acid (Mallinckrodt, Inc.)	20 ml
Phenol crystals (Mallinckrodt, Inc.)	20 g
Aniline or cotton blue (National Aniline Division)	0.05 g
Glycerol (J. T. Baker Chemical Co.)	40 ml
Distilled water	20 ml

Dissolve the aniline or cotton blue in a warm solution of phenol, lactic acid, glycerol and water.

This stain is used to prepare a wet mount of fungal cultures when the transfer of a small amount of agar is expected and in a preparation of a microslide culture mount.

2. Procedures

a. Scotch Tape Preparation

The most widely used method for the microscopic identification of the filamentous fungi is the Scotch tape preparation.

(1) Touch the adhesive side of the tape to the surface of a colony at a point intermediate between its center and periphery.
(2) Adhere the adhesive side of the tape over an area on a glass microscope slide containing two drops of the mounting medium (B,1,a) dispensed along a line on the slide.
(3) Examine microscopically for characteristic arrangement of spores with the high dry and, if necessary, oil immersion objectives.

b. Wet Mount Preparation

When the Scotch tape preparation reveals only conidia or sterile hyphae, it may be necessary to examine a wet mount preparation of the culture.

(1) With a wire hook bent at a 90° angle, remove a small portion of the colony and the supporting agar at a point intermediate between its center and periphery.
(2) Place in a drop of mounting medium (B,1,b) on a glass microscope slide and apply a no. 1 coverslip with gentle pressure to disperse the agar and hyphae.
(3) Examine microscopically.

The disadvantage of this method is that the characteristic arrangement of spores is often disrupted, but the technique is most useful when the Scotch tape preparation fails to reveal the characteristic arrangement of spores or when an organism presents distinctive diagnostic features that are easily recognized by observing the spores alone.

c. Microculture Preparation

In certain instances, the Scotch tape and wet mount preparations fail to reveal distinctive diagnostic features, and it becomes necessary to prepare a microculture to observe the organism's characteristic features in a growing culture.

(1) Select a suitable subculture medium and punch out a small agar plug with the top of an 18 × 150 mm test tube.
(2) Remove the agar plug with sterile forceps and place it on a sterile glass microscope slide.
(3) Spot inoculate the agar plug at its periphery at 90° intervals and apply a no. 1 coverslip to the surface of the plug.
(4) Place the inoculated plug onto the surface of 2% agar in a petri dish, which serves as an incubation chamber, and incubate at 30°C.
(5) Examine frequently for the appearance of growth underneath the coverslip. When sufficient growth is present, transfer the coverslip with sterile forceps to a drop of mounting medium (B,1,a) on a glass microscope slide. Also prepare a wet mount (2,b) from the growth at the periphery of the agar plug.
(6) Examine microscopically.

The microslide culture technique is the most tedious, time consuming, and expensive of the methods described and should be reserved for use in selected instances.

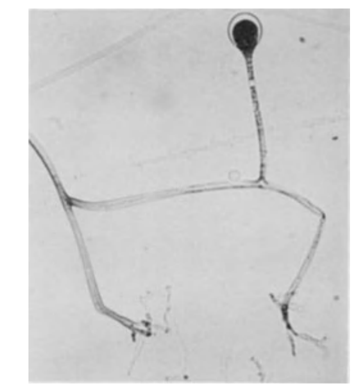
C. Laboratory Identification of Common Aseptate Hyaline Molds (Zygomycetes)

The identification of common Zygomycetes is somewhat simplified by the occurrence of only a few species in clinical specimens. Zygomycetes having spherical sporangia are easily distinguished since *Rhizopus* and *Absidia* produce rhizoids and *Mucor* does not. The rhizoids of *Rhizopus* species are produced directly below the sporangiophore while those of *Absidia* are produced intermediate between two sporangiophores. Other Zygomycetes, including *Syncephalastrum* and *Cunninghamella,* are easily recognized due to their characteristic microscopic morphological features. The characteristic features for those Zygomycetes commonly encountered are presented in Table 7–9.

D. Laboratory Identification of Common Septate Hyaline Monomorphic Molds

The septate hyaline monomorphic molds are the most commonly encountered of the filamentous fungi. Their identification is based almost exclusively on their characteristic microscopic features combined, in some

Table 7–9. Characteristics of Zygomycetes Commonly Encountered in a Clinical Microbiology Laboratory[a]

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Absidia species	Colonies are white early and later become gray and cottony.	1–5	Pyriform sporangia are produced with rhizoids formed between sporangiophores and not at their base.	Aseptate hyphae and the internodal production of rhizoids.	
Cunninghamella species	Colonies are spreading and white to silver in color and woolly in texture.	1–3	Smooth or roughened sporangiola are produced on short sporophores covering the surface of a terminal vesicle.	Aseptate hyphae and sporangiola on an enlarged vesicle.	

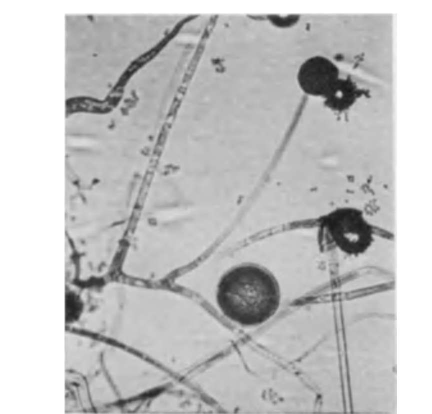
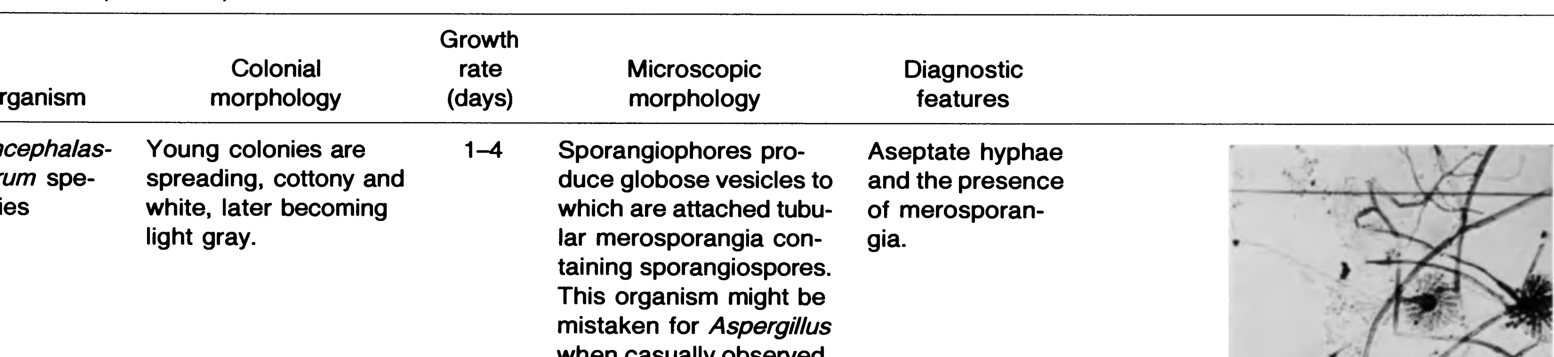
Mucor species	Young colonies are white and cottony, later becoming brown or black.	1–4	Broad, aerial, branched or unbranched sporangiophores are produced with a large spherical sporangium at the tip. No rhizoids are produced.	Aseptate hyphae, sporangia, and the absence of rhizoids.	
Rhizopus species	Young colonies are white and cottony, later becoming gray.	1–4	Unbranched sporangiophores support spherical sporangia and rhizoids are produced below the sporangiophore.	Aseptate hyphae are the nodal production of rhizoids.	

Table 7–9. (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Syncephalastrum species	Young colonies are spreading, cottony and white, later becoming light gray.	1–4	Sporangiophores produce globose vesicles to which are attached tubular merosporangia containing sporangiospores. This organism might be mistaken for *Aspergillus* when casually observed.	Aseptate hyphae and the presence of merosporangia.	

[a] From Dolan, C. T., et al.: *Atlas of Clinical Mycology* (Chicago: American Society of Clinical Pathologists, © 1976). Used by permission.

instances, with their gross colonial morphological presentation. As a general rule, they grow rapidly and produce mature colonies within 5 to 6 days. Some are pigmented and exhibit a wide variety of colors. A number of textbooks are available for the identification of the hyaline septate monomorphic molds and those most useful to our laboratory are listed in the general reference section of this chapter. In addition, Table 7–10 presents and illustrates the characteristic features of hyaline septate molds commonly encountered in a clinical microbiology laboratory. Descriptions of less frequently isolated organisms not included in this section may be found in many of the references presented in the general reference section of this chapter.

E. Laboratory Identification of Dermatophytes

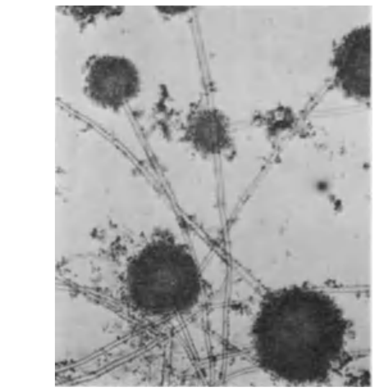
Although the dermatophytes are members of the septate hyaline monomorphic molds, they are distinctly different in regard to the sites from which they are commonly recovered and the microscopic features that they present. The clinical sources from which dermatophytes are recovered are limited to the skin, hair or nails, whereas the other hyaline monomorphic molds may be recovered from any clinical source. In general, dermatophytes possess the microscopic features that are common to the group, including raquet hyphae, spiral hyphae, antler hyphae, pectinate bodies, nodular bodies, and macroconidia that characterize the genera.

The grouping of the dermatophytes into genera is somewhat simplified by distinctive features exhibited by each. Characteristics of the three genera of dermatophytes are as follows:

1. The genus *Epidermophyton* is characterized by the presence of large clavate, multiseptate, smooth-walled macroconidia. They are usually borne in clusters of two or three but may be borne singly. No microconidia are produced.
2. The genus *Trichophyton* is characterized by the presence of multiseptate, smooth-walled, and pencil- to cigar-shaped macroconidia. However, in most instances microconidia are predominant and their shape and arrangement on the hyphae are considered as the diagnostic features.
3. The genus *Microsporum* is characterized by the presence of large, rough-walled, multiseptate macroconidia. Most are cylindrical to spindle shaped in appearance and are borne singly. Microconidia may be produced but are uncommonly found.

An additional criterion that is helpful in placing the dermatophytes into genera is their specificity for certain anatomic sites. The genus *Epidermophyton* invades only the skin and nails, while the genus *Microsporum* invades only the hair and skin. The genus *Trichophyton* is capable of invading the hair, skin, and nails.

Table 7–10. Characteristic Features of Commonly Encountered Monomorphic, Septate, and Hyaline Molds[a]

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Acremonium species	Young colonies are moist, yeastlike and spreading, later becoming floccose and pink, white or gray.	2–6	Hyphae produce slender unbranched phialides with elliptical one-celled conidia arranged in clusters at their tips.	Slender phialides with a cluster of conidia at their tips.	
Aspergillus flavus	Colonies are flat or furrowed or wrinkled and yellow-green to dark green in color.	2–6	Conidial heads consist of a spherical vesicle with or without metulae and one row of phialides producing chains of conidia. Heads are yellow-green to yellow-orange or brown in color.	Spherical, pigmented conidial heads with or without metulae and one row of phialides producing chains of conidia.	

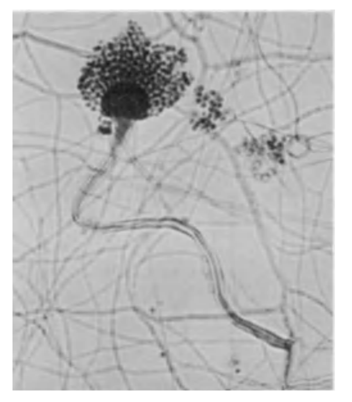
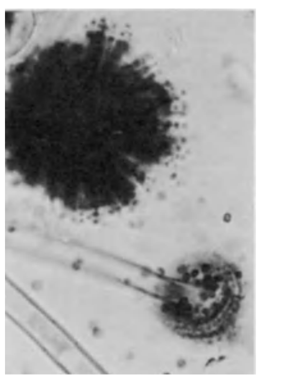
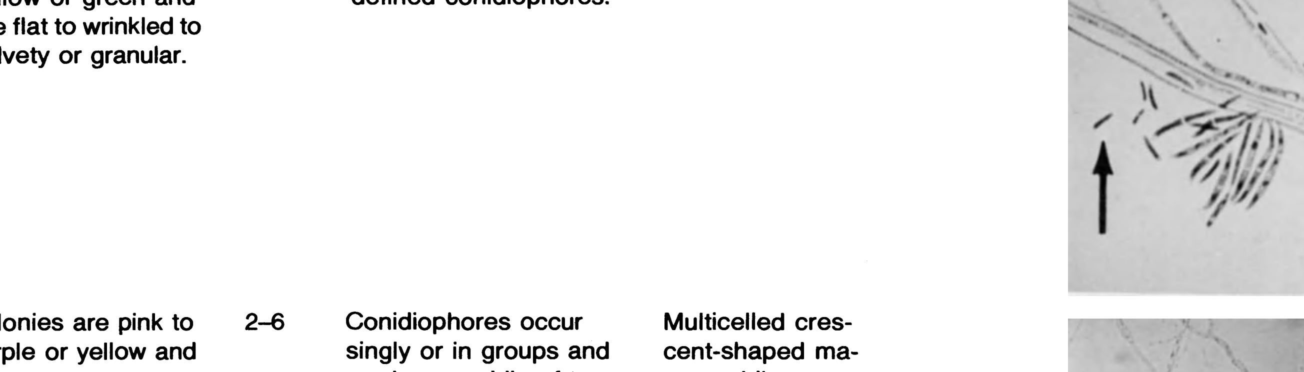
Aspergillus fumigatus	Colonies are velvety to felted and white when young, later becoming blue to gray-green in color. This organism grows well at 45°C.	2–6	Conidial heads consist of a dome-shaped vesicle having phialides on the upper one-half or two-thirds of its surface. Chains of conidia form a columnar mass on the vesicle.	Conidial heads with phialides on the upper one-half or two-thirds of the vesicle.	
Aspergillus niger	Colonies are white to yellow when young and turn black with age.	2–6	Conidial heads consist of smooth spherical vesicles entirely covered with one row of phialides producing dark conidia.	Spherical conidial heads with black conidia.	

Table 7–10 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Chrysosporium species	Colonies vary in color from white, cream, tan brown to yellow or green and are flat to wrinkled to velvety or granular.	5–14	Aleuriospores are ovoid or pyriform, usually produced singly on poorly defined conidiophores.	Pyriform or clavate conidia with a truncate base.	
Fusarium species	Colonies are pink to purple or yellow and floccose.	2–6	Conidiophores occur singly or in groups and produce conidia of two types: single celled microconidia that are usually borne in gelatinous clusters, and multicelled crescent-shaped macroconidia.	Multicelled crescent-shaped macroconidia.	

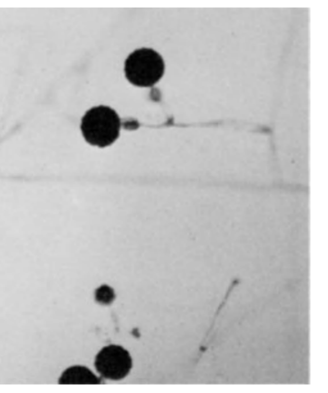
Geotrichum candidum	Colonies are moist and yeastlike when young, later developing aerial hyphae. Older colonies may be cream, green, or tan in color.	2–6	Hyphae break into rectangular arthroconidia that may remain in chains or be broken apart.	Rectangular arthroconidia.	
Gliocladium species	Colonies are white to cream or salmon to rose or green and floccose.	2–6	Conidiophores are simple or branched, arranged in whorls, and produce erect, compact heads resembling penicillium with conidia arranged in gelatinous clusters at the tip of the heads.	Penicillate heads producing conidia in gelatinous clusters or balls.	

Table 7–10 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features
Paecilomyces species	Colonies are thin, spreading, granular to velvety, becoming pink, violet, white, yellowish-brown or greenish-gold.	2–6	Single tapered phialides with chains of conidia are produced in the presence or absence of small, delicate penicillium-like heads that produce chains of conidia.	Delicate phialides and penicillium-like conidiophores producing chains of small conidia.
Penicillium species	Colonies usually are shades of green but vary to blue-green, pink, tan, yellow, orange, or white. Most colonies are wrinkled and velvety but may be granular. Some colonies produce diffusible pigments.	2–6	Conidiophores produce heads that are composed of short branches bearing phialides arranged in a "brush" fashion. Chains of conidia are produced by each phialide.	Brushlike penicillus producing chains of conidia.

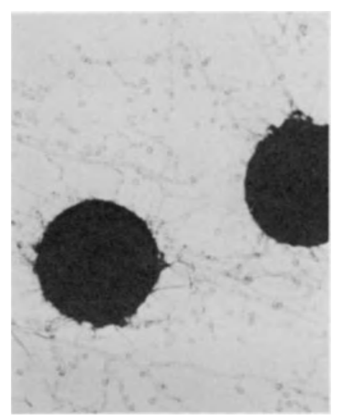
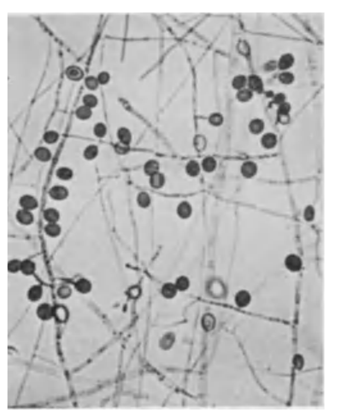
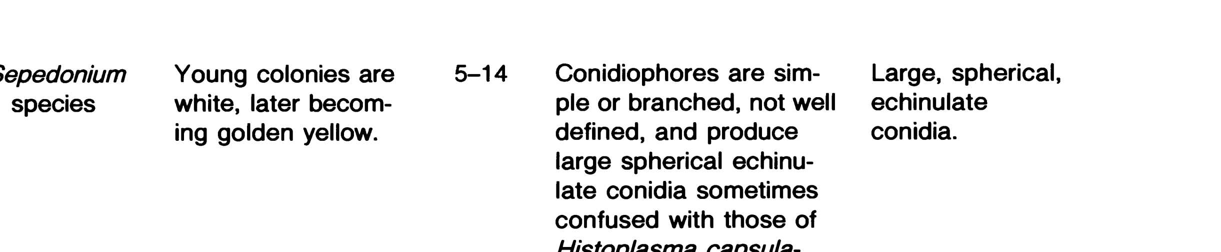
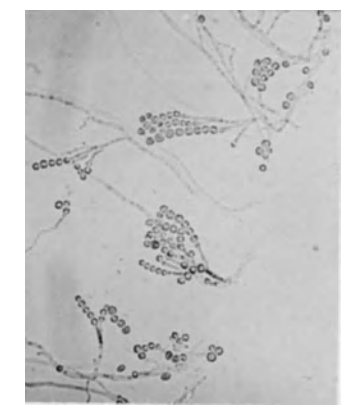
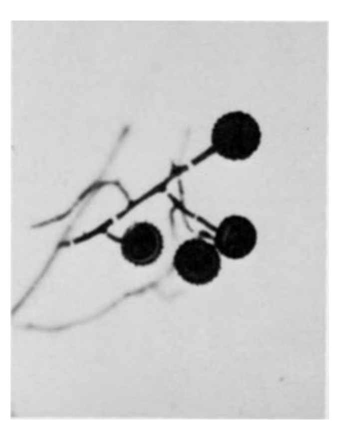
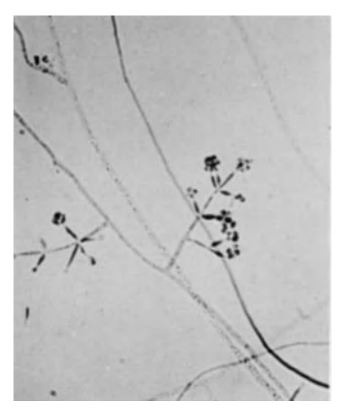
Pseudallescheria boydii (*Petriellidium boydii*)	Colonies are floccose and white to tan but most are smoky gray in color. The reverse side is gray to black in color. Ascocarps, when present, are black and granular appearing.	2–6	Hyphae are highly branched and produce simple or branched conidiophores that give rise to single or grouped pale brown conidia. Ascocarps, when present, are brown pseudoparenchymous and contain eight brown ascospores.	Usually, single conidia on short or long simple or branched conidiophores. Ascocarps may also be present.	 

Table 7–10 (*continued*)



Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Scopulariopsis species	Young colonies are white, later becoming tan or brown and granular.	2–6	Hyphae produce single phialides or branched penicillus-like structures slightly larger than those of penicillium. Broad-based echinulate conidia are produced in chains.	Large penicillate heads producing echinulate or smooth conidia in chains.	
Sepedonium species	Young colonies are white, later becoming golden yellow.	5–14	Conidiophores are simple or branched, not well defined, and produce large spherical echinulate conidia sometimes confused with those of *Histoplasma capsulatum*.	Large, spherical, echinulate conidia.	

Trichoderma species	Colonies are floccose and white when young, later becoming yellow-green to dark green with age.	2–6	Conidiophores are often verticillate producing flask-shaped phialides with clustering globose conidia at their tips.	Clusters of conidia produced on phialides attached to verticillately arranged conidiophores.	

[a] From Dolan, C. T., et al.: *Atlas of Clinical Mycology* (Chicago: American Society of Clinical Pathologists, © 1976). Used by permission.

Figure 7–3 presents a schema useful for the laboratory identification of common dermatophytes. Since many dermatophytes fail to sporulate on primary isolation media, the schema is based on the microscopic examination of cultures on cornmeal agar (p. 438). Table 7–11 presents the characteristics of dermatophytes commonly encountered in a clinical laboratory.

In addition to the microscopic examination, selected tests and media are useful for the speciation of certain dermatophytes. Their descriptions follow on page 465.

Urease Test[27]

Purpose:
To distinguish *Trichophyton mentagrophytes* from *Trichophyton rubrum.*

Principle:
Refer to p. 194.

Specimen:
An isolated, bacterial-free colony from an actively growing culture.

Reagents:
Refer to p. 194.

Procedure:

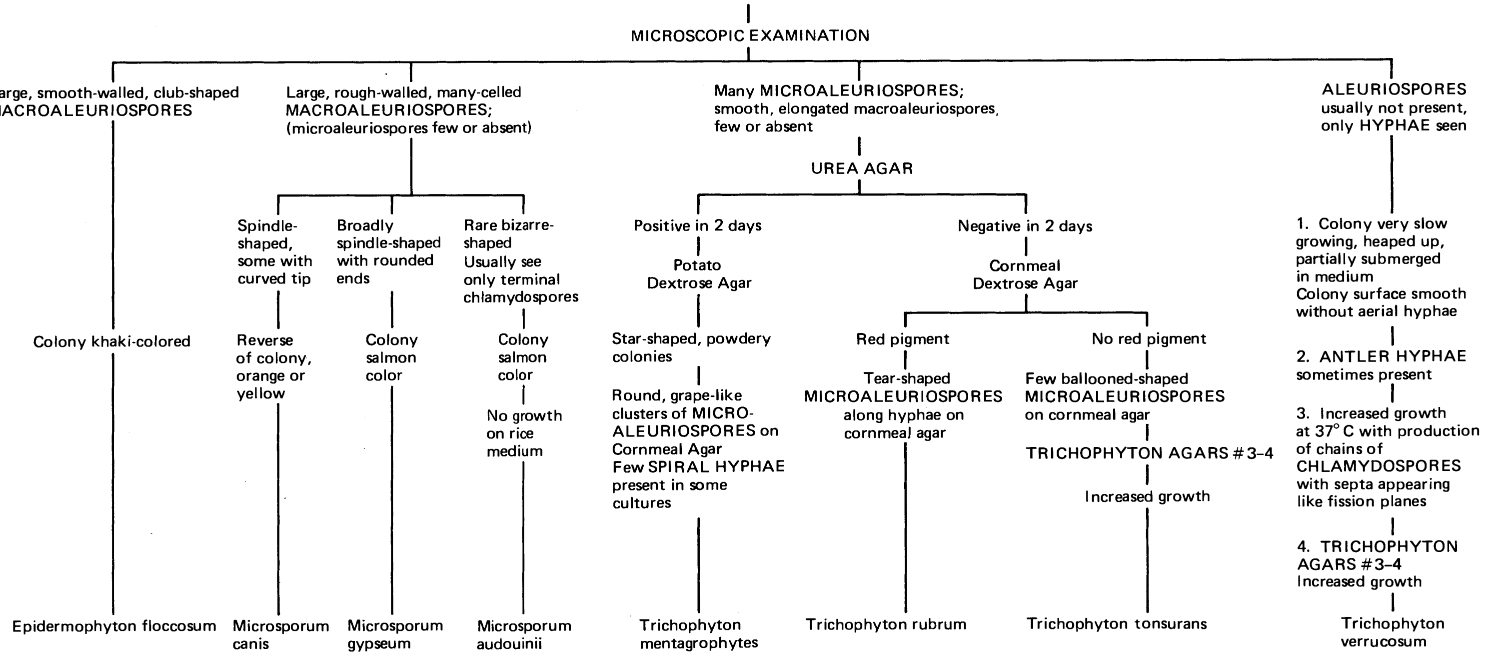
1. In instances where a culture is suspected of being *Trichophyton mentagrophytes* or *Trichophyton rubrum,* inoculate a slant of Christensen's urea agar.
2. Incubate for 2 days at 30°C.

Interpretation:
Trichophyton mentagrophytes produces urease within two days, whereas *Trichophyton rubrum* does not. Occasionally *Trichophyton tonsurans* may produce urease; however, the microscopic morphology is different from that of *Trichophyton rubrum* or *Trichophyton mentagrophytes.*

Control:

Positive control: *Trichophyton mentagrophytes*
Negative control: *Trichophyton rubrum*

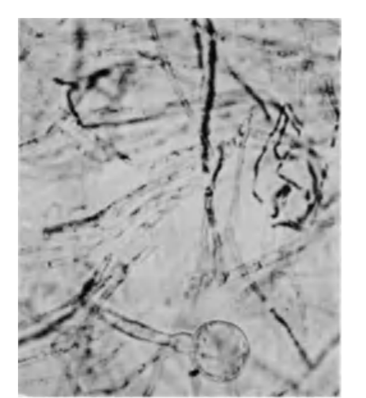
Alternative Methods:
In instances when *Trichophyton rubrum* and *Trichophyton mentagro-*



*Schema used for dermatophytes commonly recovered by Mayo Clinic Laboratory

Figure 7–3. Dermatophyte identification schema (as used for dematophytes commonly recovered by Mayo Clinic Laboratory).

Table 7–11. Characteristics of Dermatophytes Commonly Encountered in a Clinical Microbiology Laboratory[a]

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Comments	
Epidermophyton floccosum	Colonies are folded, velvety, olive-green, or khaki in color. Pleomorphism is obvious after 2–3 weeks.	5–8	Macroconidia are blunt, club-shaped, smooth-walled, and attached singly or in groups of two or three. Chlamydospores are common in older cultures. No microconidia are produced.		
Microsporum audouinii	Colonies are velvety, whitish-tan to salmon pink in color. The reverse is light salmon with reddish brown pigment.	12–21	Typical macroconidia are usually lacking but if present are bizarre-shaped. Raquet hyphae, pectinate phae, and terminal chlamydospores are present.	*M. audouinii* is the only member of the *Microsporum* group that cannot grow on polished rice.	

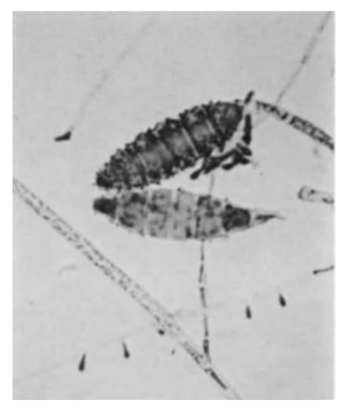
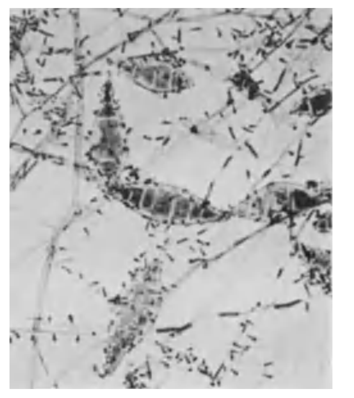
Microsporum canis	Young colonies are cottony to woolly or moist and adherent, white to buff in color. Older colonies become orange to yellow to brown. The reverse is yellow to orange.	5–7	Macroconidia are usually numerous, rough-walled, spindle-shaped, multiseptate with a curved tip, and thick-walled. Microconidia may be present.	
Microsporum gypseum	Colonies are buff to cinnamon in color and powdery. The reverse is tan.	3–6	Macroconidia are numerous, rough-walled, broadly spindle-shaped with rounded ends, and multiseptate.	

Table 7–11 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Comments
Trichophyton mentagrophytes	Colonies may be white to tan and powdery or cottony. Occasional isolates may be buff to red or yellow. The reverse is buff to reddish brown.	5–10	Granular cultures have numerous microconidia that are smooth-walled, spherical, and produced in clusters. Spiral hyphae are seen in some isolates. Macroconidia, when present, have 3–5 cells, are smooth-walled, cigar-shaped, and have a narrow attachment to the hyphae.	*T. mentagrophytes* produces urease within 2 days, whereas *T. rubrum* does not.
Trichophyton rubrum	Colonies may be white and cottony or pink to red and granular and folded. The reverse is yellow when cultures are young but turns wine red with age.	10–14	Tear-shaped microconidia are predominant and are borne laterally along the sides of small hyphae. Macroconidia usually absent, but when present, are smooth, thin-walled, and pencil-shaped.	Cultures are variable in their morphological features; some sporulate very poorly.

Trichophyton tonsurans	Colonies are commonly tan, heaped, wrinkled and suede-like in appearance. Some colonies are powdery and red or yellow. The reverse is yellow to tan to red.	10–14	Microconidia are tear-drop- or club-shaped with flat bases. They occur on open branching clusters or thickened terminal hyphae. Microconidia are often larger than those of other species and most appear swollen or distorted (balloon forms).	*T. tonsurans* grows better in the presence of thiamine.	
Trichophyton verrucosum	Colonies are small, heaped, and smooth or velvety. Most are white and appear to sink into the agar surface.	14–21	Microconidia and macroconidia are extremely rare. Most often branching hyphae resembling antler hyphae are seen.	*T. verrucosum* has a specific requirement for thiamine, and often inositol. Cultures exhibit enhanced growth at 37°C and produce chains of chlamydospores.	

[a] From Dolan, C. T., et al.: *Atlas of Clinical Mycology* (Chicago: American Society of Clinical Pathologists, © 1976). Used by permission.

phytes cannot be distinguished by the urease test, the hair perforation test will provide definitive results.[2]

Growth on Rice Grain Medium

Purpose:
To distinguish *Microsporum audouinii* from other species of the genus *Microsporum.*

Principle:
Microsporum audouinii lacks enzymes necessary for growth on polished white rice as a substrate in contrast to other species of the genus *Microsporum.*

Specimen:
A portion of an isolated colony from an actively growing culture.

Reagents:
Rice grain medium (p. 814).

Procedure:
Inoculate the cooled medium with fragments from an actively growing culture.

Interpretation:
In contrast to other members of the genus *Microsporum, Microsporum audouinii* lacks the ability to grow on polished white rice.

Controls:

Positive control: *Microsporum canis*
Negative control: *Microsporum auduoinii*

Growth on Trichophyton Agars

Purpose:
To determine the nutritional requirements of dermatophytes.

Principle:
A number of dermatophytes have essential or partial nutritional requirements necessary for growth. Important examples include *Trichophyton verrucosum,* which requires thiamine and inositol, and *Trichophyton tonsurans,* which requires thiamine. Specific nutritional requirements are necessary for the definitive identification of the two previously mentioned species.

Specimen:
A portion of a colony from an actively growing culture.

Reagents:
Trichophyton agars (p. 815)

Procedure:

1. Inoculate a very small portion of a colony onto the surface of the Trichophyton agar slants and incubate at 30°C for 2 weeks.
2. Examine the slants after 1 and 2 weeks for visible evidence of growth.
3. If cultures are contaminated with bacteria, subculture to a medium containing antibiotics (e.g., mycobiotic agar) for several generations. A very small amount of inoculum is necessary so that nutrients are not carried over from the primary culture medium onto the nutritional agars.

Interpretation:
Table 7–12 presents reactions of common dermatophytes on Trichophyton agars. Dermatophytes exhibit enhanced growth on media containing specific nutritional requirements necessary for growth.

Controls:

Positive control: *Trichophyton tonsurans*
Negative control: Uninoculated media

F. Laboratory Identification of Dimorphic Hyaline Molds

As previously mentioned, most of the dimorphic hyaline molds exhibit a slow growth rate (2 to 6 weeks) when recovered from clinical specimens; however, some may produce visible colonies within 48 hr. Regardless of the growth rate, most isolates fail to produce characteristic microscopic features on the primary isolation media and should be subcultured onto a battery of media including inhibitory mold agar (p. 813), yeast extract—phosphate agar (p. 815), and Sabouraud's dextrose (2%) agar (p. 814), to induce sporulation. Subcultures should be examined at daily intervals after visible growth is apparent. In addition, colonies that appear on the primary recovery medium should also be examined at frequent intervals for evidence of sporulation.

1. Definitive Identification of Dimorphic Hyaline Molds

A tentative identification of the dimorphic fungi can usually be made by examining a Scotch tape or wet mount preparation for the presence

Table 7–12. Reactions of Common Dermatophytes on Trichophyton Agars[a]

Organism	#1 Casein Basal Agar	#2 Casein + Inositol	#3 Casein + Inositol + Thiamine	#4 Casein + Thiamine	#5 Casein + Nicotinic Acid	#6 Casein + Ammonium Nitrate	#7 Casein + Histidine
T. verrucosum							
84%	0	±	4+	0			
16%	0	0	4+	4+			
T. schoenleinii	4+	4+	4+	4+			
T. concentricum							
50%	4+	4+	4+	4+			
50%	2+	2+	4+	4+			
T. tonsurans	± to 1+	1+	4+	4+			
T. mentagrophytes	4+			4+	4+		
T. rubrum	4+			4+			
M. ferrugineum	4+			4+			
T. violaceum	± to 1+			4+			
T. megninii						0	4+
M. gallinae						4+	4+
T. equinum	0				4+		

[a] 4+, rich abundant growth; 1+, submerged growth of approximate 10 min; ±, no growth or growth around 2 mm.

of characteristic microscopic features of the mold form as presented in Table 7–13.

The definitive identification of the dimorphic hyaline fungi, however, requires conversion of the mold form to the corresponding yeast or spherule form or the serological detection of exoantigens characteristic for the species.

a. Serological Identification of the Dimorphic Molds

Exoantigen Microimmunodiffusion Test[41]

Purpose:
To detect cell-free exoantigens produced by *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis.*

Table 7–13. Characteristics of Dimorphic, Hyaline Molds[a]

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Blastomyces dermatitidis					
Mold form	Colonies are fluffy white to tan or brown on media lacking blood enrichment. Colonies recovered on blood enriched media are often cream to tan, soft heaped, wrinkled, waxy, and yeast-like. Hyphal projections are often seen arising from the colonies.	4–≥30	Hyphae are 1–2 μm in diameter; single, round to pyriform conidia are attached to lateral conidiophores of various lengths or laterally to the hyphae. Many cultures produce only a few conidia.	Round to pyriform conidia, when present. Sometimes the microscopic morphology is nondescriptive.	
Yeast form	Colonies are tan to cream in color, waxy, and very wrinkled when grown at 35–37°C.	2–≥5	Large, thick-walled, yeast cells with single or sometimes, 2–3 buds, attached to the parent.	Large yeast with a double thick wall, flat, broad base connecting buds to the parent cell.	

Table 7–13 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Coccidioides immitis					
Mold form	Young colonies are moist and adherent to the medium. Older colonies develop cottony aerial hyphae that are unevenly distributed over the colony giving a "cobweb" appearance. Some colonies always remain flat and moist and tan in color. Most cottony colonies are white becoming tan to brown with age. Colonies on a blood enriched medium often exhibit a green color and appear yeastlike.	2–21	Hyphae are small, 1–2 μm in diameter. Young cultures are highly branched and produce raquet hyphae commonly. As the culture matures, hyphae enlarge and disassociate into barrel-shaped or elongated arthroconidia that alternate with clear cells. Some cultures fail to sporulate despite subculture or prolonged incubation. Variation in sizes and shapes of arthroconidia may occur.	Alternate arthroconidia.	

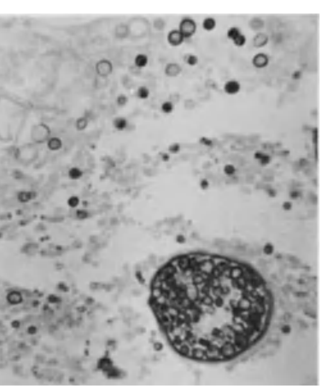
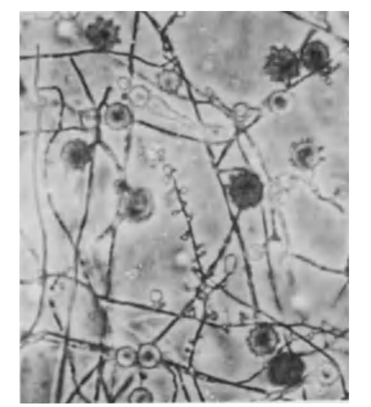
Spherule form	Not usually cultured.	—	Not usually seen in culture but is present in tissue or exudate from infected patients or animals. Round spherules 30 to 60 μm in diameter containing endospores 2–5 μm in diameter are seen. Occasionally, spherules may be confused with yeast forms; however, they do not exhibit budding.	Spherules containing endospores.	
Histoplasma capsulatum					
Mold form	Colonies are white to tan to brown and fluffy or glabrous on media lacking blood enrichment. Growth on blood-containing media appears moist, waxy, heaped, cerebriform, yeastlike, and tan to pink in color.	5–45	Hyphae are small, 1–2 μm in diameter. Macroconidia usually predominate as spherical to pyriform tuberculate spores borne on narrow conidiophores. Small, round to pyriform microconidia may be produced alone or with macroconidia.	Tuberculate macroconidia.	

Table 7–13 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Yeast form	Growth appears as rough, mucoid, and cream in color but becomes smooth, creamy, and tan with maturity	7–≥14	Small, oval budding yeast cells, 2–5 μm in size.	Small yeast cells, 2–5 μm in diameter.	
Paracoccidioides brasiliensis					
Mold form	Colonies are smooth when young but develop short aerial hyphae brown or white in color. The surface becomes flat, velvety to floccose and heaped, to cerebriform with age.	21–28	Round to pyriform conidia resembling those of *Blastomyces dermatitidis* are produced; numerous chlamydospores and swollen round cells may be found.	The mold form is usually not characteristic.	

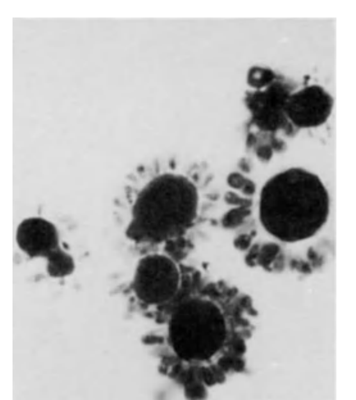
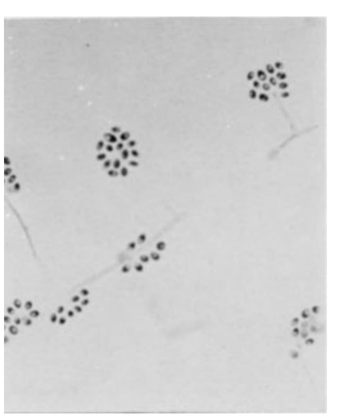
Yeast form	Colonies are smooth to cerebriform, waxy, and white to tan in color.	≥21	Large, single and multiple budding yeast cells are produced. Buds are attached to the parent cell by a narrow neck.	Large multiple budding yeast cells.	
Sporothrix schenckii Mold form	Young colonies are white to tan and yeastlike in appearance. With age they become brown to black, leathery, and wrinkled. The surface of the colony is smooth and lacks aerial hyphae. Young cultures may be mistaken for yeasts.	3–5	Hyphae are small, 1–2 μm in diameter. Slender branched conidiophores arise at right angles and bear small pyriform conidia arranged in "flowerettes" at their tips. Conidia are attached by small threadlike structures. Conidia may also be borne laterally along the hyphae as the culture matures.	Flowerette or sleeve arrangements or conidia.	

Table 7–13 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Yeast form	Colonies are white to cream, soft, and bacteria-like in appearance	5	Elongated yeast cells resembling cigars with buds are present. Occasionally oval to spherical cells with one or more buds may be found.	Cigar-shaped budding yeasts.	

[a] From Dolan, C. T., et al.: *Atlas of Clinical Mycology* (Chicago: American Society of Clinical Pathologists, © 1976). Used by permission.

Principle:
Histoplasma capsulatum, Blastomyces dermatitidis, and *Coccidioides immitis* produce cell-free exoantigens in culture that react with specific antibodies produced in the serum of patients or animals infected with histoplasmosis, blastomycosis, or coccidioidomycosis.

Specimen:
A mature culture on a Sabouraud's dextrose agar slant is optimal.

Reagents:

1. Thimerosal Solution

Sodium borate (J. T. Baker Chemical Co.)	1.4 g
Thimerosal (Eli Lilly and Co.)	1 g
Distilled water	100 ml

2. Immunodiffusion Test Medium

NaCl (J. T. Baker Chemical Co.)	0.9 g
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	0.4 g
Phenol, melted (Mallinckrodt)	0.25 ml
Glycine (J. T. Baker Chemical Co.)	7.5 g
Purified agar (Difco Laboratories)	1 g
Distilled water	100 ml

Dissolve the NaCl and $Na_3C_6H_5O_7 \cdot 2\ H_2O$ in 50 ml of distilled water, add 0.25 ml of phenol and 7.5 g of glycine, and mix thoroughly. Add 1 g of purified agar and make up to a final volume of 100 ml with distilled water. Sterilize for 5 min at 120°C.

3. Control Antisera
Control antisera for *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis* are available from Gibco Diagnostics or Scott-Nolan Laboratories.

4. Control Antigens
The antigens of *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis* are available from the Gibco Diagnostics or Scott-Nolan Laboratories.

Supplies:

Minicon concentrator, B-15 (Amicon Corporation, Lexington, Massachusetts).

Microimmunodiffusion template (L. L. Pellet Co., Dallas, Texas).

Procedure:

1. Preparation of exoantigens[16]
   a. Cover the surface of a mature fungus colony growing on a slant of Sabouraud's dextrose agar with an aqueous solution of merthiolate (Thimerosal) 1:5000 final concentration, and allow it to react for 24 hr at 25°C.
   b. Remove 5 ml of the solution and place in a Minicon B-15 concentrator and concentrate 50 × for *Histoplasma capsulatum* and *Blastomyces dermatitidis* and 5 × and 25 × for *Coccidioides immitis* antigens.
   c. Remove the concentrated antigen for use in the immunodiffusion test.
2. Microimmunodiffusion test
   a. Prepare a microimmunodiffusion plate by pipetting 6.5 ml of melted glycine-phenol agar into a plastic petri dish and allowing the agar to set for at least 30 min.
   b. Pipette 3.5 ml of molten glycine-phenol agar on top of the solidified agar base layer and lower a 17–7 well plastic microdiffusion template slowly into the agar to prevent the entrapment of air bubbles.
   c. Allow the agar to set for at least 30 min.
   d. Remove the agar plugs from the template before performing the microimmunodiffusion test.
   e. Place the control antiserum in the center well of the patterns that are to be used in testing. Allow the antisera to diffuse for 1 hr at room temperature before proceeding to Step f.
   f. Add the control antigens in the upper and lower wells and the unknown antigens in duplicate in the lateral wells on the same side of the template.
   g. Incubate the immunodiffusion plates in a moist chamber at 25°C for 24 hr.
   h. Remove the immunodiffusion template and wash the agar surface free of excess agar. Cover the agar surface with distilled water.
   i. Examine the immunodiffusion plates over an indirect light source for the presence of precipitin bands of identity with the control antigen–antibody systems. Only bands of identity are considered to be positive tests.

Interpretation:
Unknown cultures demonstrating either the IDPT, IDHL, or IDCF precipitin bands may be identified as *Coccidioides immitis.* Unknown cultures demonstrating H or M bands may be identified as *Histoplasma capsulatum.* Cultures with a B band may be identified as *Blastomyces dermatitidis.*

Controls:
Controls used in this test have been described in the previous section.

Alternate Methods:
The use of a broth culture filtrate of an unknown organism is satisfactory for use in the microimmunodiffusion test.[39,40]

---

b. *In Vitro* Conversion of the Dimorphic Hyaline Molds

Traditionally, the definitive identification of the dimorphic hyaline molds, with the exception of *Coccidioides immitis,* has been based on the *in vitro* conversion of the mold form to the corresponding yeast form. The *in vitro* conversion of *Coccidioides immitis* to its spherule form may be accomplished but is not practical for clinical laboratories. Currently, the detection of exoantigens is recommended for the identification of *Histoplasma capsulatum* and *Coccidioides immitis.* The *in vitro* conversion method should, therefore, be reserved only for the identification of *Blastomyces dermatitidis* and *Sporothrix schenckii* since conversion of these species is easily accomplished.

Brain heart infusion agar containing 10% sheep blood (p. 811) is the medium of choice for the *in vitro* conversion of *Sporothrix schenckii.* Cottonseed conversion medium[45] (p. 812) is useful only for the *in vitro* conversion of *Blastomyces dermatitidis* to its yeast form.

The *in vitro* conversion of *Blastomyces dermatitidis* and *Sporothrix schenckii* can be accomplished using the following procedure:

1. Transfer a large inoculum of the unknown filamentous culture onto the surface of a fresh, moist slant of the appropriate conversion medium. If the slant is dry, add 0.5-ml sterile distilled water before inoculation.
2. Incubate the culture at 35°C and subculture to a fresh slant as soon as growth appears. The inoculum should be taken from the portion of growth that appears most creamy in consistency.
3. Repeat the subcultures until conversion occurs. Several subcultures are usually necessary; however, conversions may be accomplished within 2 to 3 days with *Blastomyces dermatitidis* and *Sporothrix schenckii.*

G. Laboratory Identification of Dematiaceous Molds

Dematiaceous molds are easily recognized by the presence of a dark pigment in the hyphal elements; conidia may be nonpigmented. Their identification is based on the characteristic and arrangement of spores produced. Table 7–14 provides descriptions and illustrations of those dematiaceous fungi commonly found in clinical specimens.

Table 7–14. Characteristic Features of Dematiaceous Molds Commonly Encountered in a Clinical Microbiology Laboratory[a]

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Alternaria species	Colonies are gray-brown to dark green to gray-green. Older colonies are covered with gray to white hyphae. The reverse side is black.	2–6	The hyphae are septate and dark brown with simple or branching conidiophores. Conidia are dark brown and muriform with horizontal and longitudinal septae. Conidia are produced in chains but are most often seen singly because chains are easily disrupted.	Brown muriform conidia with horizontal and longitudinal septae.	
Aureobasidium pullulans	Young colonies are cream colored and yeastlike in appearance. Some colonies are mucoid resembling *Cryptococcus.* With age, colonies become heaped, wrinkled, leathery, and black in color, with a fringe of submerged hyphae.	4–8	Young hyphae are hyaline and become dark, enlarged, and thick-walled with age. Older hyphae give rise to hyaline blastoconidia on short denticles.	Dark, thick-walled, cuboid cells that give rise to hyaline blastoconidia.	

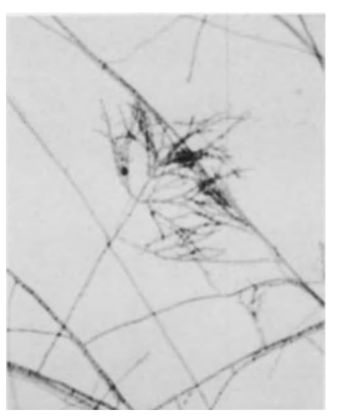
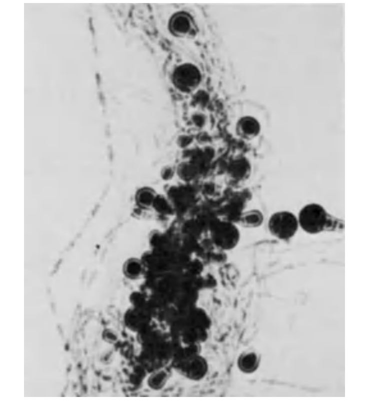
Cladosporium species	Colonies are gray-green to dark olive, velvety, folded, and heaped. The reverse side is black or gray-brown.	6–10	Conidiophores are branched, of various lengths, terminating in chains of budding conidia. Conidia are usually one-celled, oval with distinct scars on ends (disjunctors).	Darkly pigmented branched conidiophores with conidia having disjunctors.	
Curvularia species	Young colonies are gray-brown turning black to brown and floccose or velvety with age.	2–6	Conidiophores are twisted and produce curved, septate conidia with paler ends. Conidia contain 4–6 septa with the center segment larger than the others.	Dark curved conidia with paler ends.	

Table 7–14 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Drechslera species	Colonies are fluffy to velvety and gray to brown to black in color.	2–6	Conidiophores are septate, simple or branched and twisted in a zigzag arrangement. Conidia are large, cylindrical, multiseptate, and arise from the twisted areas of the conidiophore.	Zigzag arrangement of conidiophore with large multiseptate, cylindrical conidia.	
Epicoccum species	Colonies are spreading, some are moist with little aerial hyphae but produce black sporodochia; others have aerial hyphae and are yellow, yellow-brown, orange, red, or black. Some isolates produce an orange diffusible pigment.	2–6	Clusters of dark brown or black; rough-walled, muriform, multiseptate conidia (sporodochia) are found in mature cultures. Young conidia appear smooth.	Clusters of globose, muriform conidia, and pigmented hyphae.	

Exophiala jeanselmei	Young colonies are moist and yeastlike becoming gray to olive to olive-black and velvety with age.	7–14	Young cultures produce black yeasts. Mature cultures have conidiophores (annelides) that are simple or branched, cylindrical with tapered tips, and brown in color. Conidia (annelloconidia) aggregate in masses at the tip of the conidiophore.	Black yeasts in young cultures. Cylindrical conidiophores with tapered tips producing masses of conidia.
Nigrospora species	Colonies are fluffy, gray-white becoming gray and floccose with age. As the colony matures, darkened areas of sporulation are apparent.	2–6	Hyphae are hyaline but become pigmented with age. Smooth dark black conidia are produced on inflated vase-shaped conidiophores.	Dark conidia born on short vase-shaped conidiophores.

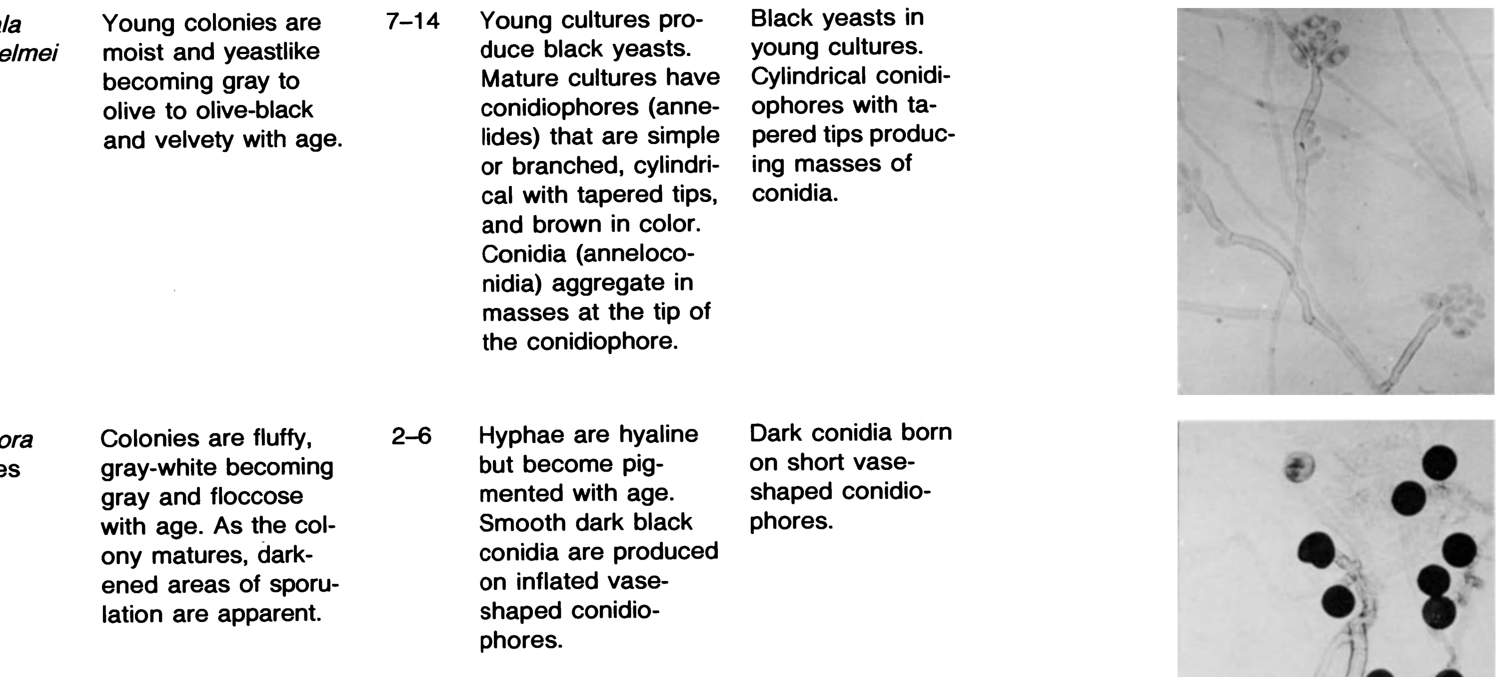


Table 7–14 (*continued*)

Organism	Colonial morphology	Growth rate (days)	Microscopic morphology	Diagnostic features	
Phialophora verrucosa	Colonies are domed, velvety and olive-gray to dark olive in color.	14–21	Conidiophores (phialides) are simple or branched, flask or vase-shaped, with a distinct collarette. Conidia aggregate in masses at the tip of the phialides.	Vase-shaped phialides having a collarette and conidia in masses at the tip.	
Wangiella dermatitidis	Young colonies are yeastlike, becoming olivaceous-gray and velvety with age.	14–21	Young cultures produce black yeasts. Mature cultures have conidiophores (phialides) that are tubular or flask-shaped and lack a collarette. Conidia aggregate in masses at the tip of phialides.	Black yeasts in young cultures, tubular conidiophores lacking a collarette that produce masses of conidia.	

[a] From Dolan, C. T., et al.: *Atlas of Clinical Mycology* (Chicago: American Society of Clinical Pathologists, © 1976). Used by permission.

V. Serodiagnosis of Fungal Infections

Fungal serological tests are useful tools for the diagnosis of mycotic infections when used with clinical findings and/or histopathological proof of etiology. Tests have been developed to detect antibodies in patients having aspergillosis, blastomycosis, candidosis, coccidioidomycosis, and histoplasmosis. A test to detect the polysaccharide capsular antigen of *Cryptococcus neoformans* is available and is highly reliable for the diagnosis of cryptococcosis.

Most of the antigens used for testing are crude and some contain common components that cross-react with other fungi to give false positive results. For example, the antigens of *Histoplasma capsulatum* are similar to those of *Blastomyces dermatitidis* and *Coccidioides immitis.* It is not uncommon for a patient with histoplasmosis to have positive serological tests to a heterologous antigen such as *Blastomyces dermatitidis.* However, in most instances the titer is greater with the homologous antigen. It is, therefore, recommended that a battery of antigens be used for testing to simplify the interpretation of cross-reactions. False negative and false positive tests can occur, but in most instances, fungal serological tests provide useful and reliable results, especially when supplemented with cultural and histopathological proof of etiology. In many instances, these tests provide the earliest and only presumptive evidence of infection.

In the past, fungal serological tests have been available only to large medical centers or reference laboratories; however, commercially prepared reagents have become available so that all laboratories now have the capability of performing these tests. The decision whether to offer them depends upon a number of factors, including demand, test volume, expiration rates and cost of reagents, and the available technical expertise.

Information concerning fungal serological tests available at the Mayo Clinic and general guidelines for their interpretation are listed in Table 7–15. Methods are not presented in this section but are described in detail by Palmer et al.[26]

VI. Antimicrobial Susceptibility Tests of Fungi

Antimicrobial susceptibility tests for fungi are performed to determine the concentration of an antifungal agent necessary to inhibit or kill the organism being tested. Antifungal susceptibility testing is not well developed and is problematical because of the lack of standardization of variables affecting the test. There is general disagreement among investigators concerning specific methods, inoculum sizes, incubation conditions, and end point determinations. These problems are further compounded by the extreme variability in growth rates of fungi.

Table 7–15. Fungal Serological Tests Available at Mayo Clinic

Disease	Antigens	Specimen for testing	Test(s)	Interpretation
Aspergillosis	*Aspergillus fumigatus*[a,b,e,f] *Aspergillus niger*[a,e] *Aspergillus flavus*[a,e]	Serum	Immuno-diffusion	One or more precipitin bands are suggestive of active infection. Precipitin bands have been shown to correlate with complement-fixation titers—the greater the number of bands, the higher the titer.[9] When cultural proof is presented in the presence of a positive test, it is diagnostic of active infection. Precipitins can be found in 90% of the sera of patients having aspergilloma and 50–70% of patients with allergic bronchopulmonary aspergillosis.[5] Antibodies are found less often in patients with invasive disease, but the usefulness of the test has been obscured by conflicting reports in the literature.[3,47]
Blastomycosis	*Blastomyces dermatitidis*[a,b,f,g] Yeast	Serum	Complement-fixation	Titers of 1:8–1:16 are highly suggestive of active infection; titers of $\geq$1:32 usually indicate infection. Cross-reactions occur in patients having coccidioidomycosis or histoplasmosis; however, titers are usually lower. A decreasing titer is indicative of regression of disease. Less than 25% of patients having blastomycosis exhibit a positive titer.
	Yeast filtrate[a]		Immuno-diffusion	Preliminary results suggested that this test was more sensitive than complement-fixation, detecting 80% of cases.[15] Studies in our laboratory, however, have shown it to be no better than the complement-fixation test. The presence of A and/or B precipitin bands are specific for antibodies to *Blastomyces dermatitidis.*

Candidosis	*Candida albicans*[c,f,g]	Serum	Immuno-diffusion	The test is difficult to interpret because precipitins are found in 20–30% of the normal population. Clinical or histopathological correlation must exist for the test to be useful. A recent study reports 89% sensitivity with a specificity of 90%.[20] There are numerous conflicting reports in the literature that obscure the value of serological tests for the diagnosis of candidosis.
Coccidioido-mycosis	Coccidioidin[a,b,f,g]	Serum or cerebrospinal fluid	Complement-fixation	Titers of 1:2 to 1:4 have been seen in active infection and should be followed by repeat testing at 2–3 week intervals. Titers >1:16 usually indicate active infection. Elevated or rising titers often indicate disseminated infection, and patients having such findings should be carefully evaluated. False positive tests may occur in patients having histoplasmosis or blastomycosis, but titers are usually higher with the homologous antigen. False negative tests may occur in patients having solitary pulmonary nodules or cutaneous infection. Titers usually parallel the severity of infection and are helpful both diagnostically and prognostically.
	Coccidioidin[a,b,f,g]	Serum	Immuno-diffusion	Antibodies may be detected within 1–3 weeks of the first appearance of symptoms. Sera must be concentrated 10 × to detect early infection, otherwise the immunodiffusion test becomes positive after 6 months. Results correlate well with the complement-fixation test.

Table 7–15 (*continued*)

Disease	Antigens	Specimen for testing	Test(s)	Interpretation
Cryptococcosis	No antigen—latex particles coated with hyperimmune anticryptococcal globulin[a,b,d,g]	Serum, cerebrospinal fluid, urine	Latex agglutination for crytococcal antigen	The presence of cryptococcal antigen is highly specific for cryptococcosis.[14,28] Positive tests have been found in the CSF of 95–98% of patients with cryptococcal meningitis and in the sera of 30% of patients with other forms of cryptococcosis. Disseminated infections usually produce positive titers in the serum. A decrease in titer correlates well with regression of disease and is a good prognostic indicator. False positive tests have been eliminated by treatment of serum or CSF with a proteolytic enzyme, pronase.[38] Rheumatoid factor controls are not necessary.
Histoplasmosis	Histoplasmin and yeast form of *Histoplasma capsulatum*[a,b,f,g]	Serum, cerebrospinal fluid	Complement-fixation	Titers of 1:8 to 1:16 indicate possible infection; however, titers of ≥1:32 usually indicate active infection. Cross-reactions occur in patients having aspergillosis, blastomycosis, and coccidioidomycosis, but titers are usually lower. Several follow-up serum samples at 2–3 week intervals should be tested. Rising titers indicate progressive infection and decreasing titers indicate regression of disease. Some patients with disseminated infections fail to produce complement-fixing antibodies. Recent skin tests in persons who have had prior exposure to *H. capsulatum* will cause an elevation in the complement-fixation titer in 17–20% of persons tested.[19] The yeast antigen gives positive titers in 75–80% of cases, while histoplasmin gives positive titers in 10–15% of cases. Ten percent of the cases have positive titers with both antigens simultaneously.

	Histoplasmin[a,b,f,g]	Serum	Immuno-diffusion	H and M bands appearing simultaneously indicate active infection. The M band may appear alone and may indicate early or chronic infection. The M band may also appear after a recent skin test. The H band appears later than the M band and disappears earlier. Its disappearance may indicate regression of the infection.
Hypersensitivity pneumonitis	*Alternaria tenuis*[e] *Aspergillus fumigatus*[a,b,e,f] *Aspergillus niger*[a,b,e,f] *Micropolyspora faeni*[e] *Thermoactinomyces vulgaris*[e] *Thermomonospora viridis*[e]	Serum	Immuno-diffusion	The presence of precipitins does not necessarily indicate hypersensitivity pneumonitis; it should be interpreted in conjunction with clinical findings. Nine percent of healthy farmers have been reported to exhibit positive tests with *Micropolyspora faeni* reacting most often (67.7% of cases).[33]
Sporotrichosis	Yeast of *Sporothrix schenckii*[g]	Serum	Agglutination	Titers of $\geq$1:80 usually indicate active infection.[23,32] Some cutaneous infections present negative tests; however, 90% of extracutaneous infections have positive tests.

[a] Available from Microbiological Associates, Walkersville, MD.
[b] Available from Meridian Diagnostics, Cincinnati, OH.
[c] Available from Hollistier-Stier Laboratories, Downers Grove, IL.
[d] Available from International Biological Laboratories, Rockville, MD.
[e] Available from Greer Laboratories, Lenoir, NC.
[f] Available from Scott Laboratories, Fiskeville, RI.
[g] Available from Gibco Laboratories, Lawrence, MA.

Despite the lack of standardization, many investigators feel that antimicrobial susceptibility tests for fungi are important, particularly to detect the development of resistance in organisms during chemotherapy. The two major drugs available for the chemotherapy of fungal infections are amphotericin B and flucytosine. Fungi are almost universally susceptible to amphotericin B so that the need for testing this agent is rarely justified. The *in vitro* testing with flucytosine is more important due to the development of resistant strains of *Cryptococcus neoformans* and *Candida* species during the course of chemotherapy. The imidazole drugs, including miconazole, clotrimazole, and ketoconazole, are available and may offer alternatives for the therapy of fungal infections. Antimicrobial susceptibility tests have been developed for the testing of these agents; however, they have not been standardized as yet and results are difficult to interpret.

Antifungal antibiotics differ greatly from antibacterial antibiotics; however, techniques used for susceptibility testing of both are similar. Methods available for antimicrobial susceptibility testing of fungi include disk diffusion,[43] Neo-Sensitab,[6] radiometry,[11] and broth dilution.[44] The broth dilution methods for testing amphotericin B and flucytosine in our laboratory are described.

Amphotericin B Susceptibility Test[44]

Purpose:
To determine the minimal inhibitory concentration (MIC) of amphotericin B for a specific fungal isolate.

Principle:
A standard amount of inoculum is added to tubes containing dilutions of amphotericin B in decreasing concentrations. After a suitable incubation period, tubes are observed for visible growth, and the lowest concentration that inhibits growth is reported as the MIC.

Specimen:
A 24- to 48-hr subculture of the organism grown on inhibitory mold agar at 30°C for 24 to 48 hr. Yeasts are more suitable for susceptibility testing since the inoculum size of the filamentous fungi is difficult to quantitate.

Reagents:

M-3 Broth Susceptibility Test Medium

Antibiotic medium no. 3 (Difco Laboratories)	17.5 g
Distilled water	1000 ml

Sterilize the medium for 15 min at 121°C.

Amphotericin B Solution

1. From its specific activity, determine the weight of amphotericin B (E. R. Squibb and Sons, Inc.) necessary to prepare a solution of 50 mg in 1 ml of 100% dimethylsulfoxide (Matheson, Coleman, and Bell Company).
2. Adjust the volume to 50 ml with sterile deionized water to obtain a solution containing 100 μg of amphotericin B per ml.
3. Dispense in 1 ml amounts into vials and freeze at −32°C until used. Discard the unused antibiotic solution after 6 months.
4. Prior to testing, prepare a 1:10 and a 1:100 dilution of amphotericin B in sterile deionized water to obtain solutions with concentrations of 10 μg/ml and 1 μg/ml, respectively.
5. Prepare a 1:5 dilution of dimethylsulfoxide in sterile deionized water just prior to testing.

Procedure:

Preparation of Inoculum

1. Collect the growth from the surface of a slant of inhibitory mold agar by washing the slant with sterile M-3 broth.
2. Adjust the optical density of the suspension at 530 nm to obtain a final concentration of 10^7 cells per ml, as shown in Table 7–16. Use 6 ml of sterile M-3 broth as the blanking solution.
3. Further dilute the inoculum suspension 1:100 with sterile M-3 broth.
4. Set up two sets of 12 test tubes (13 mm by 97 mm), each numbered 1 through 12, as shown in Table 7–17. One set is for the test organism; the other is for the control organism.
5. Incubate all tubes on a roller drum at a speed of 2 rpm for 24 hr at 30°C.
6. Examine the tubes for evidence of growth and report the lowest concentration of amphotericin B at which there is no visible growth.

Table 7–16. Optical Density Readings of Inocula of Specific Fungi for Antimicrobial Susceptibility Testing

Organism	Optical density
Candida albicans	0.20–0.25
Candida glabrata	0.15–0.20
Candida parapsilosis	0.30–0.35
Candida tropicalis	0.35–0.40
Cryptococcus neoformans	0.55–0.60
Saccharomyces cerevisiae	0.35–0.40
Filamentous fungi	0.45–0.55

Table 7–17. Protocol for Preparing Dilutions of Amphotericin B

Tube	M-3 broth (ml)	Amphotericin B (ml)		Adjusted inoculum (ml)	Amphotericin B concentration (μg/ml)
		10 μg/ml	1 μg/ml		
1	0.4	0.5	—	0.1	5
2	0.5	0.4	—	0.1	4
3	0.6	0.3	—	0.1	3
4	0.7	0.2	—	0.1	2
5	0.8	0.1	—	0.1	1
6	0.1	—	0.8	0.1	0.8
7	0.3	—	0.6	0.1	0.6
8	0.5	—	0.4	0.1	0.4
9	0.7	—	0.2	0.1	0.2
10	0.8	—	0.1	0.1	0.1
11[a]	0.8	—	—	0.1	0
12[b]	0.8	—	—	0.1	0

[a] DMSO control tube: 0.1 ml of a 1:5 solution of DMSO is added to this tube.
[b] Growth control tube: 0.1 ml of sterile deionized water is added to this tube.

Interpretation:
The lowest concentration of amphotericin B at which there is no visible growth is reported as the MIC.

Controls:
Saccharomyces cerevisiae, ATCC 9763. The MIC is 0.2–0.4 μg/ml for the control organism. Growth should be apparent in the antibiotic-free media.

Flucytosine Susceptibility Test[4,35,36]

Purpose:
To determine the minimal inhibitory concentration (MIC) of flucytosine for a specific fungal isolate.

Principle:
As previously described for amphotericin B (p. 492).

Specimen:
A 24- to 48-hr-old subculture of the organism grown on yeast nitrogen base agar supplemented with dextrose and asparagine. Yeasts are more suitable for susceptibility testing since the inoculum size of the filamentous fungi is difficult to quantitate.

Reagents:

Yeast Nitrogen Base–Dextrose–Asparagine Agar for Subcultures

Yeast nitrogen base (Difco Laboratories)	6.7 g
L-Asparagine (Difco Laboratories)	1.5 g
Dextrose (Difco Laboratories)	10 g
Distilled water	100 ml

Mix, filter sterilize, and add to a cooled sterile solution of 900 ml of distilled water containing 20 g of agar. Dispense into sterile screw-capped tubes and store prior to use as a subculture medium.

The agar solution may be dispensed in 9-ml amounts into test tubes, sterilized at 120°C for 15 min and kept refrigerated prior to use. At the time of use, 1 ml of the yeast nitrogen base–dextrose–asparagine broth may be added to a melted but cooled (50°C) tube of the molten agar. The tubes are slanted for use in subculturing test organisms prior to testing.

10 × Yeast Nitrogen Base Broth

Yeast nitrogen base (Difco Laboratories)	6.7 g
Distilled water	100 ml

Filter sterilize and store in a sterile container at 4°C until use.

1.5% L-Asparagine Solution

L-Asparagine (Difco Laboratories)	1.5 g
Distilled water	100 ml

Filter sterilize and store in a sterile container at 4°C until use.

Phosphate Buffered Saline (PBS)

Solution A:	
$NaH_2PO_4 \cdot H_2O$ (J. T. Baker Chemical Co.)	27.61 g
Distilled water	1000 ml
Solution B:	
Na_2HPO_4 (J. T. Baker Chemical Co.)	28.34 g
Distilled water	1000 ml

Add 80 ml of solution A to 140 ml of solution B. Add 8.5 g NaCl (J. T. Baker Chemical Co.) and adjust the volume to 1000 ml in a volumetric flask. Adjust the pH of the solution to 7.0 and sterilize for 15 min at 121°C.

Flucytosine Solution

1. Dissolve 160 mg of flucytosine (Roche Laboratories) in 100 ml distilled water and filter sterilize.
2. Dispense 6 ml amounts into sterile tubes and freeze at −32°C until use. Discard the unused antibiotic solution after 6 months.

Procedure:

Preparation of Inoculum

1. Collect the growth from the surface of a slant of the yeast nitrogen base–dextrose–asparagine agar by washing the slant with 6 ml of sterile PBS. Adjust the optical density at 600 nm of the suspension to obtain a final concentration of 10^7 cells per ml, as shown using Table 7–16. Use 6 ml of PBS as the blanking solution.
2. Further dilute the inoculum suspension 1:1000 with sterile PBS, transfer 9 ml to the test medium described below, and mix well before use.

Preparation of Test Medium
The following amounts of ingredients are necessary for each organism to be tested.

Yeast nitrogen base (10×)	9 ml
Dextrose (10%)	9 ml
L-Asparagine (1.5%)	9 ml
Distilled water (sterile)	36 ml
1:1000 dilution of the inoculum in PBS	9 ml

Performance of the Test

1. Prepare serial twofold dilutions by adding 5 ml of the stock antibiotic solution to 5 ml of sterile distilled water. The resulting stock solutions will have concentrations as shown in column 3, Table 7–18. Add 1 ml of the flucytosine stock solution to tubes containing 4 ml of the yeast nitrogen base–dextrose–asparagine test medium containing the inoculum. Final concentrations of the antibiotic are shown in column 5, Table 7–18.
2. Incubate at 32°C for 48 hr on a roller drum, 2 rpm.
3. Examine the tubes for evidence of growth and report the lowest concentration of flucytosine at which there is no visible growth.

Interpretation:
The concentration of flucytosine at which there is no visible growth is reported as the MIC.

Table 7-18. Protocol for Preparing Dilutions of Flucytosine (5-Fluorocytosine)

Tube	YNB and inoculum (ml)	Flucytosine stock concentration (mg/ml)	Stock flucytosine (ml)	Final flucytosine concentration (μg/ml)
1	4	800	1	160
2	4	400	1	80
3	4	200	1	40
4	4	100	1	20
5	4	50	1	10
6	4	25	1	5
7	4	12.5	1	2.5
8	4	6.25	1	1.25
9	4	3.12	1	0.63
10	4	1.56	1	0.31
11	4	0.78	1	0.16
12	4	0.39	1	0.08
13	4	0.185	1	0.04
14	4	0.092	1	0.02
15	4	0.046	1	0.01
16	4	0	0[a]	0

[a] Control tube: 1 ml deionized water added to this tube.

Controls:
Saccharomyces cerevisiae, ATCC 9763. The MIC is 0.02 μg/ml for the control organism. Growth should be apparent in the antibiotic-free media.

Alternate Methods:
Microbroth dilution[7,18] and disk-diffusion[43] methods have been developed for susceptibility testing of flucytosine.

References

1. Adams, E. D., Jr., and Cooper, B. H. Evaluation of a modified Wickerham medium for identification of medically important yeasts. *Am. J. Med. Technol.* **40**:377, 1974.
2. Ajello, L., and Georg, L. K. *In vitro* cultures for differentiating between atypical isolates of *Trichophyton mentagrophytes* and *Trichophyton rubrum. Mycol. Appl.* 8:3, 1957.
3. Bardana, E. J., Gerber, J. D., Craig, S., and Cianiulli, F. D. The general and specific humoral immune response to pulmonary aspergillosis. *Am. Rev. Resp. Dis.* **112**:799, 1975.

4. Block, E. R., Jennings, A. E., and Bennett, J. E. Variables influencing susceptibility testing of *Cryptococcus neoformans* to 5-fluorocytosine. *Antimicrob. Agents Chemother.* **4**:392, 1973.
5. Campbell, M. J., and Clayton, Y. M. Bronchopulmonary aspergillosis. *Am. Rev. Resp. Dis.* **89**:186, 1964.
6. Casals, J. B. Tablet sensitivity testing of pathogenic fungi. *J. Clin. Pathol.* **32**:719, 1979.
7. Ellis, N. S., Bartlett, M. D., and Smith, J. W. Assay for yeast susceptibility to 5-fluorocytosine and amphotericin B in a frozen microtiter system. *Am. J. Clin. Pathol.* **72**:194, 1979.
8. Fleming, W. H., III, Hopkins, J. M., and Land, G. A. New culture medium for the presumptive identification of *Candida albicans* and *Cryptococcus neoformans. J. Clin. Microbiol.* **5**:236, 1977.
9. Gerber, J. D., and Jones, R. D. Immunologic significance of *Aspergillus* antigens of six species of *Aspergillus* in the serodiagnosis of aspergillosis. *Am. Rev. Resp. Dis.* **108**:1124, 1973.
10. Goldberg, L., Kozinn, P. J., Wise, G. J., Nouri, N., and Brooks, R. B. Incidence and significance of candiduria. *J. Am. Med. Asso.* **241**:582, 1979.
11. Hopfer, R. L., Mills, K., Gröschel, D. Radiometric method for determining the susceptibility of yeasts to 5-fluorocytosine. *Antimicrob. Agents Chemother.* **15**:313, 1979.
12. Hopkins, J. M., and Land, G. A. Rapid method for determining nitrate utilization by yeasts. *J. Clin. Microbiol.* **5**:497, 1977.
13. Kaufman, C. S., and Merz, W. G. Two rapid pigmentation tests for the identification of *Cryptococcus neoformans. J. Clin. Microbiol.* **15**:339, 1982.
14. Kaufman, L., and Blumer, S. Value and interpretation of serological tests for the diagnosis of cryptococcosis. *Appl. Microbiol.* **16**:1907, 1968.
15. Kaufman, L., McLaughlin, D. W., Clarke, M. J., and Blumer, S. Specific immunodiffusion test for blastomycosis. *Appl. Microbiol.* **26**:244, 1973.
16. Kaufman, L., and Standard, P. Improved version of the exoantigen test for identification of *Coccidioides immitis* and *Histoplasma capsulatum* cultures. *J. Clin. Microbiol.* **8**:42, 1978.
17. Land, G. A., Vinton, E. C., Adcock, G. B., and Hopkins, J. M. Improved auxanographic method for yeast assimilations: A comparison with other approaches. *J. Clin. Microbiol.* **2**:206, 1975.
18. Mazens, M. F., Andrews, G. P., and Bartlett, R. C. Comparison of microdilution and broth dilution techniques for the susceptibility testing of yeasts to 5-fluorocytosine and amphotericin B. *Antimicrob. Agents Chemother.* **15**:475, 1979.
19. McDearman, S. C., and Young, J. M. The development of positive serologic tests with *Histoplasma capsulatum* antigens following single histoplasmin skin tests. *Am. J. Clin. Pathol.* **34**:434, 1960.
20. Merz, W. G., Evans, G. L., Shadomy, S., Anderson, S., Kaufman, L., Kozinn, P. J., MacKenzie, D. W. R., Protzman, W. P., and Remington, J. S. Laboratory evaluation of serological tests for systemic candidiasis: A cooperative study. *J. Clin. Microbiol.* **5**:596, 1977.
21. Mickelsen, P. A., McCarthy, L. A., and Propst, M. A. Further modifications of the auxanographic method for identification of yeasts. *J. Clin. Microbiol.* **5**:297, 1977.

22. Murray, P. R., Van Scoy, R. E., and Roberts, G. D. Should yeasts in respiratory secretions be identified? *Mayo Clin. Proc.* **52**:42, 1977.
23. Nielsen, H. S. Biological properties of skin test antigens of yeast from *Sporotrichum schenckii. J. Infect. Dis.* **118**:173, 1968.
24. Odds, F. C. *Candida and Candidosis.* Baltimore, MD, University Park Press, 1979, p. 55.
25. Paliwal, D. K., and Randhawa, H. S. Evaluation of a simplified *Guizotia abyssinica* seed medium for differentiation of *Cryptococcus neoformans. J. Clin. Microbiol.* **7**:346, 1978.
26. Palmer, D. F., Kaufman, L., Kaplan, W., and Calvallaro, J. J. *Serodiagnosis of Mycotic Diseases.* Springfield, IL, Charles C Thomas, 1977.
27. Philpot, C. The differentiation of *Trichophyton mentagrophytes* from *T. rubrum* by a simple urease test. *Sabouraudia* **5**:189, 1967.
28. Prevost, E., Newell, R.: Commercial cryptococcal latex kit: Clinical evaluation in a medical center hospital. *J. Clin. Microbiol.* **8**:529, 1978.
29. Rhodes, J. C., and Roberts, G. D. Comparison of four methods for determining nitrate utilization by cryptococci. *J. Clin. Microbiol.* **1**:9, 1975.
30. Roberts, G. D. Laboratory diagnosis of fungal infections. *Human Pathol.* **7**:161, 1976.
31. Roberts, G. D., Horstmeier, C. D., Land, G. A., and Foxworth, J. H. Rapid urea broth test for yeasts. *J. Clin. Microbiol.* **7**:584, 1978.
32. Roberts, G. D., and Larsh, H. W. The serologic diagnosis of extracutaneous sporotrichosis. *Am. J. Clin. Pathol.* **56**:597, 1971.
33. Roberts, R. C., Wenzel, F. J., and Emanuel, D. A. Precipitating antibodies in a midwest dairy farming population toward the antigens associated with farmer's lung disease. *J. Allerg. Clin. Immunol.* **57**:518, 1976.
34. Segal, E., and Ajello, L. Evaluation of a new system for the rapid identification of clinically important yeasts. *J. Clin. Microbiol.* **4**:157, 1976.
35. Shadomy, S. In vitro studies with 5-fluorocytosine. *App. Microbiol.* **17**:871, 1969.
36. Shadomy, S. Further *in vitro* studies with 5-fluorocytosine. *Infect. Immunol.* **2**:484, 1970.
37. Smith, C. D., and Goodman, N. L. Improved culture method for the isolation of *Histoplasma capsulatum* and *Blastomyces dermatitidis* from contaminated specimens. *Am. J. Clin. Pathol.* **63**:276, 1975.
38. Stockman, L., and Roberts, G. D. Specificity of the latex test for cryptococcal antigen: a rapid, simple method for eliminating interference factors. *J. Clin. Microbiol.* **17**:945, 1983.
39. Standard, P. G., and Kaufman, L. Specific immunological test for the rapid identification of members of the genus *Histoplasma. J. Clin. Microbiol.* **3**:191, 1976.
40. Standard, P. G., and Kaufman, L. Immunological procedure for the rapid and specific identification of *Coccidioides immitis. J. Clin. Microbiol.* **5**:149, 1977.
41. Standard, P. G., and Kaufman, L. Manual for the immunological identification of pathogenic fungus cultures. U.S. Department of Health, Education and Welfare, Public Health Service, Center for Disease Control, 1979.
42. Strimlan, C. V., Dines, D. E., Rogers-Sullivan, R. F., Roberts, G. D., and Sheehan, W. C. Respiratory tract aspergillus. *Minn. Med.* **63**:25, 1980.

43. Utz, C., and Shadomy, S. A diffusion disc susceptibility test for 5-fluorocytosine. *In* Williams, J. D., and Geddes, A. M. (eds.), *Chemotherapy,* Vol. 2. New York, Plenum, 1976, p. 81.
44. Utz, C. J., White, S., and Shadomy, S. New medium for *in vitro* susceptibility studies with amphotericin B. *Antimicrob. Agents Chemother.* **10**:776, 1976.
45. Weeks, R. J. A rapid simplified medium for converting the mycelial phase of *Blastomyces dermatitidis* to the yeast phase. *Mycopathol.* **22**:153, 1964.
46. Wolin, H. L., Bevis, M. L., and Laurora, N. An improved synthetic medium for the rapid production of chlamydospores by *Candida albicans. Sabouraudia* **2**:96, 1962.
47. Young, R. C., and Bennett, J. C. Invasive aspergillosis absence of detectable antibody response. *Am. Rev. Resp. Dis.* **104**:710, 1971.
48. Zimmer, B. L., and Roberts, G. D. Rapid selective urease test for presumptive identification of *Cryptococcus neoformans. J. Clin. Microbiol.* **10**:380, 1979.

General References for the Identification of Filamentous Fungi

Barnett, H. L., and Hunter, B. B. *Illustrated Genera of Imperfect Fungi.* Minneapolis, Burgess, 1972.

Barron, G. L. *The Genera of Hyphomycetes from Soil.* Baltimore, Williams and Wilkins, 1968.

Conant, N. F., Smith, D. T., Baker, R. D., and Callaway, J. L. *Manual of Clinical Mycology.* Philadelphia, Saunders, 1971.

Emmons, C. W., Binford, C. H., Utz, J. P., and Kwon-Chung, K. J. *Medical Mycology,* 3rd ed. Philadelphia, Lea and Febiger, 1977.

Gilman, J. C. *A Manual of Soil Fungi,* 2nd ed. Ames, Iowa State University Press, 1957.

Koneman, E. W., Roberts, G. D., and Wright, S. F. *Practical Laboratory Mycology.* Baltimore, Williams and Wilkins, 1978.

McGinnis, M. R. *Laboratory Handbook of Medical Mycology.* New York, Academic Press, 1980.

Raper, K. B., and Fennell, D. I. *The Genus Aspergillus.* Baltimore, Williams and Wilkins, 1965.

Rebell, G., and Taplin, D. *Dermatophytes, Their Recognition and Identification.* Coral Gables, FL, University of Miami Press, 1970.

Wilson, J. W., and Plunkett, O. A. *The Fungous Diseases of Man.* Berkeley, University of California Press, 1965.

8

Chlamydiae

Thomas F. Smith, Ph.D.

I. General Considerations

A. Description of Group

Agents belonging to the Chlamydiae are small (200 nm to 1500 nm in diameter), nonmotile, intracellular organisms. They share many properties of bacteria in that they (1) reproduce by binary fission, (2) contain both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), (3) possess enzymatic activities, (4) contain cell wall material, and (5) are susceptible to several antibiotics.[2] Although similar to viruses in their parasitism of eukaryotic cells, chlamydiae utilize their own ribosomes and enzymes for the synthesis of proteins and nucleic acids. In contrast to chlamydiae, rickettsiae are transmitted to humans by arthropod vectors, and are less dependent on the host for their energy requirements.

Chlamydiae belong to the order Chlamydiales, which contains one family, Chlamydiaceae, and one genus, *Chlamydia*, formerly called *Bedsonia*. Organisms within the genus *Chlamydia* are divided into two species (Table 8–1). *C. trachomatis*, which consists of 15 serotypes, is responsible for causing nongonococcal urethritis,[18,45,55] epididymitis,[4] and proctitis[27] in men and cervicitis[31] and pelvic inflammatory disease[5] in women, as well as trachoma, inclusion conjunctivitis in adults and children,[21] and inclusion blennorrhea and pneumonia[3] in infants. In addition, there are three serotypes which produce lymphogranuloma venereum (LGV). *C. psittaci* is thought to consist of two major serotypes, one causing an arthritis–conjunctivitis complex of disease and the other abortions in

Table 8-1. Classification of *Chlamydia*[a]

Species	Subgroup	Inclusions stained by iodine	Serotypes	Diseases
Chlamydia trachomatis	A	Yes	L1, L2, L3	Lymphogranuloma venereum
			A, B, Ba, C	Trachoma
			D, E, F, G, H, I, J, K	Inclusion conjunctivitis of neonates and adults, cervicitis, salpingitis, urethritis, epididymitis, proctitis, and pneumonia of newborns
Chlamydia psittaci	B	No		Psittacosis (ornithosis)

[a] Adapted from Schachter.[36]

Table 8-2. Tests Recommended for Diagnosis of Chlamydial Infections

Clinical manifestation	Specimen	Test method	Stain for culture confirmation
Urethritis	Urethral swab	Cell culture; IF,EIA[a]	Iodine, IF[b]
Epididymitis	Epididymal aspirate	Cell culture; IF,EIA[a]	Iodine, IF[b]
Proctitis	Rectal swab	Cell culture; IF,EIA[a]	Iodine, IF[b]
Cervicitis	Cervical swab	Cell culture; IF,EIA[a]	Iodine, IF[b]
Salpingitis	Swab, tubal biopsy	Cell culture; IF,EIA[a]	Iodine, IF[b]
Inclusion conjunctivitis, trachoma	Eye swab	Cell culture; IF,EIA[a]	Iodine, IF[b]
Infant pneumonitis	Throat swab	Cell culture; IF,EIA[a]	Iodine, IF[b]
	Serum	IFA	
Lymphogranuloma venereum	Bubo aspirate	Cell culture; IF,EIA[a]	Iodine, IF[b],IP[c]
	Serum	CF,IFA[d]	
Psittacosis	Sputum, lung tissue	Cell culture	Giemsa, IP[c]
	Serum	CF,IFA	

[a] IF, immunofluorescent stain for *C. trachomatis* inclusions in specimens (Direct Specimen Test, MicroTrak™, Syva Co., Palo Alto, CA); EIA, enzyme immunoassay for *C. trachomatis* antigen (Chlamydiazyme™, Abbott Laboratories, North Chicago, IL). *N.B.* Sensitivity of EIA relative to culture is approximately 80% and that of IF is 93%.

[b] Immunofluorescent stain for *C. trachomatis* inclusions in cell cultures. (Culture Confirmation Test, MicroTrak™, Syva Co., Palo Alto, CA)

[c] Immunoperoxidase stain for *Chlamydia* inclusions in cell culture (Cultureset™, Ortho Diagnostic Systems, Inc., Raritan NJ).

[d] CF, complement fixation; IFA, immunofluorescence test for *Chlamydia* antibody.

animals.[38] Lower respiratory infections with *C. psittaci* are acquired by airborne transmission from infected psittacine birds.

B. Source

Chlamydiae grow in columnar epithelial cells which are found in the conjunctiva, cervix, urethra, respiratory tract, and rectal mucosa. Thus, under appropriate epidemiologic conditions, chlamydiae could possibly be recovered from any area containing these cell types (Table 8–2).[37]

C. Clinical Importance

It has been clearly established that *C. trachomatis* is a major cause of sexually transmitted disease[41] and, more specifically, of nongonococcal urethritis.[18,29,33,55] While *C. trachomatis* has been recovered from 36 to 42% of individuals with urethritis, it is present in only 0 to 7% of sexually active individuals without disease[18,29,33] and in 11 to 25% of some asymptomatic high-risk populations.[30,45] *C. trachomatis* is a cause of proctitis in male homosexuals,[22,28] acute urethral syndrome in women,[46] and pelvic inflammatory disease,[25] as well as perihepatitis (Fitz-Hugh-Curtis syndrome), postpartum endometritis, and bartholinitis.[13,52,57] *C. trachomatis* has been shown to cause a distinctive pneumonia syndrome commonly associated with conjunctivitis in infants.[1,3] The infection is acquired by the neonate during passage through the birth canal. *C. trachomatis* may be present in the genital tract of as many as 13% of pregnant women. Of newborns at risk, about 35% develop conjunctivitis and 20% develop pneumonia. Based on this study, the incidence has been estimated to be 14 cases of conjunctivitis and 8 cases of pneumonia per 1000 live births.[40]

Trachoma, a chronic form of conjunctivitis recognized for several centuries, is one of the leading causes of preventable blindness in the world today. The disease is endemic in North Africa, the Middle East, and Southeast Asia, but it also exists in Australia, the Pacific Islands, and Latin America.[39]

II. Cultures for Chlamydia

A. General Considerations

Cell cultures are at least three to four times more sensitive than cultures in the embryonated hen's egg[9] for recovering *Chlamydia*. Although several different cell lines support the growth of *C. trachomatis,*[7] McCoy's

cells,[12,24] assumed to be L cells of mouse origin (ATCC CCL 1·2), have been used in the standard procedure.[37] For optimal detection of *C. trachomatis,* cells must be treated prior to inoculation by irradiation, cytochalasin B, or 5-iodo-2′-deoxyuridine. Alternatively, they can be treated following inoculation with cycloheximide, hydrocortisone, or emetine. Cycloheximide-treated cells have been found to be the most sensitive.[14] Depending upon specimen volume and equipment availability, the test can be adapted to Microtiter plates.[58]

B. McCoy's Cell Cultures

1. Preparation and Cell Transfer Procedures

a. McCoy's cell cultures are subcultured according to a schedule permitting slides to be inoculated within 72 hr of their preparation.
b. Medium is removed from two cell culture bottles; 0.5 ml of trypsin-versene solution is added to each bottle and distributed over the monolayer by gently rocking the bottle two to three times.
c. The trypsin-versene solution is removed *immediately* and replaced with 0.75 ml of the same solution.
d. The bottles are allowed to stand at 35°C for approximately 1½ min or at room temperature until the cells come off the glass surface.
e. Five milliliters of *Chlamydia* medium (p. 816) is added to each bottle and the cells dispersed by withdrawing and expelling the cell suspension (7 to 10 times) with a mechanical pipette.
f. The cells from each bottle are combined and mixed one to two times with a mechanical pipette. A 1:10 dilution in *Chlamydia* medium is made and the cells counted in a hemocytometer. Usually, the total count is approximately 4 to 5 million cells/ml. For transfer of the counted cells to vials for subsequent inoculation of specimens, the cells are diluted in *Chlamydia* medium.
g. The cells (1 ml suspension) are planted onto 12 mm round glass coverslips (Carolina Biological Supply Co.) in sterile 1-dram shell vials (Arthur H. Thomas, Co.) which are closed with a #0 rubber stopper (Bellco Glass, Inc.). Vials are planted with 125,000, 185,000, or 250,000 cells depending on how long they are to be incubated (i.e., 24, 48, or 72 hr, respectively) before they are inoculated with specimens.
h. Part of the cell suspension from (f) is diluted with *Chlamydia* medium so as to contain 2.5 to 5 million cells/15 to 20 ml of medium. Fifteen milliliters of this cell suspension is then added to new cell culture bottles (as in 1,a).

2. Inoculation of Cell Cultures

The medium is removed from the vials after the cell cultures have incubated at 35°C for 40 to 48 hr. Aliquots (0.1 ml) for each 2SP specimen

extract (Tables 3–6 and 3–10) are inoculated into each of four vials containing monolayers of McCoy's cells growing on coverslips. The vials are centrifuged at 2000 rpm (700 × *g*) for 1 hr. One milliliter of *Chlamydia* medium containing 1 μg of cycloheximide (Sigma Chemical Co.) per ml is then added to each vial. The cell cultures are then incubated at 35°C for 40 to 72 hr.

3. Growth Characteristics

a. *C. trachomatis*

Chlamydial infection starts with the attachment of the infectious elementary body to specific receptor sites on the plasma membrane of the cell. The elementary body enters the cell's cytoplasm by phagocytosis, and forms a larger, metabolically active (vegetative state), noninfectious reticulate or initial body within the first few hours of the multiplication cycle. The initial body divides by binary fission and condenses to an infectious elementary body 24 to 30 hr after infection (Figure 8–1).[8,20]

b. *C. psittaci*

Mice, embryonated eggs, and irradiated McCoy's cells are equally sensitive for culturing this species; however, its detection in cell cultures, in contrast to *C. trachomatis,* requires 7 to 10 days.[32]

4. Tests for Identification

a. General Considerations

Cell cultures have until recently been the simplest and most sensitive method for the laboratory diagnosis of *C. trachomatis* infection. Detection of iodine-stained glycogen-containing inclusions at 100 × magnification has been simple and rapid because of the contrast between the mahogany-colored inclusions and the pale-yellow background of uninfected cells. The Giemsa stain requires substantial experience to interpret accurately and hence is probably not applicable to most diagnostic facilities. Furthermore, if Giemsa-stained cells are not examined with the oil immersion objective (1000×), many inclusions may be missed. False-positive interpretations may also occur by confusing artifacts and cellular structures with inclusions. Nevertheless, the Giemsa stain will detect chlamydial infections produced by both subgroups A and B (Table 8–2).

As alternatives to the iodine and Giemsa stains, monoclonal antibodies are available for the detection of chlamydial inclusions using the culture confirmation immunofluorescence (Syva Co., Palo Alto, CA) or immunoperoxidase (Ortho Diagnostic Systems, Inc., Raritan, NJ) procedures.

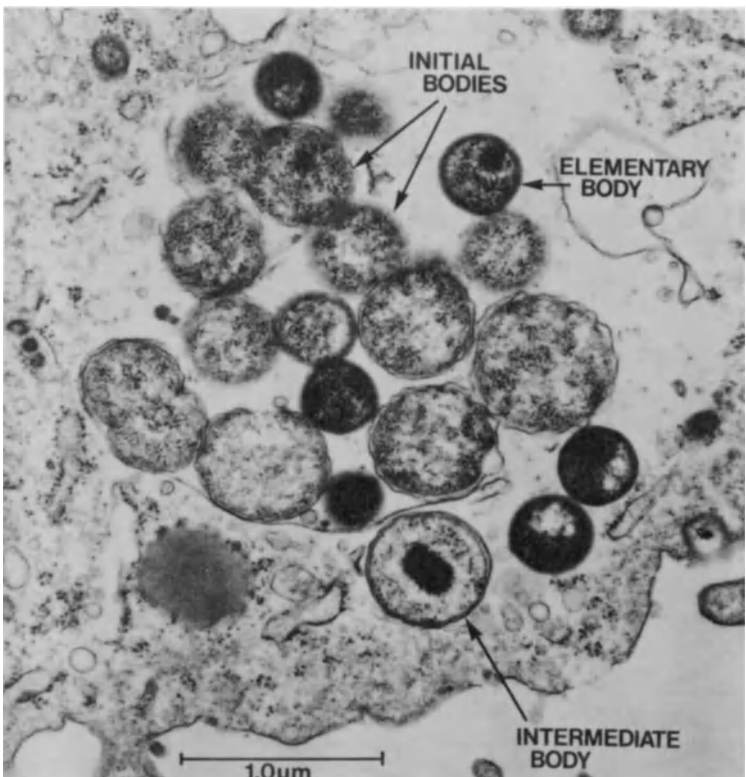


Figure 8–1. Electron micrograph of ultrathin section through a microcolony of *Chlamydia psittaci* organisms in the cytoplasm of a McCoy cell at 48 hr after infection. The various developmental forms of *Chlamydia* are *labeled* (×41,000). (From Cutlip[8])

One study, using a Microtiter plate system, reported that 98% of the positive chlamydial specimens were detected by the fluorescent monoclonal antibody compared to only 62% with the iodine stain.[47] However, a study in our laboratory indicated no difference in sensitivity between the two methods.[56] We found the fluorescence-labeled monoclonal antibody to be of very high quality with no interfering background fluorescence in infected cells. Furthermore, the time required for examination of infected cell monolayers was significantly reduced from that required for the iodine stain. In contrast to the Syva fluorescence reagent, the monoclonal antibody developed by Ortho for use in the immunoperoxidase procedure, reacts with both *C. trachomatis* and *C. psittaci.*

Immunologic tests for detecting chlamydial antigens or elementary bodies in specimens provide results within 1 to 4 hours. For the Chlamydiazyme™ (Abbott Laboratories, North Chicago, IL) assay, a swab specimen is extracted into a Chlamydiazyme Specimen Storage Reagent and

Table 8–3. Application of Diagnostic Tests for Chlamydial Infections[a]

				Inclusion conjunctivitis			
Diagnostic test[b]	Psittacosis	LGV	Trachoma	Adult	Newborn	Urethritis	Cervicitis
A. Cytology							
Iodine	−	−	±	±	+	−	−
Giemsa	−	−	++	+	++	±	+
B. Direct detection							
IF	−	−	−	−	++	++	++
EIA	−	−	−	−	++	++	++
C. Serology (IFA)	++	++	−	−	++	−	−
D. Isolation cell culture	++	++	++	++	++	++	++

[a] −, not useful; ±, rarely successful, usually not worth performing; +, often successful; ++, a useful technique.
[b] Abbreviations as in footnotes for Table 8–2.

then sent to the laboratory for enzyme immunoassay to detect antigen. We found the sensitivity and specificity of this test to be 81% and 98%, respectively. The test's high negative predictive value (95%) can be helpful in patient management in ruling out *C. trachomatis* infection.

The Direct Specimen Test (MicroTrak™, Syva Co., Palo Alto, CA) is a fluorescence assay that allows laboratories with only occasional requests for diagnosis of *C. trachomatis* infection to provide a very rapid result. For this assay, cells collected with a swab are placed on a slide, fixed with acetone included in the collection package, and then sent to the laboratory for detection of elementary bodies. Accurate results with this test depend on strict adherence to the specific staining qualities of the chlamydial elementary bodies as described by the manufacturer. A comparison of this direct test with cultures stained with iodine on specimens from 926 patients demonstrated a sensitivity of 93% and a specificity of 96%.[48]

The relative sensitivity of the laboratory tests indicate that although cell culture methodology more frequently detects chlamydial infections than do the immunologic tests, results are not available until 48 hr after receipt of the specimen (Table 8–3). Direct detection methods may thus eventually supplant the more tedious and slow cell culture methodology.

The recognition of cytologic changes of cells obtained by cervical scrapings after Papanicolaou staining was reported by Gupta et al., but culture proof of these infections was obtained in only 4 of 160 (2.5%) cases.[17] In a study at the Mayo Clinic, only 14 of 37 culture-positive patients had cytologic evidence of *C. trachomatis.* Of 450 culture-negative patients, 21 had cytologic findings suggesting chlamydial infection. Thus, of the 35 cases with suggestive cytologic findings (14 culture-positive, 21 culture-negative), only 14 (40%) were confirmed by culture. Therefore, because of the high rate of false-positive cytologic results, Papanicolaou-stained smear results suggestive for *C. trachomatis* should only serve to identify those women who should have specimens obtained for culture of the organism.[10]

b. *C. trachomatis*

Iodine Stain

Purpose:
To detect intracytoplasmic glycogen inclusions produced by *C. trachomatis.*

Principle:
C. trachomatis, but not *C. psittaci,* produces an intracytoplasmic glycogen inclusion, which when stained with iodine has a mahogany color (Figure

8–2). The McCoy's cell cultures are stained with iodine solution between 40 and 72 hr following inoculation. Fixing the cells with alcohol-formalin is not necessary, and the iodine solution can be added directly to the cells after removing the fluid medium containing cycloheximide.[42] After mounting the stained coverslip with iodine-glycerin solution on a glass microscope slide, the cells are scanned microscopically (100×) and examined, when a structure resembling an inclusion is seen, with a high dry objective (430×). The timing of staining the cells for serotypes causing lymphogranuloma venereum (LGV) can be critical in that there may be a rapid increase and disappearance of the glycogen inclusion (in 24 to 48 hr), whereas the inclusions associated with other serotypes of *C. trachomatis* (not LGV), can first be demonstrated after 48 hr of incubation and remain stainable through 96 hr.[15] The laboratory diagnosis of LGV can be facilitated when the laboratory knows that the diagnosis is suspected clinically. In such instances, staining can be performed 24 or 36 hr after inoculation of cell cultures.

Specimen:
McCoy cell cultures on coverslips.

Reagents:

Iodine Solution

Potassium iodide	5 g
Iodine crystals	5 g
Ethanol, 95%	50 ml
Distilled water, to make	100 ml

Add the iodine to the alcohol; then add potassium iodide and distilled water to make a final volume of 100 ml. Filter twice through two pieces of Whatman #1 filter paper. Dispense into brown bottles and store at room temperature. Expiration date is 6 months.

Jone's Iodine-Glycerin
Mix equal amounts of iodine solution and glycerin. Store in a brown bottle at room temperature. Expiration date is 6 months.

Procedure:

1. Remove the fluid medium from the vial.
2. Stain by adding 0.5 ml of iodine solution for 10 min.
3. Remove the coverslip from the vial and mount on a glass microscope slide with iodine-glycerin.
4. Blot excess mounting fluid and seal the edges of the coverslip.
5. Examine microscopically with the 10× objective. Suspected inclusions are examined at 430× magnification.

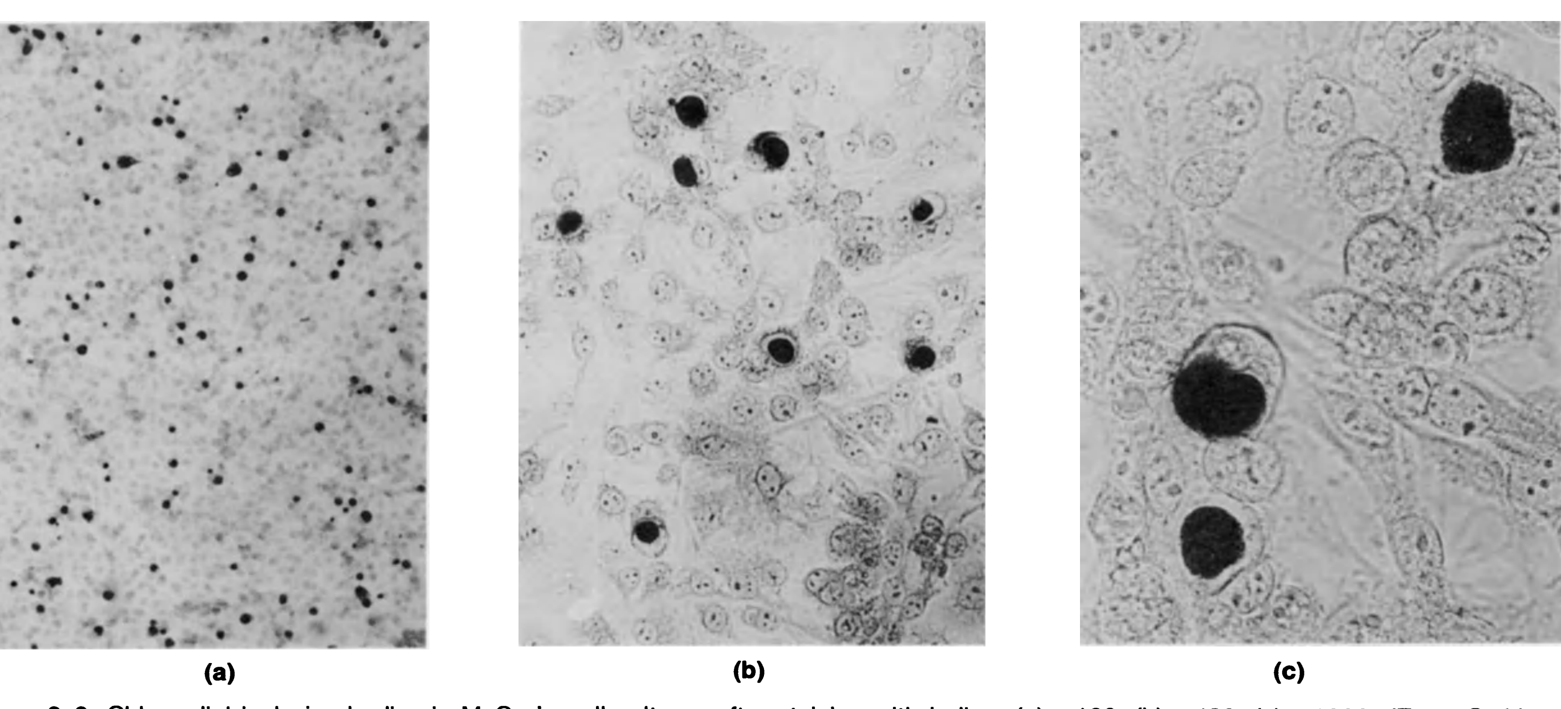


Figure 8–2. Chlamydial inclusion bodies in McCoy's cell cultures after staining with iodine. (a) ×100; (b) ×430; (c) ×1000. (From Smith et al.[44])

Interpretation:
Positive: Mahogany colored intracytoplasmic inclusions (Figure 8–2).
Negative: No intracytoplasmic inclusions.

---

c. *C. psittaci*

Organisms belonging to this species do not synthesize glycogen in infected cells. Thus, intracytoplasmic inclusions must be detected by stains other than iodine, such as Giemsa.

5. Serology

a. Immunofluorescence Test for Detection of Antibodies

A sensitive and specific microimmunofluorescence (MIF) test for detecting antibody to *C. trachomatis* was described by Wang and Grayston and is used in several major chlamydial research laboratories.[53] This test is performed using chlamydial organisms grown in the yolk sac of embryonated eggs. Suspensions of each serotype are placed on a slide in a specific pattern using separate pinpoints for each antigen. Up to nine clusters of antigen dots can be placed on each side. Serial dilutions of sera from patients are placed on the individual dots preparatory to assay with the indirect fluorescent antibody technique. However, due to the difficulty in preparation of the multiple immunotype antigens, the procedure is not practical for clinical laboratories. In addition, despite modifications to simplify the test by pooling antigens of related immunotypes,[54] the MIF was still considered to be too complicated to study large numbers of specimens.[35] Further, sera from patients with genital tract infections were found to cross-react widely with a range of serotypes,[49] a finding that lessens the epidemiological value of the test. A broadly reacting single antigen MIF test with purified reticulate bodies (RB) has been described that has sensitivity equal to that of the multiple antigen MIF tests and may be more applicable to clinical laboratories.[59]

Alternatives to the MIF test are single-antigen immunofluorescence (IF) procedures for detecting antibody by using cells infected with one strain of *C. trachomatis*.[32,34] For clinical laboratories, the IF test has several advantages over the MIF method: the inclusion is a more convenient size for detection in a fluorescence test than individual elementary bodies; antigen slides can be prepared from cell cultures without the use of fertilized hen's eggs; and only one antigen need be propagated in cell cultures. The single-antigen IF test has been used extensively to determine whether the etiology of pneumonia in infants is *C. trachomatis*.[3,50]

Serologic techniques are likely to be used more commonly for the

diagnosis of *C. trachomatis* infections with the introduction of enzyme immunoassays (EIA). The sensitivity and reproducibility of an EIA using soluble antigen from the D strain of *C. trachomatis* was found to be comparable to those of the MIF test.[11] IgM and IgG antibodies have been demonstrated, although the results of IgM EIA did not correlate well with those of the MIF.[16,26]

b. Immunofluorescence (IF) Test for Antibodies

Purpose:
To detect antibodies in serum from individuals infected with *Chlamydia.*

Principle:
Serum containing antibodies to *Chlamydia* react with LGV-1 infected cells. Chlamydial antibodies are detected by the indirect fluorescent antibody technique.

Specimens:
Two serum specimens are preferred: one obtained early in the course of disease and another 2 to 3 weeks later.

Reagents and Materials:

1. Phosphate-buffered saline (PBS), pH 7.2.
2. Fluorescein isothiocyanate (FITC)–labeled anti-human globulin (Hyland, Division Travenol Laboratories, Inc.).
3. Slides with LGV-1 infected McCoy's cells.*
   a. McCoy's cell monolayers growing on coverslips in 1-dram shell vials are infected with a LGV-1, strain 440 suspension (obtained from M. O. Beem, M.D., Univ. of Chicago) to produce 1000 to 2000 inclusions per coverslip.
   b. The cells are removed with a trypsin-versene solution after 24 hr incubation at 35°C.
   c. The cell suspension is centrifuged for 5 min. at 300 × *g*. The sedimented cells are resuspended in MEM to a concentration of 500,000 cells/ml.
   d. Each of 8 wells or circles on a slide (Roboz Surgical Instrument Co., Ind., W-800—8 circles, frosted end, 5 mm i.d. each) are seeded with 0.025 ml of the LGV-1 infected McCoy's cells.
   e. The slides are incubated at 35°C for 8 hr in a moist chamber.
   f. The slides are rinsed with PBS (pH 7.2) and fixed in cold acetone for 10 min. The cells on one slide are stained with iodine to confirm

* Commercially available from Bion Enterprises, Inc., Park Ridge, IL.

the presence of at least 30 to 40 inclusions per high-power field (430×).

g. Slides containing the same number of uninfected cells are processed as described above for controls.

Procedure:
Sera from patients are diluted 1:4 and then in fourfold increments. The indirect immunofluorescence (IF) test is performed as described on p. 268.

With sera containing antibodies to *Chlamydia,* inclusions are discrete, brightly fluorescing bodies within the cytoplasm of infected cells. These are easily detected against a background of nonfluorescing, uninfected cells (Figure 8–3, see color insert in the center of the book). The highest dilution with faint but definite fluorescence, mainly around the inside margin of the inclusion, is the titer reported. The distinctive morphology of the inclusions provides a check on the specificity of the fluorescence in the test system.[32]

Controls:

Positive control: Serum specimens known to contain antibodies to *Chlamydia.*
Negative control: Serum specimen without antibodies to *Chlamydia.*

---

6. Antibiotic Susceptibility

Most strains are inhibited by tetracycline (0.01 to 1 μg/ml) and erythromycin (0.1 to 1 μg/ml).[19,23] Rosaramicin, a macrolide antibiotic, has been found to be more active against *C. trachomatis* than tetracycline or erythromycin[43] (Table 8–4). Tetracycline hydrochloride or doxycycline

Table 8–4. Antimicrobial Susceptibility of *Chlamydia trachomatis*[a]

Drug	MIC[b] (μg/ml)
Tetracycline	0.01 to 0.5
Erythromycin	0.01 to 1.0
Sulfamethoxazole	0.5
Rifampin	0.25
Rosaramicin	0.01 to 0.1
Penicillin	0.02 to 50

[a] Data from Johannison et al.,[9] Kuo et al.,[23] Smith and Washton,[43] and Treharne et al.[51]
[b] MIC, minimal inhibitory concentration.

is the drug of choice for uncomplicated urethral, endocervical, and rectal chlamydial infections in adults; however, because of the frequent concurrence of gonococcal infection, therapy must include agents active against both organisms.[6]

References

1. Arth, C., Von Schmidt, B., Grossman, M., and Schachter, J. Chlamydial pneumonitis. *J. Pediatr.* **93**:447, 1978.
2. Becker, Y. The Chlamydia: Molecular biology of procaryotic obligate parasites of eucaryocytes. *Microbiol. Rev.* **42**:274, 1978.
3. Beem, M. O., and Saxon, E. M. Respiratory-tract colonization and a distinctive pneumonia syndrome in infants infected with *Chlamydia trachomatis. N. Engl. J. Med.* **296**:306, 1977.
4. Berger, R. E., Alexander, E. R., Monda, G. D., Ansell, J., McCormick, G., and Holmes, K. K. *Chlamydia trachomatis* as a cause of acute "idiopathic" epididymitis. *N. Engl. J. Med.* **298**:301, 1978.
5. Bowie, W. R., and Jones, H. Acute Pelvic inflammatory disease in outpatients: association with *Chlamydia trachomatis* and *Neisseria gonorrhoeae. Ann. Intern. Med.* **95**:685, 1981.
6. Centers for Disease Control. Sexually transmitted diseases treatment guidelines. *Morb. and Mort. Weekly Rpt.* (Suppl) **31**:33S, 1982.
7. Croy, T. R., Kuo, C.-C., and Wang, S.-P. Comparative susceptibility of eleven mammalian cell lines to infection with trachoma organisms. *J. Clin. Microbiol.* **1**:434, 1975.
8. Cutlip, R. C. Electron microscopy of cell cultures infected with a chlamydial agent causing polyarthritis of lambs. *Infect. Immun.* **1**:499, 1970.
9. Dawson, C. R., and Schachter, J. Tric agent infections of the eye and genital tract. *Am. J. Ophthalmol.* **63**:1288, 1967.
10. Dorman, S. A., Danos, L. M., Wilson, D. J., Noller, K. L., Malkasian, G. D., Goellner, J. R., and Smith, T. F. Detection of chlamydial cervicitis by Papanicolaou stained smears and culture. *Am. J. Clin. Pathol.* **79**:421, 1983.
11. Duc-Goiran, P., Raymond, J., Leaute, J. B., and Orfila, J. Use of the enzyme-linked immunosorbent assay for detection of antibodies to *Chlamydia trachomatis. Eur. J. Clin. Microbiol.* **2**:32, 1983.
12. Dunlop, E. M. C., Jones, B. R., and Al-Hussaini, M. K. Genital infection in association with TRIC virus infection of the eye. III. Clinical and other findings: Preliminary report. *Br. J. Vener. Dis.* **40**:33, 1964.
13. Eschenbach, D., Pollock, H. M., Schachter, J., and Rubin, S. J. Laboratory diagnosis of female genital tract infections. *Cumitech* **17**:1, 1983.
14. Evans, R. T., and Taylor-Robinson, D. Comparison of various McCoy cell treatment procedures used for detection of *Chlamydia trachomatis. J. Clin. Microbiol.* **10**:198, 1979.
15. Fan, V. S. C., and Jenkin, H. M. Glycogen metabolism in chlamydia-infected HeLa cells. *J. Bacteriol.* **104**:608, 1970.
16. Finn, M. P., Ohlin, A., and Schachter, J. Enzyme-linked immunosorbent assay

for immunoglobulin G and M antibodies to *Chlamydia trachomatis* in human sera. *J. Clin. Microbiol.* **17**:848, 1983.

17. Gupta, P. K., Lee, E. F., Erozan, Y. S., Frost, J. K., Geddes, S. T., and Donaovan, P. A. Cytologic investigations in chlamydia infection. *Acta. Cytol.* **23**:315, 1979.
18. Holmes, K. K., Handsfield, H. H., Wang, S.-P., Wentworth, B., Turck, M., Anderson, J. B., and Alexander, E. R. Etiology of nongonococcal urethritis. *N. Engl. J. Med.* **292**:1199, 1975.
19. Johannisson, G., Sernryd, A., and Lycke, E. Susceptibility of *Chlamydia trachomatis* to antibiotics in vitro and in vivo. *Sex. Transm. Dis.* **6**:50, 1979.
20. Johnson, J. E., and Smith, T. F. Comparison of *Chlamydia* subgroup A detection from clinical specimens after 40 and 64 hours of incubation in 5-iodo-2-deoxyuridine-treated McCoy's cells. *J. Clin. Microbiol.* **3**:334, 1976.
21. Johnson, J. E., Taraska, S. P., Hable-Rhodes, K., Kleinberg, F., and Smith, T. F. Inclusion blennorrhea. *Mayo Clin. Proc.* **51**:574, 1976.
22. Klotz, S. A., Drutz, D. J., Tam, M. R., and Reed, K. H. Hemorrhagic proctitis due to lymphogranuloma venereum serogroup L2. *N. Engl. J. Med.* **308**:1563, 1983.
23. Kuo, C.-C., Wang, S.-P. and Grayston, J. T. Antimicrobial activity of several antibiotics and a sulfonamide against *Chlamydia trachomatis* organisms in cell culture. *Antimicrob. Agents Chemother.* **12**:80, 1977.
24. Lewis, V. J., and Thacker, W. L. Susceptibility of McCoy's cells to infection by *Chlamydia psittaci. Can J. Microbiol.* **19**:617, 1973.
25. Mårdh, P.-A., Moller, B. R., and Paavonen, J. Chlamydial infection of the female genital tract with emphasis on pelvic inflammatory disease. A review of Scandinavian studies. *Sex. Transm. Dis.* (Suppl) **8**:140, 1981.
26. Mahony, J. B., Schachter, J., and Chernesky, M. A. Detection of antichlamydial immunoglobulin G and M antibodies by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **18**:270, 1983.
27. Quinn, T. C., Goodell, S. E., Mkrtichian, E., Schuffler, M. D., Wang, S.-P., Stamm, W. E., and Holmes, K. K. *Chlamydia trachomatis* proctitis. *N. Engl. J. Med.* **305**:195, 1981.
28. Quinn, T. C., Stamm, W. E., Goodell, S. E., Mkrtrichian, E., Benedetti, J., Corey, L., Schuffler, M. D., and Holmes, K. K. The polymicrobial origin of intestinal infections in homosexual men. *N. Engl. J. Med.* **309**:576, 1983.
29. Oriel, J. D., Reeve, P., Parvis, P., Miller, A., and Nicol, C. S. Chlamydial infection. Isolation of *Chlamydia* from patients with non-specific genital infection. *Br. J. Vener, Dis.* **48**:429, 1972.
30. Podgore, J. K., Holmes, K. K., and Alexander, E. R. Asymptomatic urethral infections due to *Chlamydia trachomatis* in male U.S. military personnel. *J. Infect. Dis.* **146**:828, 1982.
31. Rees, E., Tait, I. A., Hobson, D., and Johnson, F. W. A. Chlamydia in relation to cervical infection and pelvic inflammatory disease. *In* Hobson, D., and Holmes, K. K. (eds.), *Nongonococcal Urethritis and Related Infections.* Washington, D.C., American Society for Microbiology, 1977.
32. Richmond, S. J., and Caul, E. O. Fluorescent antibody studies in chlamydial infections. *J. Clin. Microbiol.* **1**:345, 1975.
33. Richmond, S. J., Hilton, A. L., and Clarke, S. K. Chlamydial infection. Role

of *Chlamydia* subgroup A in nongonococcal and postgonococcal urethritis. *Br. J. Vener. Dis.* **48**:437, 1972.
34. Saikku, P., and Paavonen, J. Single-antigen immunofluorescence test for chlamydial antibodies. *J. Clin. Microbiol.* **8**:119, 1978.
35. Schachter, J. Chlamydiae. *In* Rose, N. R., and Friedman, H. (eds.), *Manual of Clinical Immunology.* Washington, D.C., American Society for Microbiology, 1976, P. 494.
36. Schachter, J. Chlamydial infections (First of three parts). *N. Engl. J. Med.* **298**:428, 1978.
37. Schachter, J. Chlamydial infections (Third of three parts). *N. Engl. J. Med.* **298**:540, 1978.
38. Schachter, J., Banks, J., Sugg, N., Sung, M., Storz, J., and Meyer, K. F. Serotyping of *Chlamydia:* Isolates of bovine origin. *Infect. Immun.* **11**:904, 1975.
39. Schachter, J., and Dawson, C. R. Human chlamydial infections. Littleton, Massachusetts, PSG Publishing, 1978.
40. Schachter, J., Grossman, M., Holb, J., Sweet, R., Goodner, E., and Mills, J. Prospective study of chlamydial infection in neonates. Lancet **2**:377, 1979.
41. Schachter, J., Hanna, L., Hill, E. C., Massad, S., Sheppard, C. W., Conte, J. E., Cohen, S. N., and Meyer, K. F. Are chlamydial infections the most prevalent venereal disease? *J. Am. Med. Asso.* **231**:1252, 1975.
42. Smith, T. F., Brown, S. D., and Weed, L. A. Diagnosis of *Chlamydia trachomatis* infections by cell cultures and serology. *Lab. Med.* **13**:92, 1982.
43. Smith, T. F., and Washton, H. E. In vitro susceptibility of 30 strains of *Chlamydia trachomatis* to rosamicin. *Antimicrob. Agents Chemother.* **14**:493, 1978.
44. Smith, T. F., Weed, L. A., Segura, J. W., Pettersen, G. R., and Washington, J. A., II. Isolation of *Chlamydia* from patients with urethritis. *Mayo Clin. Proc.* **50**:105, 1975.
45. Stamm, W. E., Koutsky, L. A., Benedetti, J. K., Jourden, J. L., Brunham, R. C., and Holmes, K. K. *Chlamydia trachomatis* urethral infections in men. *Ann. Intern. Med.* **100**:47, 1984.
46. Stamm, W. E., Running, K., McKevitt, M., Counts, G. W., Turck, M., and Holmes, K. K. Treatment of the acute urethral syndrome. *N. Engl. J. Med.* **304**:956, 1981.
47. Stamm, W. E., Tam, M., Koester, M., and Cles, L. Detection of *Chlamydia trachomatis* inclusions in McCoy cell cultures with fluorescein-conjugated monoclonal antibodies. *J. Clin. Microbiol.* **17**:666, 1983.
48. Tam, M. R., Stamm, W. E., Handsfield, H. H., Stephens, R., Kuo, C.-C., Holmes, K. K., Ditzenberger, K., Krieger, M., and Nowinski, R. C. Culture-independent diagnosis of *Chlamydia trachomatis* using monoclonal antibodies. *N. Engl. J. Med.* **310**:1146, 1984.
49. Thomas, B. J., Reeve, P., and Oriel, J. D. Simplified serological test for antibodies to *Chlamydia trachomatis. J. Clin. Microbiol.* **4**:6, 1976.
50. Tipple, M. A., Beem, M. O., and Saxon, E. M. Clinical characteristics of the afebrile pneumonia associated with *Chlamydia trachomatis* infection in infants less than 6 months of age. *Pediatrics* **63**:192, 1979.
51. Treharne, J. D., Day, J., Yeo, C. K., Jones, B. R., and Squires, S. Susceptibility of Chlamydiae to chemotherapeutic agents. *In* Hobson, D., Holmes, K. K. (eds.), *Nongonococcal Urethritis and Related Infections.* Washington, D.C., American Society for Microbiology, 1977, p. 214.

52. Wang, S.-P., Eschenbach, D. A., Holmes, K. K., Wager, G., and Grayston, J. T. *Chlamydia trachomatis* infection in Fitz-Hugh-Curtis syndrome. *Am. J. Obstet. Gynecol.* **138**:1034, 1980.
53. Wang, S.-P., and Grayston, J. T. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. *Am. J. Ophthalmol.* **70**:367, 1970.
54. Wang, S.-P., Grayston, J. T., Alexander, E. R., and Holmes, K. K. Simplified microimmunofluorescence test with trachoma-lymphogranuloma venereum (*Chlamydia trachomatis*) antigens for use as a screening test for antibody. *J. Clin. Microbiol.* **1**:250, 1975.
55. Weed, L. A., Smith, T. F., Pettersen, G. R., and Segura, J. W. Urethritis associated with *Chlamydia. Minn. Med.* **59**:228, 1976.
56. Wilson, D. J., Smith, T. F., and Ilstrup, D. M. Comparison of iodine- and fluorescein-labeled monoclonal antibodies for detection of *Chlamydia trachomatis* inclusions in cells grown in glass vials. *Diag. Microbiol. Infect. Dis.* **2**:17, 1984.
57. Wolner-Hanssen, P., Mårdh, P.-A., Moller, B., and Westrom, L. Endometrial infection in women with chlamydial salpingitis. *Sex. Transm. Dis.* **9**:84, 1982.
58. Yoder, B. L., Stamm, W. E., Koester, C. M., and Alexander, R. Microtest procedure for isolation of *Chlamydia trachomatis. J. Clin. Microbiol.* **13**:1036, 1981.
59. Yong, E. C., Chinn, J. S., Caldwell, H. D., and Kuo, C.-C. Reticulate bodies as single antigen in *Chlamydia trachomatis* serology with microimmunofluorescence. *J. Clin. Microbiol.* **10**:351, 1979.

9

Mycoplasmas

Thomas F. Smith, Ph.D.

I. General Considerations

A. Description of Group

The mycoplasmas belong to the class Mollicutes comprising three distinct families, Mycoplasmataceae, Acholeplasmataceae, and Spiroplasmataceae, and four genera, *Mycoplasma* (over 60 species), *Acholeplasma* (eight species), *Spiroplasma* (four species), and *Ureaplasma* (one or two species).[21,45] There are at least 14 serovars of *Ureaplasma*.[45] In contrast to other bacteria, mycoplasmas are small organisms (0.3 to 0.8 μm in diameter and up to 150 μm in length) and contain no cell wall. Unlike cell wall deficient L-forms or protoplasts, which may revert to their parent bacterium, mycoplasmas have no other form. Characteristic colonies resembling a fried egg develop on solid agar medium as the result of growth of the central portion of the colony into the agar and a surrounding light area of surface growth. Exceptions are colonies of *Mycoplasma pneumoniae,* which usually lack the halo of surface growth and have a finely granular appearance.[20] With the exception of *Acholeplasma,* all large colony mycoplasmas require sterol for growth. *Ureaplasma urealyticum* (T-strain *Mycoplasma*) are small spherical organisms averaging 330 nm in diameter (size range: 100 nm to 850 nm). These organisms are the smallest known free-living organisms and form minute colonies (15 μm to 60 μm in diameter) on agar adjusted to pH 6.0.

B. Source

Mycoplasma pneumoniae is primarily a respiratory pathogen involving the nasopharynx, throat, trachea, bronchi, bronchioles, and alveoli.[10] *Ureaplasma urealyticum* is commonly found with increasing age and sexual activity in the male and female genital tracts. Other mycoplasmas may occur, especially in the upper respiratory tract; however, they are regarded as part of the normal microbial flora of the oral cavity (Table 9–1) and are not etiologically associated with clinical symptoms.[37]

C. Clinical Importance

Mycoplasma pneumoniae produces respiratory tract disease, ranging from minor febrile illness, with or without pharyngitis, to severe bronchitis and pneumonia, most commonly in children and young adults (ages 2 to 19 years).[32] Other clinical manifestations which have been associated with infection by this organism[10,26,33] include those of the cardiovascular,[26,29] dermatologic,[5,7] and central nervous systems.[24] Long-term surveillance studies of infants and children have shown that 74% of infections with *M. pneumoniae* are asymptomatic and that reinfection may occur.[13] Naturally acquired immunity to infection with *M. pneumoniae* appears to be of limited duration (2 to 3 years).[15] These findings suggest that more severe disease, more common about the age of 10 years, may be a manifestation of an immune response to reinfection with the organism.[13]

It has been difficult to draw firm conclusions regarding the role of *U. urealyticum* in causing non- or postgonococcal urethritis (NGU) because of the frequency of urethral and vaginal colonization by *U. urealyticum* in sexually promiscuous, though asymptomatic, populations and because of the recognized association between this disease and *Chlamydia trachomatis*.[44] Bowie et al.[2] treated men with NGU in a randomized, double-blind study with either sulfisoxazole (active against *C. trachomatis* but not *U. urealyticum*) or an aminocyclitol (active against *U. urealyticum*

Table 9–1. Usual Habitat of Large Colony *Mycoplasma* Species Isolated from Humans

Species	Source
M. pneumoniae	Oropharynx, lung
M. orale (*M. pharyngis*)	Oropharynx
M. buccale (*M. orale* II)	Oropharynx
M. faucium (*M. orale* III)	Oropharynx
M. salivarium	Oropharynx
M. hominis	Genitourinary tract, oropharynx
M. fermentans	Genitourinary tract

but not *C. trachomatis*). Aminocyclitol therapy produced a clinical response in men with ureaplasmal but not chlamydial urethritis, while men with chlamydial but not ureaplasmal urethritis responded to sulfisoxazole. These data provided circumstantial evidence for the etiological role of both *C. trachomatis* and *U. urealyticum* in urethritis. Additional support for a pathogenic role for *U. urealyticum* was derived from other therapeutic trials,[1] as well as from studies of antibody levels,[4] postgonococcal urethritis,[3] and experimental inoculations.[46]

The role of *U. urealyticum* as a cause of infertility continues to be controversial,[6,19] probably owing to many factors, including lack of properly controlled studies, different laboratory procedures used for isolation of the organisms, variations in prevalence of certain strains of *Ureaplasma*, and selection of patients.[47,48] Studies of the effects of *Ureaplasma* during pregnancy on fetal outcome have also yielded divergent results.[25,34] Specific strain markers will be needed to resolve these problems.

Mycoplasma hominis is primarily a sexually transmitted organism that colonizes the vagina of women and the urethra of men.[30] The organism has been recovered from a number of sites of the urogenital tract in adults, central nervous system, and respiratory tract of neonates.[28] *M. hominis* may also cause arthritis, skin ulcers, conjunctivitis, and bloodstream invasion in immunocompromised patients.[31] Nevertheless, because of the high frequency of colonization of *M. hominis* in individuals without any disease manifestations (similar to *U. urealyticum*), the etiologic role of this agent has also been difficult to assess.[16]

II. Identification

A. General Considerations

1. Large Colony Mycoplasmas

Although cells can be observed by dark field or phase contrast microscopy of broth cultures, identification of mycoplasmas requires isolation of the organism on a complex agar selective medium, usually containing penicillin, thallium acetate, and amphotericin B. Among the mycoplasmas, *M. pneumoniae* has several unique characteristics, including fermentation of glucose, optimal growth aerobically, reduction of tetrazolium salts, and hemadsorption of guinea pig erythrocytes by colonies on agar (Table 9–2).

2. *Ureaplasma urealyticum*

In addition to their small colony size and their inhibition by thallium acetate, these organisms can be distinguished from other mycoplasmas by their ability to hydrolyze area. Other urease-producing microorgan-

Table 9–2. Properties of *Mycoplasma* Species Isolated from Humans

Species	Glucose fermented	Slow growth (aerobic incubation)	Tetrazolium reduced aerobically	Guinea pig erythrocytes hemadsorbed	Guinea pig erythrocytes hemolyzed (β-hemolysis)	Growth on 0.002% methylene blue agar	Arginine hydrolyzed
M. pneumoniae	+	+	+	+	+	+	−
M. orale (*M. pharyngis*)	−	−	−	−	−	−	+
M. buccale (*M. orale* II)	−	−	−	−	−	−	+
M. faucium (*M. orale* III)	−	−	−	−	−	−	+
M. salivarium	−	−	−	−	−	−	+
M. hominis	−	+	−	−	−	−	+
M. fermentans	+	−	−	−	−	−	+

isms will not grow on *Mycoplasma* agar selective medium containing penicillin and amphotericin B.

B. Growth Characteristics

1. Large Colony Mycoplasmas

Maximum yield of *M. pneumoniae* isolates is obtained by inoculation of both *Mycoplasma* selective agar plates and extraction of swabs in diphasic broth.[9] Because of the slow growth of some strains of *M. pneumoniae*, two subcultures are made at 4 to 5 day intervals. The primary transfer is made with 0.1 ml from the diphasic broth culture after incubation for 4 to 6 days at 35°C to *Mycoplasma* selective agar (Table 3–7). The secondary transfer is made 8 to 12 days after the first transfer. The agar plates are sealed with tape to prevent desiccation and incubated at 35°C in air. The diphasic broth cultures are incubated at 35°C for 30 days before being discarded as negative. Growth of *M. pneumoniae* in the diphasic broth tubes is indicated by the presence of faint turbidity or a change in the phenol red indicator in the medium from red to yellow, indicating acidity due to the fermentation of glucose. Plates are observed for at least 10 days after the second subculture before being discarded as negative.

Agar plates are inverted and examined microscopically (50×) twice weekly for the presence of typical colonies of *M. pneumoniae* which have a greenish golden hue, a regular spherical shape (10 μm to 100 μm in diameter), fine surface granularity imparting a berrylike appearance, and little or no colored surface fringe surrounding a dense colony core (Figure 9–1). Typically, growth is slow, and a minimum of 5 to 7 days is required usually before colonies can be observed.

Modified New York City (NYC) medium, consisting of a phosphate-buffered proteose peptone–cornstarch agar base supplemented with serum, yeast dialysate, dextrose, and antibiotics, has been reported to provide more isolates and more rapid growth of *M. pneumoniae* than Hayflick medium.[17,18,20] These results have not been confirmed in our laboratory.[11]

Colonies of *Mycoplasma* species other than *M. pneumoniae* appear in 1 to 3 days and are typified by the classic "fried egg" appearance. These colonies are often larger than those of *M. pneumoniae* (Figure 9–2). Artifacts or "pseudocolonies," closely resembling colonies of *Mycoplasma*, may occur on the surface of the agar and apparently arise from components of the medium such as serum (Figure 9–3).

2. *Ureaplasma urealyticum*

Specimens are extracted in 1.8 ml of U-9B broth (original extract) (Table 3–10). A 10^{-1} dilution of this extract is made by transferring 0.2 ml to

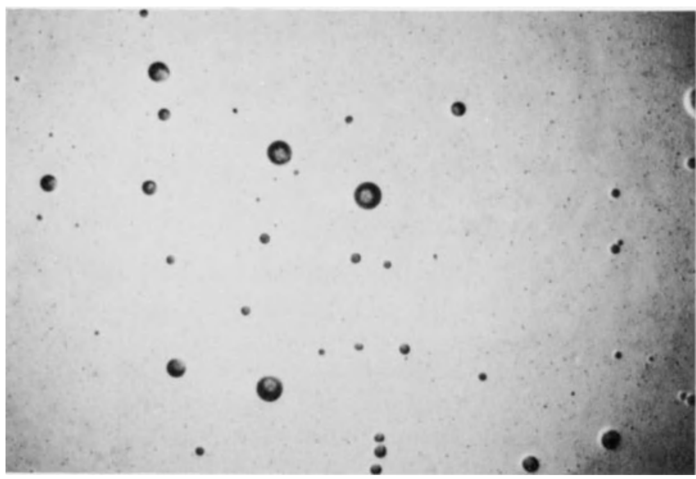


Figure 9–1. Colonial morphology of *Mycoplasma pneumoniae* on agar medium (×50).

a vial containing 1.8 ml of U-9B broth.[39,40,41] Both the original extract and the 10^{-1} dilution of that extract are incubated at 35°C and observed for a pH change from acid to alkaline, indicating hydrolysis of urea with the release of ammonia. When *U. urealyticum* is present, the medium will usually become alkaline within 12 to 24 hr. No turbidity should be observed. Since *Ureaplasma* can be inactivated under alkaline conditions, subculture should be made within 4 hr after the pH change has been observed in the broth. If inactivation should occur, a frozen (−70°C)

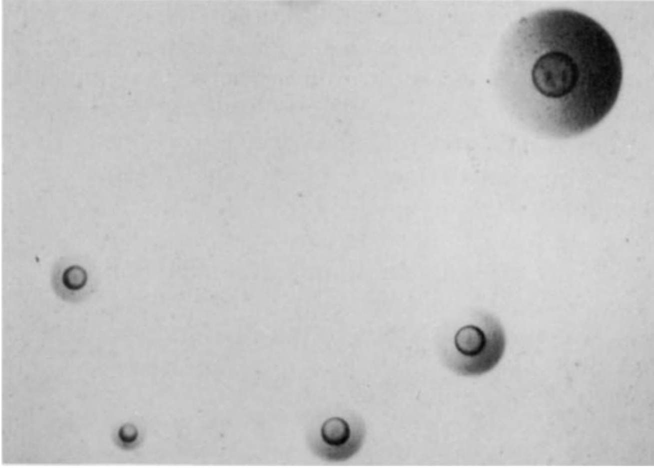


Figure 9–2. Colonial morphology of *Mycoplasma salivarium* on agar medium (×50).

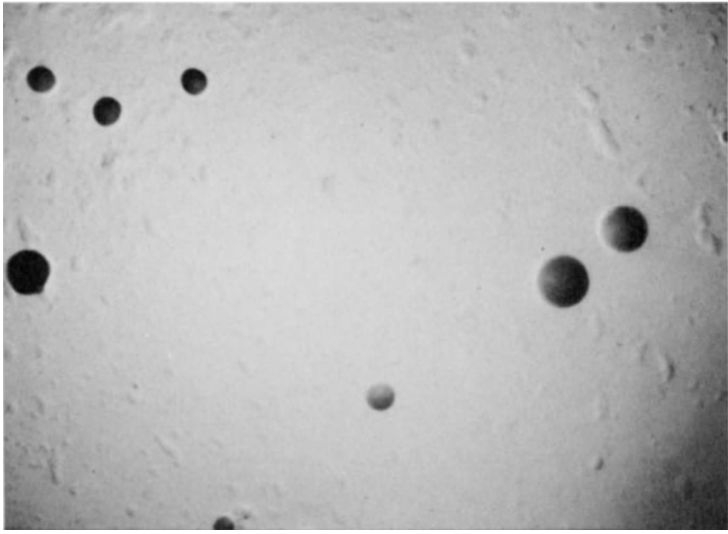


Figure 9–3. "Pseudocolonies" resembling *Mycoplasma* colonies on agar medium (×50).

aliquot of the original extract may be thawed and used for isolation of the organism.

When either the original extract or its 10^{-1} dilution have produced an alkaline shift in U-9B broth, 0.1 ml of the broth is spread over the surface of an A-7B agar plate and a 1:10 dilution of the original extract in a A-7B broth is spread on another A-7B agar plate.[38] (If the U-9B broth with the original extract and its 10^{-1} dilution have both become

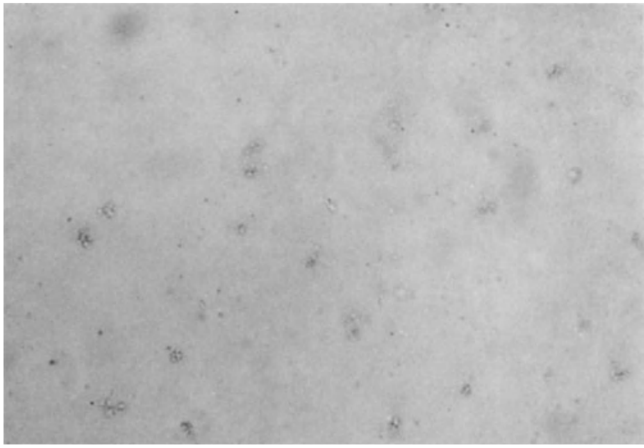


Figure 9–4. Colonial morphology of *Ureaplasma urealyticum* on agar medium (×50).

alkaline, only the 10^{-1} dilution is subcultured.) Incubate the A-7B agar plates at 35°C in an anaerobic jar (GasPak, BBL Microbiology Systems).

U. urealyticum colonies appear within 12 to 36 hr and are very small (15 μm to 60 μm in diameter), although colony size is inversely proportional to the degree of crowding. The colonies are generally circular but irregular in outline, diffuse, and grow into the agar medium[14] (Figure 9–4). *U. urealyticum* colonies will be surrounded by a red zone in agar containing urea and phenol red.[48]

C. Tests for Identification

1. *M. pneumoniae*

Hemadsorption[27]

Purpose:
To distinguish between hemadsorbing colonies of *M. pneumoniae* and nonpathogenic large colony mycoplasmas which are common inhabitants of the upper respiratory tract.

Principle:
Guinea pig erythrocytes will attach to colonies of *M. pneumoniae* growing on agar. This property is unique for *M. pneumoniae* among the mycoplasmas that infect man, although other strains of *Mycoplasma* that infect animals and fowl also have this property.

Specimen:
Isolated (but not crowded) colonies on agar plate incubated for 4 to 9 days.

Reagent:
A 0.4% suspension of washed guinea pig erythrocytes in *Mycoplasma* broth. Erythrocytes are stable for 1 week.

Procedure:

1. Flood the agar plate with 3 ml of the erythrocyte suspension.
2. Incubate the plate at 35°C for 30 min; rock the plate occasionally to redistribute the erythrocytes over the surface of the agar.
3. Remove unadsorbed erythrocytes by washing the agar surface three times with *Mycoplasma* broth (2 to 3 ml/wash) by agitating and rotating the plate with each wash to remove loose cells.
4. Examine colonies at 50× to 100× magnification.

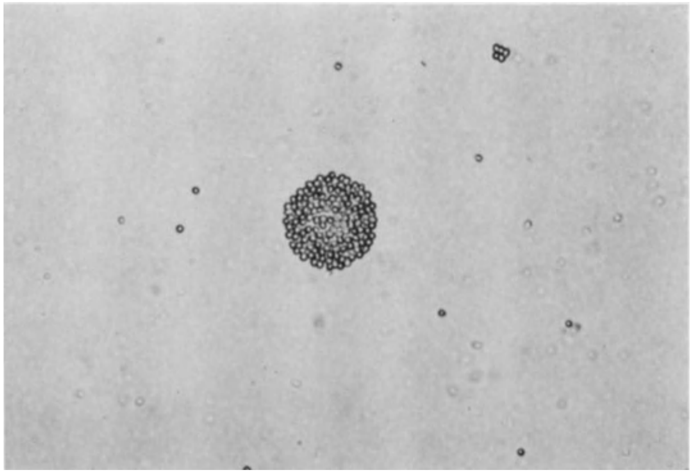


Figure 9–5. Hemadsorption of guinea pig erythrocytes on surface of colony of *Mycoplasma pneumoniae* (×200).

Interpretation:

Positive test: Hemadsorption (Figure 9–5).
Negative test: No hemadsorption.

Controls:

Positive control: *Mycoplasma pneumoniae*
Negative control: *Mycoplasma* species

Comments:
Closely spaced colonies of *M. pneumoniae* and those requiring ≥10 days for growth may yield false-negative results.

Tetrazolium Reduction[22,49]

Purpose:
To distinguish between colonies of *M. pneumoniae*, which reduce tetrazolium under aerobic conditions, and the nonpathogenic large-colony *Mycoplasma* species, which do not.

Principle:
M. pneumoniae reduces the colorless compound 2-(*p*-iodophenyl)-3-nitrophenyl-5-phenyl tetrazolium chloride (INT) to formozan, which is red.

Specimen:
Isolated colonies on agar plate.

Reagent:

2-(*p*-Iodophenyl)-3-nitrophenyl-5-phenyl, tetrazolium chloride (Aldrich Chemical Co.)	0.21 g
Distilled water	100 ml

Sterilize by filtration or by autoclaving. Cover reagent bottle with foil and store at room temperature in the dark. Reagent is stable for 3 years.

Procedure:

1. Flood the agar surface with 2 ml of the tetrazolium chloride solution.
2. Incubate the plate for 15 to 60 min at 35°C.
3. Examine colonies at 50× to 100× magnification.

Interpretation:

Positive test: *M. pneumoniae* colonies will darken, becoming pink after 15 min and purple to black after 3 to 4 hr (Figure 9–6).

Negative test: Colonies remain unstained.

Controls:

Positive control: *Mycoplasma pneumoniae*
Negative control: *Mycoplasma* species

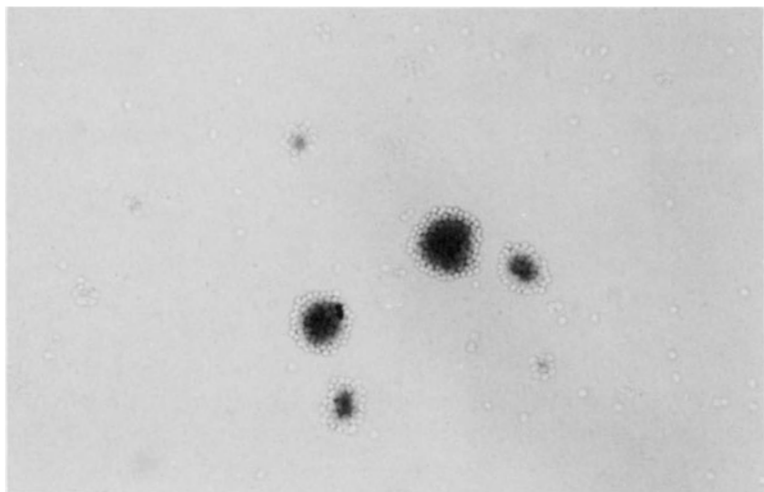


Figure 9–6. *Mycoplasma pneumoniae* colonies on agar medium after hemadsorption of guinea pig erythrocytes and reaction with tetrazolium (×100).

Comments:
Crowding of colonies does not influence the results of the test. The iodonitro derivative (INT) of 2,3,5-triphenyltetrazolium chloride (TTC) should be used because it is more rapidly stably reduced than the parent compound (TTC).

The results of the two tests for *M. pneumoniae*, hemadsorption and tetrazolium reduction, are comparable. However, if both tests are used, the tetrazolium test can be performed after hemadsorption on the same agar plate.

M. pneumoniae has also been identified by a growth inhibition test on agar plates with paper disks impregnated with dried antiserum;[42] however, this test requires subculture of the organism and examination in 4 to 6 days to obtain results. Moreover, the concentration of colonies is critical, several control strains of *Mycoplasma* are required, and the antiserum-impregnated disks remain active for only a few months. Correlation between the tetrazolium reduction and hemadsorption tests with the disk inhibition test is excellent.

2. *Ureaplasma urealyticum*

Manganous Chloride-Urea[41]

Purpose:
To distinguish between colonies of *U. urealyticum* and those of classical, large colony mycoplasmas.

Principle:
The urease activity of *U. urealyticum* is detected following the addition of a solution of $MnCl_2$ to the agar by the formation of a dark, golden-brown color due to the deposition of manganese dioxide on colonies of *U. urealyticum*.

The reaction is summarized as follows:

$$2\ NH_3 + 2\ H_2O \rightleftharpoons 2\ NH_4OH \rightarrow 2\ NH_4^+ + 2\ OH^-$$
$$MnCl_2 + 2\ OH^- \rightarrow MnO_2 + 2\ HCl$$

Specimen:
Isolated colonies on agar plate.

Reagent:

Urea (Schwarz/Mann)	1.0 g
$MnCl_2$ (Fisher Scientific Co.)	0.8 g
Distilled water	100 ml

Sterilize by filtration, dispense in 4 ml aliquots and freeze at −20°C. Discard reagent after use. Expiration date is 1 year.

Procedure:

1. Flood the plate with 2–3 ml of the $MnCl_2$ solution.
2. After 1 to 5 min at room temperature, examine the colonies at 50× to 100× magnification.

Interpretation:

Positive test: *U. urealyticum* colonies are stained a dark, golden-brown color (Figure 9–7).
Negative test: Colonies remain unstained.

Controls:

Positive control: *Ureaplasma pneumoniae*
Negative control: *Mycoplasma hominis*

Comments:
The $MnCl_2$ solution must be made fresh daily and discarded after 1 day's use or stored frozen (−20°C) in aliquots sufficient for single tests. Strains of Proteeae and other urealytic bacteria are completely unreactive in this direct test for urease.

It is important that the urease test be applied to young colonies. The

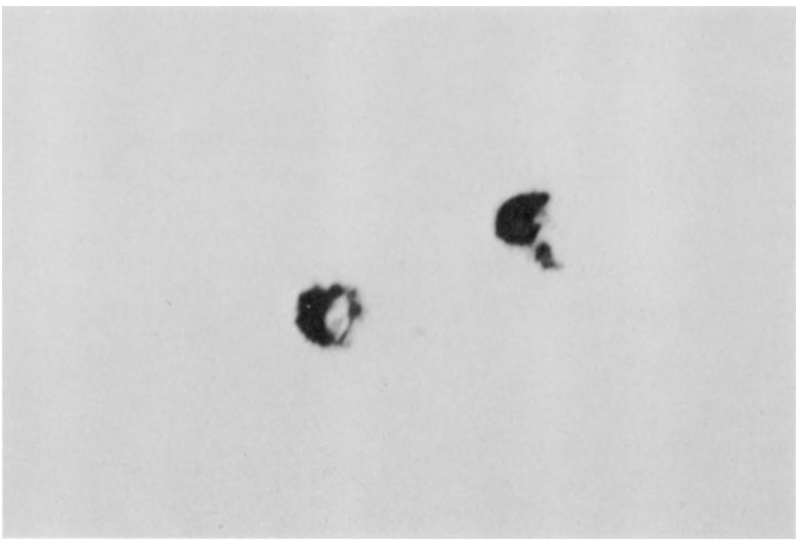


Figure 9–7. *Ureaplasma urealyticum* colonies on agar medium after reaction with manganous chloride (×100).

test may be unreliable if applied to colonies that have been incubated for greater than 48 hr at 35°C.

D. Serology

Serological tests for the diagnosis of *M. pneumoniae* infections are important since the organism is fastidious in its growth requirements and requires a minimum of 4 to 6 days to be detected on agar. *M. pneumoniae* is generally recovered in about 75% of the instances in which the complement-fixation (CF) test indicates that infection has occurred.[12]

The CF test is more sensitive and specific than the cold agglutinin test. Cold agglutinin titers of ≥1:32 occur in only about 50% of the patients infected with *M. pneumoniae.* Furthermore, cold agglutinins occur in response to a number of viral, chlamydial, bacterial, and protozoan infections. The metabolic-inhibition test is both sensitive and specific for measuring antibody responses to *M. pneumoniae,* but antibiotics in patients' sera may produce false positive results.[43]

A fourfold rise in complement-fixing antibody between acute and convalescent sera indicates infection; however, a single titer of 1:64 has been considered highly suggestive, while a titer of 1:128 has been considered diagnostic of recent *M. pneumoniae* infection by some investigators.[8] Conversely, low or undetectable levels of antibody in serum specimens from patients with respiratory infections of 1 to 2 weeks' duration suggest that other etiological agents may be involved.

1. Complement Fixation Test (CF)

The CF test is performed by the microtiter adaptation of the Laboratory Branch Complement Fixation test. The method for extracting the lipid antigen from *M. pneumoniae* for use in the test is described below.[23] Antigen may be obtained commercially (Flow Laboratories, McLean, VA, or M.A. Bioproducts, Walkersville, MO).

a. Inoculate *M. pneumoniae* into 200 ml of complete *Mycoplasma* broth with methylene blue and phenol red.
b. Incubate at 35°C until the medium becomes acidic (indicated by a dark green color).
c. Transfer 50 ml of the actively growing culture to approximately 350 ml of *Mycoplasma* broth (as in 1,a above).
d. Incubate at 35°C with continuous stirring.
e. After adequate growth has occurred, centrifuge (at 34,800 × *g*, or approximately 20,000 rpm in a Beckman Model L preparative ultracentrifuge) the suspension for 30 min; wash the sedimented pellet

twice with 0.02 *M* phosphate-buffered saline (PBS), pH 7.2, and then resuspend in 4 ml of PBS.

f. Extract the liquid antigen with 150 ml of chloroform–methanol (2:1) and vigorous shaking in a separatory funnel. Add 37.5 ml of 0.1 *M* KCl, shake the mixture again, and then chill to 4°C to accelerate separation of the heavier chloroform phase, containing the lipid antigen, from the aqueous methanol phase.
g. With a flash evaporator (Buchler Instruments), take the chloroform phase to dryness; suspend the residue in 4 ml of 95% ethyl alcohol.
h. Add the lipid antigen (one part) *slowly* to three parts of the 5% bovine albumin in PBS. Store at −70°C.

2. Cold Agglutinin Test

Cold agglutinin determinations are performed with human group O erythrocytes, according to a method described elsewhere.[36] Briefly, human group O erythrocytes are mixed with dilutions of serum and incubated for 1 hr at 4°C. The cold agglutinin titer is recognized as the highest dilution of serum which produces a uniform layer of erythrocytes coating the bottom of the test tubes. The next highest dilution of serum (no agglutination) should contain a compact button of cells at the bottom of the tube.

E. Antimicrobial Susceptibility

1. *M. pneumoniae*

Minimal inhibitory concentrations (MIC) for several antibiotics are: erythromycin, <0.1 μg/ml; tetracycline, 1 μg/ml; clindamycin, 3.1 μg/ml; kanamycin, 5 μg/ml; chloramphenicol, 10μg/ml; and streptomycin, 10 μg/ml. Penicillin, cephalothin, bacitracin, and polymyxins are completely ineffective.[26] Erythromycin-resistant strains have been reported.[35]

2. *Ureaplasma urealyticum*

Most strains are inhibited by <1 μg/ml of tetracycline and 1.25 μg/ml of erythromycin. About 75% of these organisms have an MIC of ≤2.5 μg/ml of gentamicin.

References

1. Bowie, W. R. Etiology and treatment of nongonococcal urethritis. *Sex. Transm. Dis.* 5:27, 1978.
2. Bowie, W. R., Alexander, E. R., Floyd, J. F., Holmes, J., Miller, Y., and Holmes,

K. K. Differential response of chlamydial and ureaplasma-associated urethritis to sulphafurazole (sulfisoxazole) and aminocyclitols. *Lancet* **2**:1276, 1976.

3. Bowie, W. R., Alexander, E. R., and Holmes, K. K. Etiologies of postgonococcal urethritis in homosexual and heterosexual men: Roles of *Chlamydia trachomatis* and *Ureaplasma urealyticum*. *Sex. Transm. Dis.* **5**:151, 1978.
4. Bowie, W. R., Wang, S.-P., Alexander, E. R., Floyd, J., Forsyth, P. S., Pollock, H. M., Lin, J.-S. L., Buchanan, T. M., and Holmes, K. K. Etiology of nongonococcal urethritis. *J. Clin. Invest.* **59**:735, 1977.
5. Brettle, R. P., Gray, J. A., Sangster, G., Murdoch, J. McC., and Dick, H. M. The mucocutaneous syndromes—Erythema multiforme, Stevens-Johnson and Ectodermosis erosiva pluriorificialis. *J. Infect.* **4**:149, 1982.
6. Cassell, G. H., Younger, J. B., Brown, M. B., Blackwell, R. E., Davis, J. K., Marriott, P., and Stagno, S. Microbiologic study of infertile women at the time of diagnostic laparoscopy. *N. Engl. J. Med.* **308**:502, 1983.
7. Cherry, J. D., Hurwitz, E. S., and Welliver, R. C. *Mycoplasma pneumoniae* infections and exanthems. *J. Pediatr.* **59**:369, 1975.
8. Cockcroft, D. W., and Stilwell, G. A. Lobar pneumonia caused by *Mycoplasma pneumoniae. Can. Med. Assoc. J.* **124**:1463, 1981.
9. Craven, R. B., Wenzel, R. P., Calhoun, A. M., Hendley, J. O., Hamory, B. H., Gwattney, J. M., Jr. Comparison of the sensitivity of two methods for isolation of *Mycoplasma pneumoniae. J. Clin. Microbial.* **4**:225, 1976.
10. Denny, F. W., Clyde, W. A., Jr., and Glezen, W. P. *Mycoplasma pneumoniae* disease: Clinical spectrum, pathophysiology, epidemiology, and control. *J. Infect. Dis.* **123**:74, 1971.
11. Dorman, S. A., Wilson, D. J., and Smith, T. F. Comparison of growth of *Mycoplasma pneumoniae* on modified New York City and Hayflick media. *Am. J. Clin. Pathol.* **79**:235, 1983.
12. Evans, A., Allen, V., and Sveltmann, S. *Mycoplasma pneumoniae* infection in University of Wisconsin students. *Am. Rev. Resp. Dis.* **96**:237, 1967.
13. Fernald, G. W., Collier, A. M., and Clyde, W. A., Jr. Respiratory infections due to *Mycoplasma pneumoniae* in infants and children. *Pediatrics* **55**:327, 1975.
14. Furness, G. T-mycoplasmas: Some factors affecting their growth, colonial morphology, and assay on agar. *J. Infect. Dis.* **128**:703, 1973.
15. Foy, H. M., Kenny, G. E., Sefi, R., Ochs, H. D., and Allan, I. D. Second attacks of pneumonia due to *Mycoplasma pneumoniae. J. Infect. Dis.* **135**:673, 1977.
16. Freundt, E. A. *Mycoplasma hominis:* Historical outline and taxonomy. *Sex. Transm. Dis.* **10**:226, 1983.
17. Granato, P. A., Poe, L., and Weiner, L. B. Use of modified New York City medium for growth of *Mycoplasma pneumoniae. Am. J. Clin. Pathol.* **73**:702, 1980.
18. Granato, P. A., Poe, L., and Weiner, L. B. New York City medium for enhanced recovery of *Mycoplasma pneumoniae* from clinical specimens. *J. Clin. Microbiol.* **17**:1077, 1983.
19. Gump, D. W., Gibson, M., and Ashikaga, T. Lack of association between genital mycoplasmas and infertility. *N. Engl. J. Med.* **310**:937, 1984.
20. Hayflick, L., and Channock, R. M. *Mycoplasma* species of man. *Bacteriol. Rev.* **29**:185, 1965.

21. Howard, C. J., and Gourley, R. N. Proposal for a second species within the genus *Ureaplasma, Ureaplasma diversum* sp. nov. *Int. J. Syst. Bacteriol.* **32**:446, 1982.
22. Johnson, J. E., and Smith, T. F. A simplified tetrazolium reduction test for *Mycoplasma pneumoniae. Med. Lab. Sci.* **33**:235, 1976.
23. Kenny, G. E., and Grayston, J. T. Eaton pleuropneumonia-like organism (*Mycoplasma pneumoniae*) complement-fixing antigen: Extraction with organic solvents. *J. Immunol.* **95**:19, 1965.
24. Klimek, J. J., Russmand, B. S., and Quintiliani, R. *Mycoplasma pneumoniae* meningoencephalitis and transverse myelitis in association with low cerebrospinal fluid glucose. *Pediatrics* **58**:133, 1976.
25. Kundsin, R. B., Driscoll, S. G., Monson, R. R., Yeh, C., Biano, S. A., and Cochran, W. D. Association of *Ureaplasma urealyticum* in the placenta with perinatal morbidity and mortality. *N. Engl. J. Med.* **310**:941, 1984.
26. Levine, D. P., and Lerner, M. The clinical spectrum of *Mycoplasma pneumoniae* infections. *Med. Clin. North Am.* **62**:961, 1978.
27. Manchee, R. J., and Taylor-Robinson, D. Haemadsorption and haemagglutination by mycoplasmas. *J. Gen. Microbiol.* **50**:465, 1968.
28. Mardh, P.-A. *Mycoplasma hominis*—A neglected human pathogen. *Eur. J. Clin. Microbiol.* **2**:303, 1983.
29. Maresh, H., Klimek, J. J., and Quintiliani, R.: Myocardial dysfunction and hemolytic anemia in a patient with *Mycoplasma pneumoniae* infection. *Chest* **71**:410, 1977.
30. McCormack, W. M. Epidemiology of *Mycoplasma hominis. Sex. Transm. Dis.* **10**:261, 1983.
31. McCormack, W. M., Moller, B. R., and Mardh, P.-A. *Mycoplasma hominis*—A human pathogen. *Sex. Transm. Dis.* **10**:160, 1983.
32. Murphy, T. F., Henderson, F. W., Clyde, W. A., Jr., Collier, A. M., and Denny, F. W. Pneumonia: An eleven-year study in a pediatric practice. *Am. J. Epidemiol.* **113**:12, 1981.
33. Murray, H. W., Masur, H., Senterfit, L. R., and Roberts, R. B. The protean manifestations of *Mycoplasma pneumoniae* infection in adults. *Am. J. Med.* **58**:229, 1975.
34. Naessens, A., Foulan, W., Volckaert, M., Amy, J. J., and Lauwers, S. Cervical and placental colonization by *Ureaplasma urealyticum* in pregnant women and fetal outcome. *J. Infect. Dis.* **148**:333, 1983.
35. Niiter, Y., Hasegawa, S., Svetake, T., Kukota, H., Konnatsu, S., and Horitawa, M. Resistance of *Mycoplasma pneumoniae* to erythromycin and other antibiotics. *J. Pediatr.* **76**:438, 1970.
36. Purcell, R. H., and Chanock, R. M.: Mycoplasma of human origin. *In* Lennette, E. H., and Schmidt, N. J. (eds.), *Diagnostic Procedures for Viral and Rickettsial Infections,* 4th ed. New York, American Public Health Association, 1969, p. 786.
37. Sackel, S. G., Alpert, S., Fiumara, N. J., Donner, A., Laughlin, C. A., and McCormack, W. M. Orogenital contact and the isolation of *Neisseria gonorrhoeae, Mycoplasma hominis,* and *Ureaplasma urealyticum* from the pharynx. *Sex. Transm. Dis.* **6**:64, 1979.
38. Shepard, M. C., and Combs, R. S. Enhancement of *Ureaplasma urealyticum*

growth on a differential agar medium (A7B) by a polyamine, putrescine. *J. Clin. Microbiol.* **10**:931, 1979.

39. Shepard, M. C., and Lunceford, C. D.: Urease color test medium U-9 for the detection and identification of "T" mycoplasmas in clinical material. *Appl. Microbiol.* **20**:539, 1970.
40. Shepard, M. C., and Lunceford, C. D. Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T Mycoplasmas) in primary cultures of clinical material. *J. Clin. Microbiol.* **3**:613, 1976.
41. Shepard, M. D., and Howard, D. R. Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. *Ann. N.Y. Acad. Sci.* **174**:809, 1970.
42. Stanbridge, E., and Hayflick, L. Growth inhibition test for identification of *Mycoplasma* species utilizing dried antiserum-impregnated paper discs. *J. Bacteriol.* **93**:1392, 1967.
43. Smith, T. F., and Herrmann, E. C., Jr. Possible influence of antibiotic therapy on usefulness of metabolic inhibition test for diagnosis of *Mycoplasma pneumoniae* infections. *Appl. Microbiol.* **21**:160, 1971.
44. Stamm, W. E., Koutsky, L. A., Benedetti, J. K., Jourden, L., Brunham, R. C., and Holmes, K. K. *Chlamydia trachomatis* urethral infections in men. *Ann. Intern. Med.* **100**:47, 1984.
45. Razin, S., and Freundt, E. A. The Mycoplasmas. Section 10. *In* Krieg, N. R. (ed.), *Bergey's Manual of Systematic Bacteriology,* Vol. 1. Baltimore, 1984, Williams and Wilkins, p. 740.
46. Taylor-Robinson, D., Csonka, G. W., and Prentice, M. J. Human intraurethral inoculation of ureaplasmas. *Quart. J. Med.* **46**:309, 1977.
47. Upadhyaya, M., Hibbard, B. M., and Walker, S. M. The effect of *Ureaplasma urealyticum* on semen characteristics. *Fertil. Steril.* **41**:304, 1984.
48. Williams, M. H., and Taylor-Robinson, D. Aids to the detection of T-strain Mycoplasma. *Ann. N.Y. Acad. Sci.* **143**:394, 1967.
49. Woods, L. L., and Smith, T. F. Tetrazolium agar overlay in test for *Mycoplasma pneumoniae. Appl. Microbiol.* **24**:148, 1972.

10

Viruses

Thomas F. Smith, Ph.D.

I. General Considerations

A. Description of Viruses

Virus particles comprise a nucleic acid (either DNA or RNA, but never both in the same particle) surrounded by a protein shell (capsid), the components or structural units of which are called capsomeres. Collectively, the capsid surrounding the nucleic acid constitutes the nucleocapsid, which, in turn, may be surrounded by an envelope, usually passively derived from the host as the virus emerges from the cell. Viruses generally have either of two types of symmetry, icosahedral or helical. Particles with icosahedral symmetry contain capsomeres that are arranged in equilateral triangles. The icosahedron has 20 triangular facets, 12 corners (apices), and 30 edges. Rod-shaped viruses have helical symmetry in which the capsomeres follow the helical form of the nucleic acid and extend to the outside of the particle. Poxviruses are morphologically unrelated to any of the other viruses and have a complex symmetry resulting in a brick-shaped particle. Viruses are smaller (20 to 300 mm) than the *Chlamydia* and *Rickettsia* but, like these organisms, require a living cell for replication.

Viruses are classified into two major groups according to their nucleic acid content, i.e., DNA or RNA. The criteria used for subdividing viruses within these groups include: (1) symmetry of the nucleocapsid, (2) presence or absence of an envelope, (3) number of strands of nucleic acid, (4) size and shape of the viron, and (5) number of capsomeres of icosahedral viruses or the diameter of the helix of helical viruses. Some of these

Table 10–1. Viral Isolates at Mayo Clinic (1961 through 1982)

Nucleic acid type	Group	Virus		Number Isolated
DNA	Adenovirus	Adeno		1201
	Herpesvirus	Herpes simplex		3880
		Cytomegalo		557
		Varicella-zoster		422
	Poxvirus	Vaccinia		8
			Subtotal	6068
RNA	Myxovirus	Influenza A		1148
		Influenza B		203
	Paramyxovirus	Mumps		294
		Parainfluenza		1113
	Metamyxovirus	Respiratory syncytial		289
	Picornavirus	Coxsackie A		381
		Coxsackie B		556
		Echo		461
		Polio		96
		Entero (untyped)		647
		Rhino		764
			Subtotal	5952
			Total	12,020

properties, such as size, symmetry, and the presence or absence of an envelope, can be exploited in the laboratory by the diagnostic virologist. A list of the medically important viruses capable of replicating in cell cultures in our laboratory is presented in Table 10–1.

B. Source

Most viruses are isolated from respiratory sources but may also be found in feces, urine, cerebrospinal fluid, blood, dermal tissue, or in the eye. In disseminated infections viruses may directly involve the brain, lung, or kidney.

C. Clinical Importance

The association of viruses with upper respiratory disease has been clearly established[45,73]; however, for the past 10 years we have noted a decrease in the percentage of specimens from the upper respiratory tract of ambulatory outpatients (80% to 38%) relative to the percentage of specimens

from other sources [skin, urine, and cerebrospinal fluid (CSF)] from inpatients suffering from serious sequelae of viral infections,[126,126] examples of which are patients hospitalized with suspected herpes simplex virus (HSV) or varicella-zoster (VZ) infections complicating neoplastic disease, enteroviruses causing central nervous system (CNS) infections, or cytomegalo-viruses (CMV) associated with renal transplant recipients. The isolation of such viruses can provide more direct prognostic and therapeutic effect on patient management.[126]

II. Processing of Specimens and Cultures

Throat, rectal, and dermal swabs (Culturette, Marion Laboratories) are immersed in 2 ml of serum-free medium containing anitbiotics in 7 ml vials as described in Table 3–5. A portion (0.5 ml) of the serum-free medium extract is pipetted into the swab container to collect any residual virus, and then removed and returned to the vial containing the serum-free extract. Body fluids (cerebrospinal fluid and urine) and tissue are processed and inoculated as described in Tables 3–4, 3–5, and 3–7.

MRC-5 cell cultures are inoculated before CMK cell cultures to avoid contamination of the MRC-5 cell cultures by simian viruses which may be present in the CMK cultures.[63] The screw-caps on inoculated culture tubes are never removed except when cytopathic effects (CPE) are observed and a subpassage of the viral-infected cell culture is made.

Inoculated and uninoculated (control) cell cultures are examined microscopically (125×) three times weekly for the presence of CPE. However, cell cultures inoculated with genital specimens are examined each weekday for a total of seven days. The degree of CPE is recorded by a scoring system as follows: O = no CPE; ± = suggestion of beginning CPE; 1+ = 25% or less of the cells in the monolayer affected; 2+ = 25 to 50% of cells in the monolayer affected; 3+ = 50 to 75% of cells in the monolayer affected; and 4+ = 75 to 100% of cells in the monolayer affected or sloughed off the glass surface.

III. Cell Cultures

A. MRC-5 (Medical Research Council)

1. Description and Sources

The MRC-5 cells (Viromed Laboratories, Minneapolis, MN) are the human diploid fibroblast cell line of choice[66] because of uncertain supply of WI-38 cells and the proved equivalence of viral recovery rates from both cell lines.[35,126] A typical monolayer of MRC-5 cells is shown in Figure 10–1.

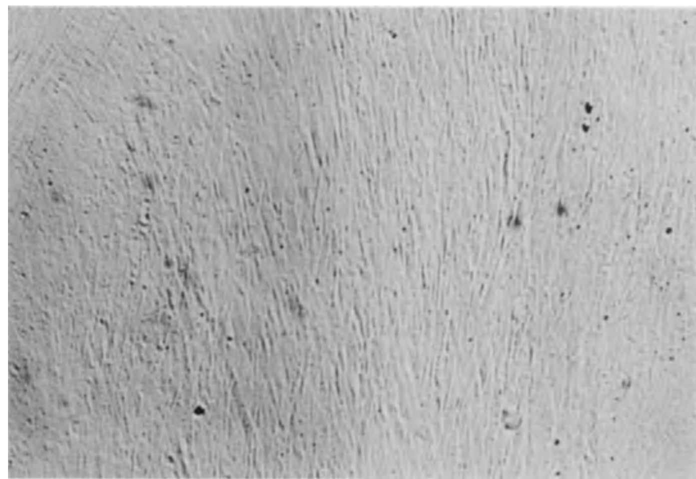


Figure 10–1. Uninfected human embryonic diploid fibroblast (MRC-5) cell culture (×100).

2. Trypsinization for Subculturing Cells

a. Aspirate the medium from a monolayer in a glass prescription bottle (16 or 32 oz.) with a sterile transfer pipette attached to a vacuum source. (On receipt of cells from a commercial source, they are transferred to glass prescription bottles.)
b. Add trypsin-EDTA solution to the culture (1 ml/16 oz. culture) and tilt the bottle to distribute the solution over the entire surface of the monolayer.
c. Remove the trypsin-EDTA solution and replace it with fresh trypsin-EDTA solution (1.25 ml/16 oz. culture).
d. Incubate the bottle for 2 to 3 minutes at 35°C or until the cells have detached from the glass surface.
e. Add 10 ml of growth medium (containing serum which stops the enzyme action) to the bottle. Disperse the cells by aspirating and expelling the suspension from a pipette several times so that no cellular clumps remain.
f. Adjust the volume of the cell suspension with sufficient growth medium to allow seeding of new cell culture tubes. (For example, the cell suspension is usually adjusted to about a 70-ml volume: 0.5 ml is transferred to each of 64 16 × 125 mm tubes in a rack and the remainder is returned to the original bottle containing the culture (A,2a). Since diploid cells that have been subpassaged *in vitro* more than 30 times do not grow as rapidly compared with younger cells, the number of culture tubes which may be seeded with each subculture of the diploid cells depends on their passage level.)

3. Incubation and Maintenance of Culture Tubes

a. Shake the culture tubes vigorously to disperse the cells and incubate at a 5° slant in stationary racks at 35°C.
b. after monolayers develop or the pH of the medium reaches approximately 6.8 (usually after 3 to 5 days of incubation), add 0.5 ml of maintenance medium.
c. After an additional 3 to 5 days, aspirate the medium from the cell cultures and replace with 1 ml of maintenance medium. (The medium is replaced every 2 weeks if the cell cultures are not used.)
d. Add 1 ml of maintenance medium 3 days before the cultures are inoculated with virus specimens. Confluent cell sheets of MRC-5 cells may be maintained under these conditions for at least 1 month.

4. Comments

MRC-5 cells, which are multiplying rapidly and producing considerable acid, usually require subpassage twice weekly; however, one must continuously assess the rate of growth (according to the time required to reach a monolayer) throughout the phase and make adjustments in subsequent cell subcultures.

The techniques of trysinization of MRC-5 cells are very critical: The trypsin-EDTA solution must be in contact with cells only until they are detached from the glass surface, since further contact decreases the number of viable cells in the suspension; all media and solutions must be equilibrated at 35°C because clumping and aggregation of the cells occurs at lower temperatures, resulting in isolated cell clones instead of uniform monolayers.

The suspension of MRC-5 cells (after trypsinization) may be returned to the original culture bottle (rather than to a fresh culture bottle) to avoid loss of cells due to transfer techniques.

B. Primary Cynomolgus (CMK) or Rhesus (RMK) Monkey Kidney Cells

1. Description and Source

Individual CMK cells are obtained from intact kidneys, which are removed from cynomolgus monkeys, cut into small fragments, and incubated in trypsin. A monolayer culture is shown in Figure 10–2. Cell cultures are obtained commercially (Flow Laboratories) each week.

A few or, sometimes all, of the cell cultures prepared from the original cell suspension are contaminated with simian viruses (SV), depending on the source of the monkeys, their contacts with humans and animals,

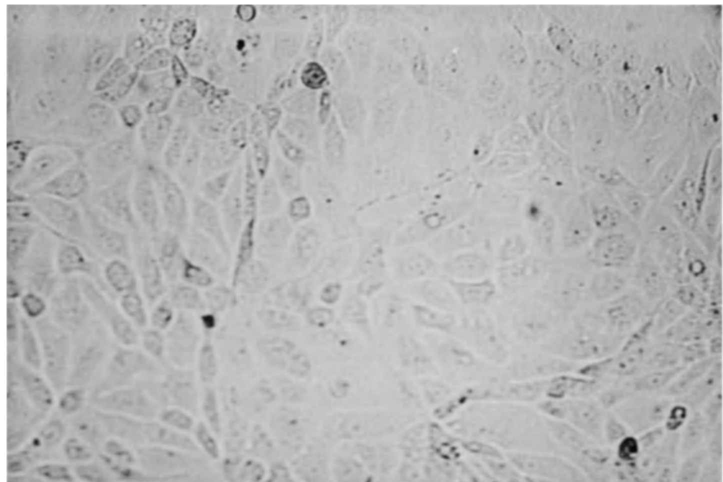


Figure 10–2. Uninfected primary monkey kidney (CMK) cell culture (×100).

and their quarantine conditions.[64] It is essential to recognize the presence of these viruses in CMK cell cultures. Primary CMK cells are extremely important for the isolation of orthomyxoviruses and paramyxoviruses, adenoviruses, and some enteroviruses.[60,125] CMK and RMK cells are equally susceptible to these viruses.[55]

2. Preparation

a. Aspirate the medium from commercially available tubes when they are received and replace with 3 ml of serum-free medium. (Serum contains inhibitors that may decrease the recovery of orthomyxoviruses and paramyxoviruses from specimens.)
b. Incubate the cell cultures at 35°C until used.

C. Human Embryonic Kidney (HEK) Cells

These cells (Figure 10–3) are obtained commercially (M. A. Bioproducts) as monolayers in cell culture tubes. They may be useful as a supplementary cell culture system to MRC-5 and CMK cells for recovering herpes simplex and adenoviruses. During 1 year in our laboratory, 2300 specimens were inoculated into MRC-5, CMK or RMK, and HEK cell cultures, Of 30 isolates of adenovirus, 10 were recovered in HEK cells only and 1 in CMK or RMK cells only. On the other hand, of 126 isolates of HSV, 8 were recovered in MRC-5 cells only and 4 in HEK cells only. Thus, HEK cells were of limited benefit in our laboratory.

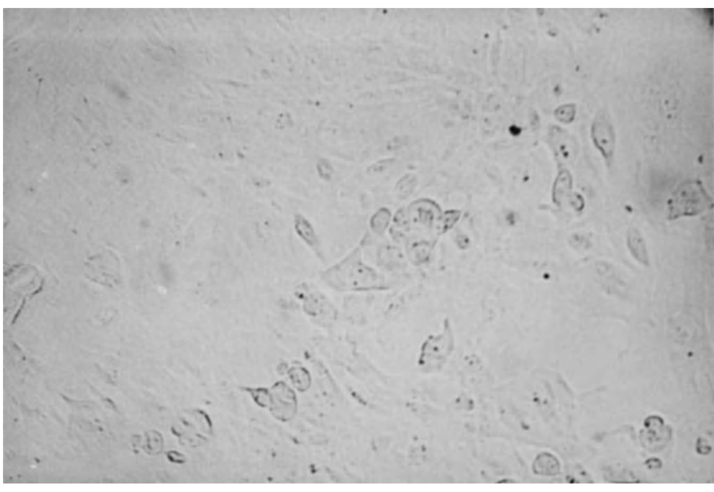


Figure 10–3. Uninfected primary human embryonic kidney (HEK) cell culture (×100).

IV. Identification of RNA-Containing Viruses

A. Picornaviruses

1. Description

Picornaviruses are small (18 nm to 30 nm in diameter), RNA-containing viruses which have similar morphological (icosahedral symmetry), physical, and chemical properties. The picornaviruses are subdivided into the enteroviruses and the rhinoviruses. Collectively, these two subgroups consist of over 160 serotypes, of which about 100 are rhinoviruses. The enterovirus serotypes include more than 30 for echovirus, more than 24 for coxsackievirus A, 6 for coxsackievirus B, and 3 for poliovirus.[84] Although both the enteroviruses and the rhinoviruses lack an outer lipid envelope, exposure to acidic conditions (pH 3) will destroy the infectivity of the rhinoviruses but not the enteroviruses.[74]

2. Source

Most enteroviruses (15 to 20% of all viruses isolated over several years in our laboratory) are isolated from throat specimens from individuals with upper respiratory tract infections (URI); however, they can also be excreted in the feces for several days following URI.[73,145] Enteroviruses, most commonly coxsackievirus B and echoviruses in the United States, can be recovered from the CSF of patients with central nervous system

(CNS) disease, from dermal lesions (hand, foot, and mouth disease), and from the eye (conjunctivitis). Enterovirus infections occur almost exclusively between the months of May through November, with the exception of probable vaccine strains of poliovirus which occur throughout the year.[55]

3. Clinical Importance

The most serious complication of enterovirus infection is meningitis. Although the prognosis of meningitis is generally excellent,[79,109] neurological deficits have been recognized and fatal cases reported in children who were infected during the first year of life.[46,76,120] Furthermore, persistent and fatal disease has been recognized in adults and children with agammaglobulinemia.[141] The isolation of virus from the CSF is of etiological significance; however, its recovery only from throat or stool specimens from such patients suggests, but does not prove, the cause of the disease.[79,87]

Echoviruses have been associated with a variety of other clinical problems, such as hemorrhagic conjunctivitis[88,106] and rashes.[55] Coxsackievirus B, in our experience, has not been involved in CNS disease as frequently as echoviruses[55]; however, it may cause primary myocardial disease in adults as well as children.[44] At least two studies have shown an association of coxsackievirus B with the hemolytic-uremic syndrome.[3,78] Apparently, neurological sequelae due to CNS disease by this virus in the neonatal period are not common.[33]

Herpangina, upper respiratory tract infections, and hand, foot, and

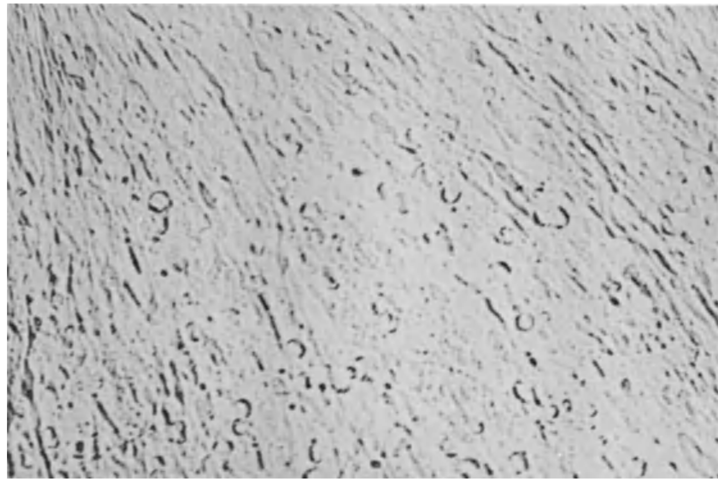


Figure 10–4. CPE produced by enteroviruses in MRC-5 cell culture (×100).

mouth disease occur predominantly in children as a result of coxsackievirus A infection.[55] Because of the infrequent involvement of this virus in CNS disease, our laboratory has discontinued the routine inoculation of suckling mice, a procedure required for the recovery of most serotypes of coxsackievirus A.

The finding of poliovirus in throat or stool specimens of children can be explained in most instances by the recent administration of the live vaccine. Although rare in the United States, CNS disease resulted from vaccination of immunodeficient individuals.[23]

Like enteroviruses, rhinoviruses are most often associated with upper respiratory tract disease. Rhinovirus can, however, produce transient peripheral airway abnormalities in previously normal young adults,[7] precipitate attacks of asthma,[86] and cause other severe lower respiratory tract infections.[67,107]

4. Growth Characteristics

a. Enteroviruses

(1) MRC-5

The CPE produced in MRC-5 cell cultures by coxsackievirus types A9 and A16 (and occasionally some other serotypes), echovirus, and poliovirus are very similar. Infected fibroblasts become enlarged; subsequent cytolysis results in irregular shaped and disintegrated cells, ultimately involving the entire monolayer (Figure 10–4). Only 3 to 6% of coxsackievirus B strains produce CPE on primary isolations in this system.[55]

(2) CMK

All enteroviruses produce enlarged, tear-shaped cells with prominent and refractile cell membranes in CMK. The infected cells occur randomly scattered throughout the cell sheet (Figure 10–5). Less than 50% of all of the cells in the culture are usually infected, with the exception of strains of poliovirus and coxsackievirus B which produce cytolysis and cause the cells to detach from the glass surface.

b. Rhinovirus

(1) MRC-5

Discrete foci or plaques of swollen, rounded cells of various sizes appear in a field of normal fibroblasts. The plasma membrane of each infected cell is prominent and refractile, except that in some of the smaller cells it may appear as very large granules with no visible cytoplasm. At times the entire cell sheet can become involved, so that the CPE can be confused with that of the enteroviruses (Figure 10–6). The conditions of

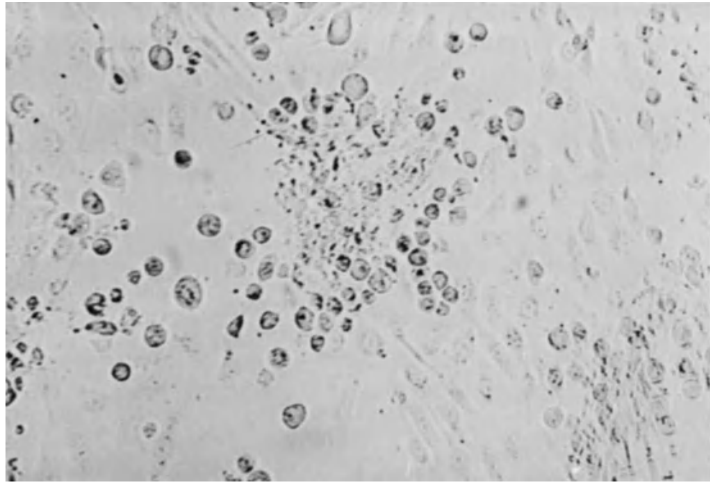


Figure 10–5. CPE produced by enteroviruses in CMK cell culture (×100).

acidic pH and lower temperatures recommended for rhinovirus isolations[105] are not critical when human diploid cell strains are used.[51]

(2) CMK

Few isolates of rhinovirus are recovered in CMK cells. Incubation of cell cultures in this laboratory at 35°C in a medium with pH 7.0 to 7.6 tends to prevent the isolation of "M" (monkey cell affinity) or "H" (human cell affinity) strains.

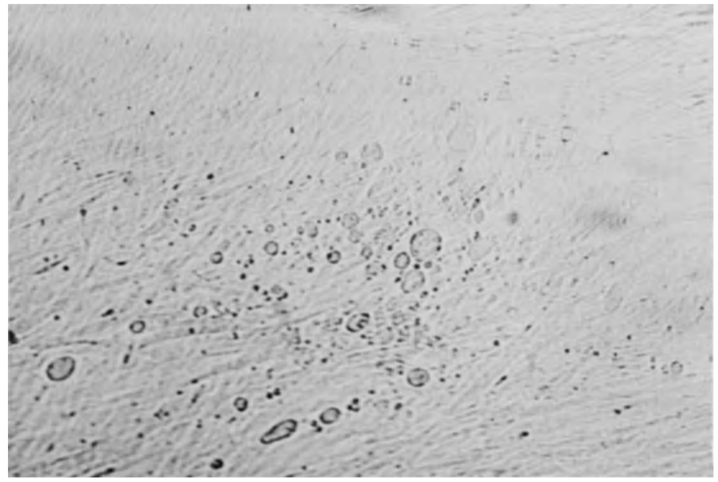


Figure 10–6. CPE produced by rhinovirus in MRC-5 cell culture (×100)

5. Tests for Identification

a. Virus Neutralization

For general purposes in clinical virology, neutralization of viral infectivity is not usually required. However, when a specific isolate, such as an enterovirus from a suspected case of poliomyelitis must be identified, it is necessary to determine neutralization by reference antisera.

Neutralization of Infectivity

Purpose:
To identify a viral isolate by inhibiting its infectivity in cell cultures by specific antiserum.

Principle:
Specific antibodies react with virus particles forming complexes which are noninfective in cell cultures.

Specimen:
A 10^{-2} dilution of a virus suspension harvested with CPE grade 3+ or 4+ in cell cultures.

Reagent:
Reference antisera for enterovirus serotypes. (M. A. Bioproducts.)

Procedure:

1. Add 0.3 ml of antiserum, diluted in tryptose phosphate broth to contain 100 units (p. 548), to 0.3 ml of virus suspension, diluted to contain approximately 100 tissue culture infective doses ($TCID_{50}$) of virus (p. 549).
2. Mix the virus-antiserum mixture thoroughly and incubate for 1 to 1.5 hr at 20°C.
3. Inoculate cell cultures (use the most sensitive cell for the virus in question) as follows: one tube, uninoculated control; one tube, 0.1 ml of undiluted virus; one tube, 0.1 ml of the virus dilution used in the neutralization test; and two tubes, 0.2 ml of virus-antiserum mixture.
4. Incubate the cultures at 35°C in a stationary rack (slanted at 5°) and examine daily at ×125 magnification for 10 days for evidence of CPE.

Interpretation:

Positive test: A specific viral serotype is identified by the absence of CPE in cell cultures inoculated with the virus-antiserum complex.

Negative test: Presence of CPE in cell cultures inoculated with the virus-antiserum complex.

Controls:

Positive: Known enterovirus serotype
Negative: Uninoculated cell culture

Detailed methodology for virus titration, antiserum dilution, and intersecting serum scheme.

1. Virus Titration
Conventional neutralization tests use exactly 100 tissue culture infective doses ($TCID_{50}$). A typical titration is described below:

a. Add 0.9 ml of tryptose-phosphate broth to seven sterile test tubes (25 by 100 mm, Curtin Scientific Company).
b. Add 0.1 ml of the virus suspension to the first dilution tube and mix at least five times on a Vortex mixer (Scientific Industries, Inc.).
c. With a new 1.0 ml pipette, remove 0.2 ml from this tube, add 0.1 ml to the second tube, and discard the remainder. Mix the suspension and continue this process with all seven tubes.
d. Inoculate each of two to five cell cultures (the most sensitive host cell system) with 0.1 ml of each dilution of virus (use a separate pipette for each dilution). Incubate the cultures at 35°C in a stationary rack (slanted at 5°) and examine daily for CPE.
e. Determine the titration endpoint by the method of Reed and Muench.[112] An example of the determination of the 50% $TCID_{50}$ is shown in Table 10–2. In the example, 1 $TCID_{50} = 10^{-4.375}$; therefore, 100 $TCID_{50} = 10^{-2.375}$. To obtain 100 $TCID_{50}$ virus per 0.1 ml. the suspension is diluted to 10^{-2} (1:100) and then, because the antilog of 0.375 is 2.37, it is further diluted to 1:2.37.

This procedure is obviously time consuming and expensive, especially when all viral isolates are specifically identified by neutralization tests. Alternatively, certain virus dilutions are used such as a 10^{-2} dilution for enteroviruses as described previously.

2. Antiserum Dilution
Antiserum titers are expressed as units/volume. One unit of antiserum is defined as the highest dilution of antiserum that neutralizes the infectivity of 100 $TCID_{50}$ of homologous virus, e.g., 1:320/0.1 ml or, expressing the titer in $\log_{10}$ form, $10^{2.5}$ (antilog of 0.5 is 3.2). Since 100 units of antibody are used in standard viral neutralization tests, a 1:3.2 dilution of this antiserum would be required. Other examples of this calculation are as follows:

Dilution of Original Antiserum

Antiserum titer/0.1 ml	To contain 100 units
$10^{-2.3} = 1/200$	1:2
$10^{-2.806} = 1/640$	1:6.4
$10^{-3.107} = 1/1{,}280$	1:12.8

3. Intersecting Serum Scheme

The echoviruses can be identified by using the "intersecting serum scheme" of Schmidt et al.[119] This system involves preparation of serum pools containing antisera for each echovirus serotype. Antisera for each echovirus serotype are included in each of two pools. An unknown echovirus is then tested against the antiserum pools. The serotype of the echovirus is determined by specific neutralization of viral infectivity in two of

Table 10–2. Example of Determination of $TCID_{50}$

Negative log of virus dilution	Data: Number of tubes, With CPE[a]	Data: Number of tubes, No CPE[b]	Data: Total cultures, With CPE	Data: Total cultures, No CPE	CPE ratio[c]	Cultures with CPE (%)
−1	5	0	19	0	19/19	100
−2	5	0	14	0	14/14	100
−3	5	0	9	0	9/9	100
−4	4	1	4	1	4/5	80
−5	0	5	0	6	0/6	0

[a] Sum from bottom line up.
[b] Sum from top line down.
[c] Ratio of number of cultures with CPE to total accumulated number of cultures at each dilution.

Calculations

From data table, 50% end point ($TCID_{50}$) is between negative logs −4 and −5. The proportionate distance above −4 is given by:

$$\frac{\text{\% with CPE at next dilution} >50\% - 50\%}{(\text{\% with CPE at next dilution} >50\% - \text{\% with CPE at next dilution} <50\%)} = \frac{80\% - 50\%}{80\% - 0\%} = \frac{30}{80} = 0.375$$

The negative log of the $TCID_{50}$ is given by:

Negative log of next dilution with >50% CPE + (proportionate distance factor × dilution factor) = −4.0 + (0.375 × negative log 10) = −4.375 $TCID_{50} = 10^{-4.375}$

Table 10–3. Composition of Antiserum Pools with Common Serotypes of Echovirus

Pool #	1	2	3	4
5	12	3	7	6
6	9	11	16	28
7	22	17	30	32

the pools and can be identified as the serotype common to both pools. For example, an echovirus type 17 would be neutralized by pools 2 and 7 (Table 10–3). Since some echovirus serotypes have rarely, if ever, been isolated in the Rochester, Minnesota, area, we have prepared 2 antiserum schemes. One set of pools (1 through 7) contains those serotypes which are isolated most commonly (Table 10–3), while pools 8 through 16 contain the less frequently isolated serotypes (Table 10–4).

The preparation of echovirus antiserum pools must be accurate and the pool must be nontoxic for cell cultures. In our experience, pools containing 50 to 75 units of each type-specific antiserum produce reliable results with no toxicity problems. The serum pools are prepared as follows:

If the commercial echovirus antiserum has a titer of 640 (640 units/ 0.1 ml), then 1 ml (or 6400 units) is added to the pool (Table 10–5). Since the final volume of this pool will be adjusted to 3 ml, the concentration of this antiserum will be 2100 units/ml, or 210 units/0.1 ml. Before use in the echovirus neutralization tests, the pool is diluted 1:3 with tryptose-phosphate broth (71 units/0.1 ml) and mixed with 0.3 ml of the virus suspension.

Neutralization tests are performed as described previously. Two cell cultures each are inoculated with 0.2 ml of each virus-antiserum mixture. If the echovirus cannot be specifically identified by neutralization with two of the antiserum pools, then other interpretations are possible. If there is neutralization by only one of the pools, more than one echovirus serotype is present but only one serum pool contains antisera to both viruses. If there is no neutralization by any pool, then (1) more than

Table 10–4. Composition of Antiserum Pools with Infrequently Isolated Serotypes of Echovirus

Pool #	8	9	10	11
12	10	2	5	8
13	1	4	13	14
14	15	18	19	20
15	21	23	24	25
16	26	27	29	31

Table 10–5. Example of Preparation of Echovirus Antiserum Pool

Echovirus antiserum type	Antiserum/0.1 ml	Volume of antiserum used in pool[a] (ml)
1	640	1.00
2	1850	0.34
3	2000	0.32
31	2800	0.23
5	3980	0.16
6	1250	0.50
Normal rabbit serum	0	0.45
Total		3.00

[a] Note that echovirus type 2 antiserum has a titer of 1850 and therefore contains 2.9 times as many units as antiserum type 1. If 1 ml of an antiserum with a titer of 640 is required, then 0.34 ml (1 ml ÷ 2.9) is needed of antiserum type 2.

one echovirus serotype is present and each serum pool contains antiserum to only one echovirus, (2) the virus isolate is not an echovirus, or (3) the antibody titer in the combined pool is too low.

With coxsackievirus type B and poliovirus isolates, complete neutralization sometimes is not observed, but a delay of 3 to 4 days in appearance of the CPE in the tubes with the homologous antiserum identifies the virus. If a delay of just a day or two occurs, then a higher dilution of virus should be used or quantitative infectivity titrations can be carried out.

pH 3 Test

Purpose:
To distinguish between rhinoviruses and enteroviruses.

Principle:
Among viruses without a lipid envelope, the rhinoviruses are uniquely sensitive (infectivity of the virus is destroyed) to pH 3.

Specimen:
0.1 ml of a viral suspension.

Reagent:
Eagle's medium (BME, pH 2.2) without bicarbonate or Tris buffer.

Procedure:

1. Add 0.1 ml of viral suspension to 0.9 ml of Eagle's medium without bicarbonate or Tris buffer; incubate the mixture at room temperature for at least 10 min.
2. Inoculate cell cultures (the most sensitive cell cultures for the isolate are used) with 0.1 ml of the acid-treated viral mixture. Another cell culture is inoculated with a similar dilution of virus made in BME buffered to pH 7.6.
3. Incubate the cell cultures at 35°C. Observe daily at ×125 magnification for the presence of CPE in cell cultures.

Interpretation:
Presence of CPE: Enterovirus.
Absence of CPE: Rhinovirus.

Controls:

Positive: Viral suspension buffered to pH 7.6
Negative: Cell culture medium without virus

Chloroform Test

Purpose:
To distinguish between enveloped and nonenveloped viruses.

Principle:
Chloroform removes the lipid-containing envelopes of viruses such as herpes-, ortho-, and myxoviruses and destroys their infectivity. The infectivity of nonenveloped viruses, such as adeno- and picornaviruses, remains intact. Rhinoviruses and enteroviruses are resistant to chloroform but may be distinguished from one another on the basis of the pH 3 test (Table 10–6) and from other viruses by characteristics, such as characteristic CPE and hemadsorption.

Specimen:
0.1 ml of a viral suspension.

Procedure:

1. Add 0.05 ml of chloroform to a 15 ml screw-cap glass centrifuge tube (Bellco Glass Company) containing 0.1 ml of viral suspension diluted with 0.9 ml of BME with bicarbonate and Tris buffer.
2. Shake the tube vigorously for 10 min and then centrifuge at 35 × *g* for 5 min.

Table 10–6. Response of Picornaviruses to pH 3 and Chloroform Tests

Virus	Test	
	pH 3	$CHCl_3$
Coxsackievirus type A	–	–
Coxsackievirus type B	–	–
Echovirus	–	–
Poliovirus	–	–
Rhinovirus	+[a]	–

[a] No active virus.

3. Inoculate separate cell cultures (the most sensitive cell cultures for the isolate are used) with 0.1 ml of the chloroform-free fraction (upper phase) and with 0.1 ml of a tenfold dilution of the virus suspension not treated with chloroform.
4. Incubate the cell cultures at 35°C. Observe daily at a ×125 magnification for the presence of CPE in cell cultures.

Interpretation:
Presence of CPE indicates virus does not have lipid envelope. (Picornaviruses lack lipid envelopes.)

Controls:

Positive: Viral suspension without chloroform treatment
Negative: Uninoculated cell culture

Inoculation of Suckling Mice

Purpose:
To distinguish between coxsackievirus A and other enteroviruses.

Principle:
Since only coxsackievirus serotypes A9 and A16 regularly produce CPE in cell cultures, suckling mice must be inoculated to detect most coxsackieviruses. This procedure is done only on specific request in instances when these viruses are suspected to have caused CNS disease.

Deaths occurring within the first 24 to 36 hr are attributed to trauma associated with the inoculation or to something other than virus in the specimen. Mice infected with coxsackievirus type A develop a flaccid paralysis characterized by general weakness and limp reactions, usually

Figure 10–7. Paralysis in infant mice infected with coxsackievirus type A.

of their legs. Frequently, the hind legs are extended backward (Figure 10–7). Coxsackievirus type A is presumed to be present if the mice develop flaccid paralysis (usually within 3 to 7 days) in the absence of typical CPE in the cell cultures.

Specimen:
0.1 ml of a viral suspension (0.03 ml per animal).

Procedure:

1. Fill a 1-ml tuberculin syringe, fitted with a 1-cm 27-gauge needle, with the specimen fluid. The technologist should wear disposable surgeon's gloves when handling the mice so that the mother accepts the infant mice (approximately 24 hr old) after the inoculation.
2. Place the mother mouse in a separate cage during the inoculation procedure.
3. Swab the abdomen of each infant mouse with 95% ethyl alcohol. Inoculate at least five mice intraperitoneally with 0.03 ml of the specimen.
4. At the same time, inoculate thioglycollate broth with 0.1 ml of the specimen to determine if the specimen is contaminated with bacteria. Swab the injection site on each mouse with an alcohol sponge to remove specimen leakage. Return the infant mice and the mother to a prepared nest in a cage.
5. Examine the mice 6 days each week for paralysis and mortality. Check the thioglycollate broth tubes for evidence of bacterial growth.
6. If paralysis occurs, kill one of the paralyzed mice with chloroform

and suspend the torso in serum-free medium containing antibiotics for homogenization and clarification as for tissue specimens.
7. Reinoculate portions (0.03 ml) of the homogenate intraperitoneally into infant mice to ascertain the virus's transmissibility.

Interpretation:

Positive test: Flaccid paralysis of mice 3 to 7 days after inoculation.
Negative test: No paralysis.

Controls:

Positive: Known coxsackievirus A
Negative: Cell culture medium without virus

B. Ortho-, Para-, and Metamyxoviruses

1. Description

The orthomyxovirus group contains the three types of influenza virus (A, B, and C), which are differentiated by the specificity of a soluble antigen (S antigen) associated with the internal ribonucleoprotein component of the virion. The paramyxovirus group includes measles virus (rubeola), mumps virus, and the four serotypes of parainfluenza virus. Respiratory syncytial virus (RSV) is classified in the metamyxovirus group.[84] All members of these groups contain RNA, have helical symmetry, and possess outer lipoprotein envelopes. With the notable exception of RSV, myxoviruses induce the synthesis and incorporation of viral hemagglutinin in the host cell plasma membrane. The interaction of erythrocytes with either viral particles or hemagglutinin associated with the cell during viral maturation (hemadsorption) or when virus has been released from the host cell (hemagglutination) and the inhibition of these reactions by specific antibody provide the basis for tests used in the identification of each virus.

2. Source

Influenza, parainfluenza, and respiratory syncytial viruses are respiratory tract pathogens which are commonly recovered from nasopharyngeal washings and throat specimens. In addition, influenza virus has been recovered from lung tissue.[128,131] Mumps virus involves primarily the parotid and related salivary glands and can optimally be recovered from Stensen's duct; however, infection can lead to CNS disease and accumulation of the virus in CSF. Urine specimens may yield the virus late in the course of the disease when other specimens are unproductive.[108]

3. Clinical Importance

Influenza virus infections occur every year in the United States, but they vary greatly in incidence, severity, and geographical distribution. Although influenza viruses A and B are responsible for only a portion of all respiratory tract disease, they cause periodic widespread outbreaks of respiratory tract disease in both adults and children.[40,114] The type of nucleocapsid protein indicates whether the influenza virus is type A, B, or C. Influenza A viruses can be classified into subtypes of strains on the basis of two antigens, hemagglutinin (H) and neuraminidase (N), which are located on the surface of the virus particle. Pandemics of influenza virus type A infections have occurred at 10 to 20 year intervals since 1890. Apparently, widespread disease with influenza virus type A results from alteration in the composition of the hemagglutinin and neuraminidase antigens. For example, the influenza strain prevalent from 1946 to 1958 was designated H_1N_1. Abrupt changes in both antigens produced a strain with altered immunological surface characteristics referred to as H_2N_2 (antigenic shift). Because these changes were substantial from an immunological standpoint, the population at risk was susceptible to infection and widespread disease. Later, in 1968, another antigenic shift occurred in the virus particle, but in this instance only the hemagglutinin changed significantly from that of the preceding strain. The new influenza virus isolate was designated H_3H_2. Since then, many minor changes (antigenic "drift" rather than "shift") have been noted, but most of these new strains have not caused pandemic disease. In recent years, influenza virus strains which were prevalent many years ago have been isolated. Of these, swine influenza virus ($H_{sw1}N_1$) has generally been associated with inapparent infection and limited person-to-person transmission[29,130] In 1977, A/USSR/77 (H_1N_1), appeared in China and Russia. This strain was closely related to influenza viruses that had circulated throughout the world in the 1950s and between January and April, 1978, spread throughout the United States, causing outbreaks in several schools and colleges, and to a lesser extent, in young persons in the general community. Two or three influenza virus types are generally isolated during a seasonal outbreak. For example, during the period from November, 1982 to April, 1983, the predominant agent (79% of isolates) recovered in the United States was influenza type A (H_3N_2) (similar to the A/Bangkok/79). H_1N_1 (similar to A/Brazil/78) and some type B strains (similar to B/Singapore/79) were also recovered.[34,92]

Clinically, influenza virus infections produce upper respiratory tract disease, which is usually indistinguishable from other febrile respiratory diseases and is accompanied by fever, cough, sore throat, headache, and myalgia. Nasal congestion, nonpleuritic chest pain, malaise, and hoarseness are less common symptoms. Normally, influenza is a self-limited disease, lasting for 3 to 7 days; however, about 10% of patients develop

small areas of lobar pulmonary consolidation. Although extensive pneumonia is rare,[39] this complication accounts for most deaths from influenza. Severe pneumonias due to influenza virus without bacterial superinfection do occur, but secondary bacterial pneumonias are the major cause of death. Other complications of influenza virus type A infections have included febrile convulsions and gastrointestinal symptoms,[39] parotitis,[9] and pneumonias with pleural effusion.[83] The virus can also cause widespread disease within hospitals[70] and nursing homes.[42] Influenza virus type B can also cause serious lower respiratory tract disease and has been associated with Reye's syndrome in children (encephalopathy with liver impairment).

An inactivated (killed) vaccine containing the influenza virus strains which currently are circulating in the world [A/Philippines/82 (H_3N_2), A/Chile/83 (H_1N_1) and B/USSR/83] is recommended for all individuals at increased risk to infections of the lower respiratory tract, including those with heart disease, chronic pulmonary disease, renal disease, diabetes mellitus, severe anemia such as that associated with sickle cell disease, and other conditions that compromise the immune mechanism, including certain malignancies and immunosuppressive therapy.[93]

Amantadine (Symmetrel) is also recommended for the prophylaxis and therapy of influenza type A infections.[21] Chemoprophylaxis should be combined with vaccine administration to ensure protection after treatment.[100] The drug is not active in the prevention of type B infections.

Of the four serotypes of parainfluenza virus, types 1, 2, and 3 are the most frequent cause of respiratory tract disease, most commonly pharyngitis and tonsillitis and croup.[24,49,54] Most infections are mild and occur in children, usually by the age of 5 years.[41] Acute parotitis resembling that associated with mumps, has been associated with parainfluenza type 3 infections of children.

The most common clinical features of mumps are fever and unilateral or bilateral swelling of the parotid glands. The sublingual and submaxillary glands may also be involved. Most cases of mumps virus infection occur in children; however, the disease's incidence has declined dramatically.[94] The frequency of orchitis or oophoritis increases with age of onset of the infection.[108] Mumps virus has been one of the most important causes of viral CNS disease (meningitis and encephalitis) in the United States; however, administration of the mumps virus vaccine has greatly reduced its incidence. Our laboratory has not recovered mumps virus from a clinical specimen since 1974.

Measles is another common childhood infection that has been effectively controlled in most communities by vaccination progams,[2,56] although many cases are still imported to the United States. The illness is characterized by the abrupt onset of fever, followed by a rash. Headache and abdominal pain are common symptoms, and many affected children develop pneumonia.[144] "Atypical measles" has been detected in previ-

ously immunized children following exposure to measles, most frequently in those who initially received inactivated (killed) measles viral vaccines but also in several cases who received only attenuated (live) vaccines.

Central nervous system complications, the most common of which is encephalomyelitis (0.1%), may occur after the acute phase of measles infection subsides. Prognosis for complete recovery in these patients is poor: between 10% and 30% of all cases are fatal, and 20% to 50% of the survivors develop significant motor, intellectual, sensory, or emotional sequelae.[75,98]

Subacute sclerosing panencephalitis (SSPE) is a rare, degenerative disease of the central nervous system which occurs in children and young adults and is caused by a variant of measles virus. It is characterized clinically by a decrease in intellectual skills and progresses to an almost complete loss of brain function and death. Although the virus has been recovered from brain tissue and other sites, laboratory diagnosis is most easily achieved by finding antibody to measles virus in the cerebrospinal fluid of affected patients.[121]

Respiratory syncytial virus is recognized as the most important cause of bronchiolitis and pneumonia in infants and young children,[48,132] especially in those less than 6 months of age, suggesting that serum antibody (obtained passively from the mother) may react immunologically with RSV in the lungs and contribute to the development and severity of the symptoms. Adults can acquire the virus and perhaps contribute to its spread among hospitalized infants and other susceptible groups.[47,139]

4. Growth Characteristics

a. Influenza Virus

(1) MRC-5

Influenza virus replicates poorly, if at all, in these cells.

(2) CMK

CPE is characterized by the formation of large, somewhat irregularly shaped granular cells occurring randomly throughout the culture (Figure 10–8). The cell sheet becomes discontinuous, and much cell debris is found floating in the medium.

Influenza virus infection progresses rapidly to involve all the cells in the culture. However, when the virus (H_3N_2 strains) is subpassaged to fresh CMK cell cultures, the CPE involves fewer cells in the monolayer than when the virus was initially isolated. Initial CPE of influenza virus can be confused with that of adenovirus in CMK cells.

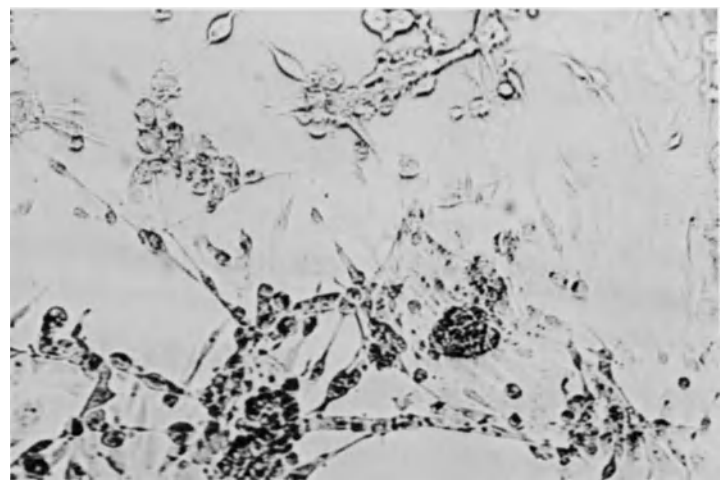


Figure 10–8. CPE produced by influenza virus in CMK cell culture (×100).

b. Parainfluenza Virus

(1) MRC-5

Parainfluenza virus replicates poorly in these cells compared with CMK cell cultures.

(2) CMK

Usually, little or no CPE is observed in cells infected with parainfluenza viruses, with the exception of parainfluenza virus type 2 or 3 which frequently causes the formation of syncytial cells (Figure 10–9) which can be easily confused with the CPE produced by a simian virus (SV5) (Figure 10–10). The other parainfluenza virus serotypes can produce a subtle, fine, granular, somewhat "lacy" appearance in the cell sheet which differs from that of the uninoculated control culture.

c. Mumps Virus

(1) MRC-5

These cells are inferior to CMK cells for the primary isolation of mumps virus.

(2) CMK

Mumps virus produces a characteristic, large, granular, syncytial cell formation which, however, usually does not involve more than half of the cells in the culture (Figure 10–11).

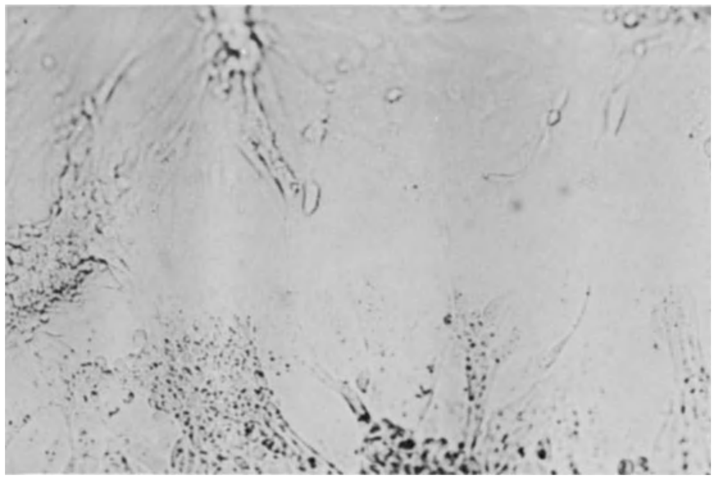


Figure 10–9. CPE produced by parainfluenza virus in CMK cell cultures (×100).

d. Measles Virus (Rubeola)

(1) MRC-5

These cells are inferior to CMK cells for the primary isolation of measles virus.

(2) CMK

Measles virus produces vacuolated syncytial cells in both cell cultures like that of respiratory syncytial virus; however, measles virus is rarely

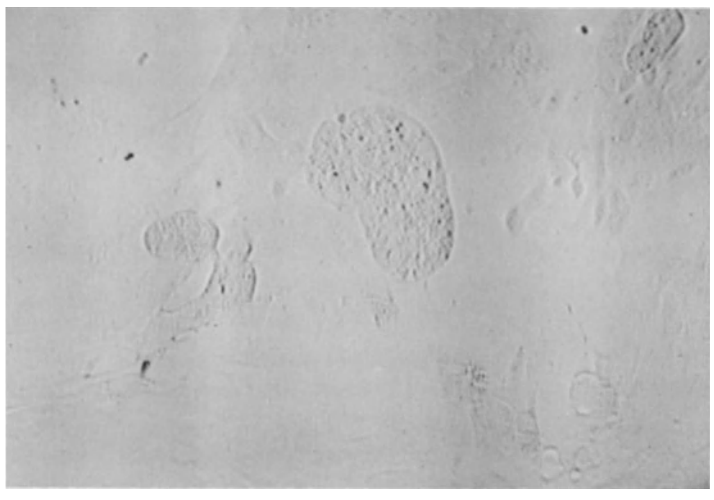


Figure 10–10. CPE produced by SV5 in monkey kidney cell culture (×100).

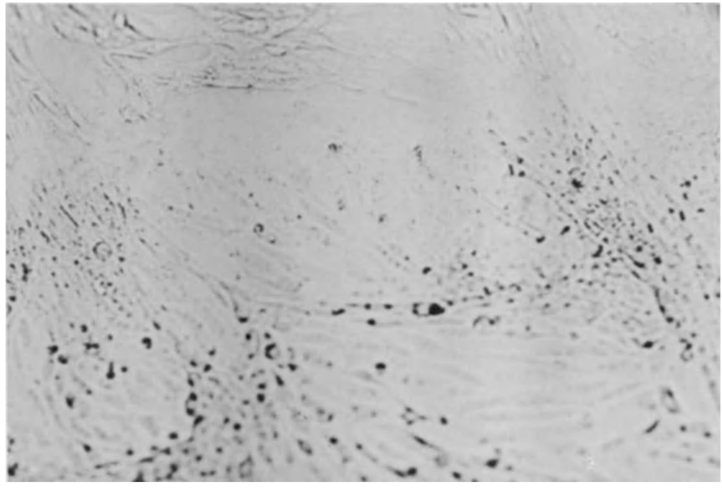


Figure 10–11. CPE produced by mumps virus in monkey kidney cell culture (×100).

isolated in the diagnostic virology laboratory because the disease is so readily recognized by physicians.

e. Respiratory Syncytial Virus

(1) MRC-5

CPE typically involves the entire monolayer, causing elongated syncytial and some rounded cells (Figure 10–12).

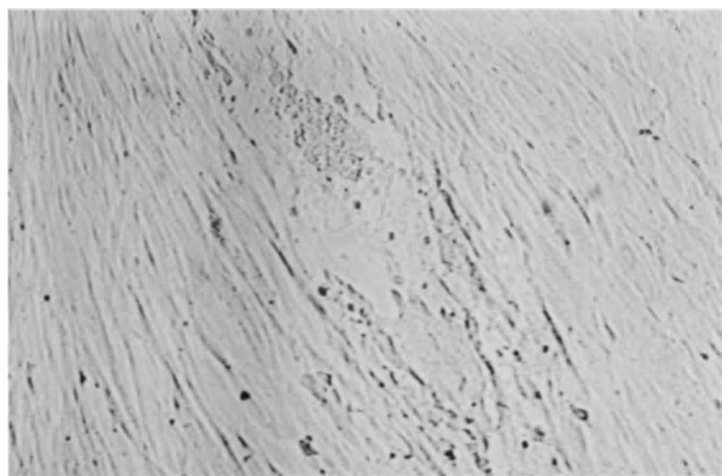


Figure 10–12. CPE produced by respiratory syncytial virus in MRC-5 cell cultures (×100).

(2) CMK
CPE is frequently produced in CMK cells; however, that produced in MRC-5 cell cultures is more specific with this virus.

5. Tests for Identification

Hemadsorption (HAd)

Purpose:
To detect ortho- and paramyxovirus infection in monkey kidney cell cultures.

Principle:
The hemagglutinin antigen of orthomyxoviruses (influenza virus) and paramyxoviruses (parainfluenza and mumps viruses) is incorporated into the

Table 10–7. Hemadsorption Patterns and Cytopathic Effects with Orthomyxoviruses and Paramyxoviruses

	Hemadsorption, 4°C to 20°C (% total isolates)				
Virus	Same	Increased	Decreased	Presence of CPE	Virus reported
Parainfluenza					
Types 1, 2, 3	17–28	2–9	58–80	Sometimes; granular lacy appearance of cells	Parainfluenza virus
Type 4	25	58	17	Syncytial cells	Mumps virus or parainfluenza virus[a]
Mumps	38	60	2	Syncytial cells	(Mumps virus or parainfluenza virus[a])
Influenza[b]					
A_2	91	2	7	Enlarged, rounded refractile cells of various sizes and shapes	Influenza virus
B	96	0	4	(Enlarged, rounded refractile cells of various sizes and shapes)	(Influenza virus)

[a] The clinical history is important because parainfluenza virus type 4 (although infrequently isolated) can also produce this HAd pattern.
[b] Erythrocyte agglutination usually is present in the fluid phase.

plasma membrane of the host cell just prior to the assembly of infectious virus.[62,97] The surfaces of such infected cells thus contain virus particles or antigenic components thereof to which erythrocytes of certain species attach. The resulting HAd forms the basis of a test described by Shelokov and colleagues[122,140] and provides a rapid and convenient method of detecting infection by these viruses in cell cultures, especially before CPE is apparent. Furthermore, the proportion of cells with attached erythrocytes at 4°C compared with that at 20°C frequently provides some indication as to whether or not the isolate is a parainfluenza virus rather than a mumps or influenza virus. Erythrocytes frequently elute from CMK cells infected with parainfluenza types 1, 2, and 3 (in contrast to mumps and influenza viruses), but HAd is best for type 4 at 20°C. Parainfluenza virus type 4 is seldom isolated in the laboratory (Table 10–7).

Guinea pig erythrocytes are smaller than fowl erythrocytes; therefore, many of these erythrocytes attach to one infected monkey kidney cell. The pattern of HAd formed by the guinea pig erythrocytes is much more uniform and easier to interpret than that of fowl erythrocytes.

Specimen:
Monkey kidney cell cultures of respiratory tract or urine (mumps) specimens.

Reagent:
0.4% suspension of washed guinea pig erythrocytes in serum-free medium. Erythrocytes are stable for 1 week.

Procedure:

1. Deliver 0.6 ml (0.2 ml/ml medium) of the erythrocyte suspension to each cell culture tube with an automatic Cornwall pipette (Becton, Dickinson and Company).
2. Place the tubes in racks slanted at a 5° angle for 1 hr at 4°C.
3. Examine the cultures at ×125 magnification for the presence of HAd or adherence of the erythrocytes to the cell surface. Determine whether agglutination of erythrocytes is present in the fluid phase.
4. Incubate the cultures for 1 hr at 20°C.
5. Examine the cell cultures again. Specific HAd is observed as rosettes and short chains of erythrocytes on the cell surface (Figure 10–13).

Interpretation:

Positive test: Hemadsorption is recorded on a scale of 1+ to 4+, similar to that used for CPE: ±, questionable; 1+, less than 25% of the cells have HAd; 2+, 25 to 50% of the cells have HAd; 3+, 51 to 75% of the cells have HAd; 4+, 76 to 100% of the cells have HAd.

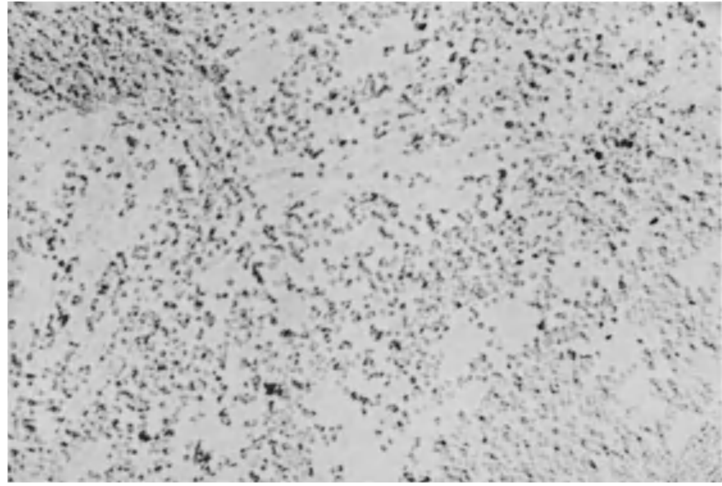


Figure 10–13. Hemadsorption of guinea pig erythrocytes on monkey kidney cells infected with parainfluenza virus (×100).

The erythrocytes may be arranged in the form of rosettes or short chains on the monkey kidney cells.

Negative test: Guinea pig erythrocytes floating freely in the medium; no areas of the cell monolayer on which aggregates of erythrocytes have collected.

Controls:

Positive: Known parainfluenza virus
Negative: Uninoculated cell culture

Fluorescent Antibody Test

Purpose:
Identification of parainfluenza isolates.

Principle:
Parainfluenza antigens can be detected using the indirect fluorescent antibody test.

Specimen:
Primary monkey kidney cell cultures infected for 2 to 3 days with a suspected parainfluenza virus isolate.

Reagents:
Antisera prepared in horses directed against the serotypes of parainfluenza virus. Fluorescein-conjugated antihorse serum is used as the conjugate.

Procedure:
Elute hemadsorbed erythrocytes in the cell culture infected with the suspected parainfluenza virus by incubation of the tube at 35°C overnight.

1. Infect 2 to 3 primary cell cultures with 0.1 ml of the suspension containing the paramyxovirus isolate.
2. After 2 to 3 days, scrape the cells in each culture into the fluid medium. Mix the cell suspension by placing the tube on a Vortex mixer.
3. Combine the contents from each tube and centrifuge the cell suspension at 700 × *g* for 5 min.
4. Wash the pelleted cells two times (5 ml/wash) with sterile PBS without Ca^{2+} and Mg^{2+}. Sediment the cells at 700 × *g* for 5 min.
5. Resuspend the washed cells in PBS without Ca^{2+} and Mg^{2+} using approximately 0.5 ml/cell culture harvested.
6. Add 1 drop of the cell suspension to 5 wells on a slide. Allow the cell suspension to dry at room temperature.
7. Fix the cells by placing the slide in acetone for 10 min at 4°C.
8. Use the indirect fluorescent antibody technique to identify the isolate as follows:
   a. Add specific parainfluenza and influenza virus type B antisera to the fixed cells deposited on the glass slide.
   b. Incubate the slide in a moist chamber at 35°C for 45 min.
   c. Place the slide in two washes of PBS without Ca^{2+} and Mg^{2+} (10 min each).
   d. Dry the slide in the incubator at 35°C.
   e. Add FITC-labeled antispecies antiserum to each of the areas on the slide containing cells.
   f. Incubate the slide in a moist chamber at 35°C for 45 min.
   g. Place the slide in two washes of PBS without Ca^{2+} and Mg^{2+} (10 min each).
   h. Dry the slide in the incubator at 35°C.
   i. Add a thin film of buffered glycerol solution to the slide and overlay with a coverslip.
   j. Examine the slide at 200 × magnification using a fluorescence microscope.

Interpretation:
The parainfluenza virus isolate is identified by specific fluorescence especially located at the outer plasma membrane of the infected cells. Because of the antigenic similarities among the parainfluenza viruses, crossreac-

tions occur; however, a quantitative difference in fluorescence in the homologous reaction compared with the other antisera should enable a specific identification to be made.

Influenza type B generally does not produce sufficient hemagglutination in microtiter plate assays to perform hemagglutination-inhibition tests for identification of this virus. The fluorescence procedure, therefore, provides a convenient alternative for identifying influenza type B isolates. Influenza virus type A can usually be identified by its ability to agglutinate fowl erythrocytes at a high titer.

Controls:

Positive control: known parainfluenza virus
Negative control: uninfected cells processed in the same way as infected cells.

Comment:
The hemadsorption-inhibition (HAd-I) test can be used as an alternative to the fluorescent antibody test for identification of parainfluenza viruses.[136] However, the HAd-I test requires greater quantities of reagents, and results are not as definitive as the fluorescence procedure. In addition, the identification of influenza type B isolates by the HAd-I method is not reliable.

Hemagglutination Test (HA) for Myxoviruses

Purpose:
To standardize the number of viral hemagglutinin units to be used in the hemagglutination-inhibition test (HAI).

Principle:
Certain viruses can react with specific receptor sites on the surface of red blood cells (RBCs) to produce a visible agglutination reaction.

Specimen:
0.025 ml of fluid from viral-infected cell culture.

Reagent:
0.5% washed goose RBCs (GRBC) in HEPES-saline-albumin-gelatin buffer (HSAG), pH 6.2 (Grand Island Biological Company). GRBCs are stable for 1 week.

Procedure:

1. To each of 10 consecutive wells in a row in a plastic microtiter plate (Lindbro Chemical Company) with U-shaped wells, add 0.025 ml of HSAG.
2. Transfer 0.025 ml of virus suspension with a calibrated loop or minidiluter (Cooke Engineering Company) into the first well (antigen dilution 1:2) of the microtiter plate; rotate the loop several times to ensure complete mixing of the contents. (*N.B.* Loops should be tested on delivery test cards to ensure that they deliver exactly 0.025 ml. When transferring fluid, the loops should be examined to make sure they are filled.)
3. Transfer a loopful of the contents of the first well to the second well and rotate the loop for mixing.
4. Continue serial dilution of the virus as described in Step 3. The antigen will be diluted 1:1024 in the last well.
5. With a calibrated dropper (Cooke Engineering Company), add 0.025 ml of HSAG buffer (0.025 ml) and then 0.025 ml of a 0.5% suspension of GRBC in HSAG buffer to each well.
6. Prepare three control wells on the same microtiter plate by adding 0.025 ml of 0.5% of GRBC suspension to 0.05 ml of HSAG buffer.
7. Tap the side of the plate firmly several times to disperse the GRBCs.
8. Record the HA end point promptly at 1 hr; the agglutination patterns are unstable due to elution of the virus at 20°C to 25°C.

Interpretation:

Positive test: The highest dilution of virus that produces 1+ to 2+ agglutination of the GRBCs is considered to contain one hemagglutinating unit (HAU). For example:

Virus dilution	HA result	HAU/0.025 ml
2	4+	
4	4+	
8	4+	
16	4+	4
32	4+	2
64	2+	1
128	−	
256	−	

A 4+ pattern consists of a uniform layer of GRBCs coating the entire bottom of the well.

Negative test: Nonagglutinated GRBCs settle in a compact sharply outlined button at the bottom of the microtiter plate well.

Controls:

Positive: Known influenza virus
Negative: Fluid from uninoculated cell culture

Comment:
Generally, the suspension containing the unknown influenza virus serotype is diluted to contain 4 HAU/0.025 ml. However, if the HA titer is only 1:4, the undiluted virus should be used in the HAI test in order to obtain 4 HAU of virus. Cellular debris is often present (due to CPE produced by influenza virus in CMK cell cultures) that can interfere with the HAI test; therefore, subpassage of some influenza isolates in the allantoic sac of embryonated eggs may be required to increase the HA titer. Subpassage of A/influenza virus isolates (H_3N_2) in monkey kidney cell cultures results in a loss of virus, as measured by HA, with concomitant loss of infectious virus on subpassage.

Hemagglutination-Inhibition (HAI)

Purpose:
To identify influenza virus isolates.

Principle:
HA produced by influenza virus can be inhibited by specific antisera as follows:

HA: Virus + RBCs → agglutination of RBCs

HAI: Virus + antibody → virus antibody reaction + RBCs (no further reaction)

Reagents:

1. 0.5% of washed goose RBCs (GRBCs) in HSAG buffer. GRBCs are stable for 1 week.
2. Immune chicken serum (Centers for Disease Control, Atlanta, Georgia), prepared by immunization of chickens with an influenza virus strain which was found to be prevalent in the United States during the previous year. The serum contains not only specific antibody but also a variety of nonspecific inhibitors which react with influenza virus and which when present will prevent HA by the virus. These nonspecific inhibitors can be selectively removed from reference sera containing influenza antibody by treating with receptor destroying enzyme (RDE) of *Vibrio cholerae.*[28]

a. Procedure for removal of nonspecific inhibitors

i. Add one volume (0.5 ml) of serum to four volumes (2.0 ml) of RDE. (Reconstitute RDE with 5 ml of sterile distilled water. Dispense in 1 ml aliquots and freeze at −20°C. Expiration date is 1 year.) Incubate overnight (12 to 18 hr) in a water bath at 35°C.

ii. Add three volumes (1.5 ml), based on original serum volume, of 2.5% sodium citrate solution. Heat at 56°C for 30 min.

Sodium citrate ($Na_3C_6H_5O_7$—$2H_2O$)	2.5 g
Distilled water to make	100 ml

Expiration date is 1 year.

iii. Add two volumes (1 ml), based on original serum volume, of HSAG to raise the starting serum dilution to 1:10. [If nonspecific inhibitors have not been completely removed by the above procedure, use a more concentrated RDE solution (prepare lower dilution of RDE stock in calcium saline solution).]

b. Procedure for removal of GRBC agglutinins. All influenza virus antisera should be adsorbed with GRBCs to remove agglutinins:

i. To each reference serum in a centrifuge tube, add five drops (0.025 ml GRBCs/0.1 ml serum used) of 50% GRBC suspension. Shake the tubes vigorously and store overnight at 4°C.

ii. Centrifuge the sera at 800 *g* for 20 min at 10°C (hemolysis of the GRBC will occur in the presence of GRBC agglutinins if warm).

iii. Decant the sera into vials (2 dram, screw-cap) and store at −20°C. The pH of the serum is 8.3. Final dilution of serum is 1:10.

Procedure:

1. Add 0.025 ml of HSAG buffer to each of nine consecutive wells starting with *the second well.* Leave the first well in each row empty. The number of rows to which buffer is added depends on the suspected occurrence of subtypes or strains in the community. For example, six rows would be required in the following situation:

Row	Antiserum	Antigen
1	A/Brazil/78 (H_1N_1)	A/Brazil/78 (H_1N_1)
2	A/Philippines/82 (H_3N_2)	A/Brazil/78 (H_3N_2)
3	A/Philippines/82 (H_3N_2)	A/Philippines/82 (H_3N_2)
4	A/Brazil/78 (H_1N_1)	A/Philippines/82 (H_3N_2)
5	A/Brazil/78 (H_1N_1)	Isolate to be identified
6	A/Philippines/82 (H_3N_2)	Isolate to be identified

2. Add 0.05 ml of the specific RDE-treated goose-adsorbed reference antisera to the first well in each of six rows as shown in the example above.
3. Dilute the antisera serially through well number 10 (1:1024) using a microtiter loop.
4. Add 0.025 ml of the standardized virus suspension containing 4 HAU (determined by the HA test) to each well.
5. Prepare the following controls, each in a final volume of 0.075 ml (0.025 ml of each component):
   a. Virus + GRBC + HSAG
   b. Serum + GRBC + HSAG
   c. GRBC + HSAG (.05 ml)
6. After the plates have incubated for 15 min at 20°C, add 0.025 ml of 0.5% GRBC suspension to each well.
7. Mix the contents of each well by tapping the side of the plate. Incubate the plates for 1 hr at 20°C.

Interpretation:

Positive test: The endpoint in the HAI test is the reciprocal of the highest dilution of serum that completely prevents the agglutination of GRBCs by virus. The identity of the unknown influenza virus isolate, presumed in the example to be influenza virus type A (H_3N_2), is determined by the relative amount of homology it shows (compared with other strains of influenza virus A) in reacting against the reference antisera. The results and interpretation of such a test follow:

Row	End-point	Conclusion
1	160	High homologous specificity.
2	20	Low heterologous cross-reaction.
3	80	High homologous specificity.
4	10	Low heterologous cross-reaction.
5	10	Low heterologous cross-reaction between A/Brazil/78 (H_1N_1) antiserum and A/Philippines/82 (H_3N_2) virus.
6	80	High titer specific inhibition with low cross-reaction with A/Brazil/78 (H_1N_1) antiserum. The isolated virus is therefore identified as A/Philippines/82 (H_3N_2).

Negative test: Partial or complete agglutination of GRBCs coating the bottom of the wells. (When neither influenza virus type A reference antiserum reacts with the unknown isolate, then reference antisera for other influenza viruses of type A or type B should be used.)

Controls:

Positive: Known influenza virus strains and their homologous antisera
Negative: Fluid from uninoculated cell culture

Comment:
Elimination of nonspecific inhibitors of influenza virus agglutination in sera other than from humans or chickens varies according to the specific strain of virus. Therefore, treatment of the serum with potassium periodate, or a combination of trypsin/periodate, or kaolin may be required.[30]

C. Togaviruses

1. Description

More than 350 agents belong to this group, which contains single-stranded RNA as its genetic material, is enveloped, possesses cubic icosahedral symmetry, and ranges in size from 35 nm to 40 nm in diameter. Togaviruses are classified into groups A and B on the basis of HAI testing. However, group C, or the Bunyamwera group, containing California virus, is classified on the basis of complement-fixation serology, which shows greater cross-reactivity among members of the group than does HAI. Rubella virus is classified with the togaviruses (rubivirus), although it resembles them more closely on the basis of morphological characteristics than in certain epidemiological features (such as the mode of transmission) and the clinical disease produced.

Arthropods, especially mosquitos and ticks, serve as reservoirs and vectors in the cycle of transmission (with the notable exception of rubella virus) of togaviruses. Several species of birds, rodents, and mammals may serve as intermediate hosts. Most agents in the togavirus group are endemic only in tropical areas.

2. Source and Growth Characteristics

Arboviruses can be recovered from the blood and less often from the throat and CSF only during the initial stages of diseases. Generally, the likelihood of viral isolation decreases once symptoms appear. Serological tests, rather than viral isolation, are recommended for laboratory diagnosis because of the risk of infection to laboratory personnel with these agents.[110] Furthermore, viral isolation requires the inoculation of infant mice, instead of cell cultures, and tedious subpassages of the isolate for specific identification.

Similarly, rubella virus can be recovered from the nasopharynx of

adults and products of conception from an infected fetus, but specialized cell culture techniques are required, such as African green monkey kidney cells and superinfection of infected cell cultures with a virus cytopathogenic for these cells. Alternatively, HAI antibodies to rubella virus develop rapidly and persist for life so that this serological test may be used to measure changes in antibody levels during acute infection, as well as to determine the immune status of women of child-bearing age.

3. Clinical Importance

Most infections with togaviruses are subclinical, but the clinically inapparent to apparent ratio varies. Generally, there are 100 to 1000 individuals with antibody to a togavirus for every clinically apparent case. While the majority of agents within the group are endemic only in tropical areas, the Western, Eastern, and Venezuelan equine encephalitis viruses, St. Louis encephalitis virus, and California encephalitis virus are most prevalent in the United States.

All reported cases of California virus encephalitis have been children, but relatively few cases occur among those less than 1 year of age in contrast to Western equine and St. Louis encephalitis viruses. The clinical presentation of California virus encephalitis ranges from mild fever, headache, and malaise to the sudden onset of focal or generalized seizures. Although California virus infections were at one time the most frequent cause of encephalitis in Rochester, Minnesota,[27] the prevalence of arboviral encephalitis has varied from year to year. Generally, the most common type of togavirus infection in the United States is encephalitis due to St. Louis virus,[80] followed closely by the California, Western equine, and Eastern equine viruses; however, only 3 confirmed cases of St. Louis encephalitis were reported in 1983, and California virus predominated as the cause of arboviral encephalitis.[90] Most cases of St. Louis virus infections present clinically as encephalitis (63%) or aseptic meningitis (15%),[22] but the spectrum of disease can range from one with very mild, almost imperceptible neurological signs to an extremely severe illness leading to death within days after onset.

Western equine virus generally causes an acute febrile illness characterized by inflammation of the meninges, brain, and spinal cord. Although the virus is widely distributed throughout the United States and Canada, disease occurs almost exclusively in the western states and Canadian provinces. The relative absence of disease in eastern United States probably reflects a paucity of the vector species, *Culex tarsalis,* and possibly a lower pathogenicity of local viral strains. Infections are common in children under 5 years of age, and infants under 1 year of age are most susceptible to develop severe encephalitis. The ratio of inapparent to apparent CNS disease is 50:1 in children and 1000:1 in adults. The case fatality rate is 3 to 4%.[15]

The association of congenital defects and rubella viral infection of the mother, especially during the first trimester of pregnancy, has focused considerable attention on this virus. Following the 1964 rubella virus epidemic in the United States, some 20,000 to 30,000 infants were born with the rubella syndrome. Rubella virus infections of pregnant women may result in a spontaneous abortion, stillbirth, or the expanded rubella syndrome. The major congenital defects are heart lesions, cataracts, deafness, microcephaly, and mental retardation. Other manifestations include thrombocytopenic purpura, bone lesions, hepatitis, hemolytic anemia, growth retardation, low birth weight, and diverse abnormal neurological findings.

4. Tests for Identification

Hemagglutination (HA) Test for St. Louis and Western Equine Viruses

Purpose:
To standardize the number of viral hemagglutinin units (HAU) to be used in the hemagglutination-inhibition (HAI) test.

Principle:
St. Louis and Western equine viruses can react with specific receptor sites on the surface of red blood cells (RBCs) to produce a visible agglutination reaction.

Specimen:
0.025 ml of serum.

Reagents:

1. 1.6% suspension of washed goose red blood cells (GRBCs) in dextrose veronal gelatin (DVG) buffer (prepared by making a 1:35 dilution of a 50% suspension). GRBCs are stable for 1 week.
2. Phosphate-buffered saline (PBS)

a. NaCl (1.5M)	87.67 g
Distilled water to make	1000 ml
b. Na_2HPO_4,anhydrous	283.96 g
Distilled water to make	1000 ml
c. $NaH_2PO_4 \cdot H_2O$	276.02 g
Distilled water to make	1000 ml

Stock Solution A	
NaCl	100 ml
Na_2HPO_4	100 ml
Distilled H_2O	800 ml

Stock Solution B	
NaCl	100 ml
NaH_2PO_4	100 ml
Distilled H_2O	800 ml

Solutions A and B are mixed in varying proportions, depending on the final pH desired in the working solution as follows:

Proportion of Stock Solutions Required to Produce Designated Final pH

Final pH[a]	Solution A(ml)	Solution B(ml)
5.8	3.0	97.0
6.0	12.5	87.5
6.2	22.0	78.0
6.4	32.0	68.0
6.6	45.0	55.0
6.8	55.0	45.0
7.0	64.0	36.0
7.2	72.0	28.0
7.4	79.0	21.0

[a] The final pH is determined by mixing equal volumes of PBS and 0.4% bovalbumin-borate-saline (BABS) solution, pH 9.0. The actual pH of the resulting mixture must be determined before use in the test.

3. Bovine-albumin-borate saline (BABS) solution, 0.4%

a. Borate solution, pH 9.0, 0.5 *M*

H_3BO_3	30.92 g
Hot distilled water (dissolve, then cool)	700 ml
Distilled water to make	1000 ml

b. Borate-saline solution, pH 9.0

NaCl	80 ml
H_3BO_3, 0.5 *M*. (solution a)	100 ml
NaOH, 1 *N*	24 ml
Distilled water to make	1000 ml

Determine pH and adjust to pH 9.0 if necessary.

c. 4% bovalbumin (Fraction V)

Bovalbumin	4.0 g
Borate-saline solution (solution b) to make	100 ml

d. 0.4% bovine-albumin-borate-saline (BABS solution

4% bovalbumin (solution c)	100 ml
Borate-saline (solution d)	900 ml

All antigen dilutions should be made in BABS solution. Sera are diluted 1:5 in borate-saline solution before kaolin absorption. Expiration date is 6 months.

4. Antigens. These are obtained from the Centers for Disease Control, Atlanta, GA. Rehydrate each vial of antigen with 1 ml distilled H_2O and store for 1 hr at 4°C before use. Prior to the test, dilute the antigen with BABS solution and store at 4°C for 1 hr to dissolve the antigen completely.

Procedure:

1. Add 0.025 ml of BABS solution to each of the wells in six rows. The first two rows are labeled pH 5.8; the next two, pH 6.0; and the last two, pH 6.2.
2. Add 0.025 ml of a 1:10 dilution of antigen (in BABS solution) to the first well in each row.
3. Serially dilute the antigen from 1:2 to 1:2048 with the calibrated microdiluter loops.
4. Add 0.025 ml of BABS solution to each well.
5. Add 0.025 ml of adult white male goose (G) RBCs (GRBC), diluted 1:35 [adjusted to pH 5.8, 6.0, or 6.2 with the PBS-BABS mixture (p. 573)] to the appropriate rows. For example, the first two rows will receive GRBCs suspended in a buffer of pH 5.8.
6. Tap the plates so that the GRBCs are uniformly distributed within the wells.
7. For *each* specific pH tested, at least three wells with RBC controls are included. Each control well will contain two drops of BABS solution and one drop of RBC suspension.
8. Cover the plate with plastic tape and incubate for 1 hr at 35°C.

Interpretation:

Positive test: The end point of the HA titration is the highest dilution of virus which produces a 2+ to 4+ agglutination of the GRBCs. This dilution contains one unit of antigen. The optimal pH to be used in the test is based on a uniform hemagglutination pattern of the GRBCs. Four to eight units of antigen are used in the test. (See p. 566 for influenza virus titration.)

Negative test: Nonagglutinated GRBCs settle in a compact, sharply outlined button at the bottom of the microtiter plate well.

Comment:
In contrast to the myxoviruses, optimal hemagglutinating activity of togaviruses is pH dependent. Furthermore, the togaviruses vary in the pH required for maximum HA titers. Therefore, it is necessary to titrate the antigen in solutions of different pH to determine the dilution of antigen to be used in the HAI procedure.[124]

Hemagglutination-Inhibition (HAI) for Measuring Antibodies to St. Louis and Western Equine Viruses

Purpose:
To measure antibodies to St. Louis or Western equine virus in serum.

Principle:
Antibodies in serum combine with antigen to prevent agglutination of GRBC.

HA: Virus + RBCs → agglutination of RBCs

HAI: Virus + nonspecific inhibitors (removed by kaolin treatment) and antibody → virus antibody reaction + RBCs (no further reaction)

Reagents:

1. 1.6% suspension of washed goose RBCs (GRBCs) in dextrose veronal-gelatin (DVG) buffer (prepared by making a 1:35 dilution of a 50% suspension). GRBCs are stable for 1 week.
2. Antisera. Reference antisera prepared to St. Louis and Western equine viral antigens are obtained from Centers for Disease Control, Atlanta, GA. Rehydrate each vial of antiserum with 1 ml distilled H_2O and store for 1 hr at 4°C before use.
3. Kaolin. Prepare by adding 100 g of kaolin (BDH Chemicals Ltd., Poole, England) to 400 ml of 1 *N* HCl and stirring the mixture for 1 hr. Collect the kaolin on filter paper in a Buchner funnel and wash it with distilled water until no trace of acid is detected in the filtrate. Dry the washed kaolin at room temperature overnight and then grind it to a fine powder with a mortar and pestle. Add 25 g of the acid-washed kaolin to 100 ml of borate-saline solution, pH 9.0 (p. 574). After thorough stirring, measure the pH of the suspension. If the pH

is less than 8.5, centrifuge the kaolin at 250 × *g* for 5 min, discard the supernatant fraction, resuspend the sedimented kaolin in fresh borate-saline solution, pH 9.0, and measure the pH again. If the pH is still less than 8.5, repeat the procedure until pH 8.5 is achieved. The suspension is stored at 4°C. The pH of the kaolin suspension is rechecked prior to each test. Expiration date is 1 year.

Specimen:
0.025 ml of serum. Because normal human serum contains nonspecific inhibitors of togavirus hemagglutination and agglutinins for certain species erythrocytes, the test serum and reference antisera must be treated to eliminate these interfering agents.

1. Procedure for removal of nonspecific inhibitors.
   a. Add 0.4 ml borate saline solution, pH 9.0, and 0.5 ml kaolin to each 0.1 ml of serum (including control sera) in a plastic centrifuge tube.
   b. Cover the top of each tube with plastic tape and hold the mixture at 20°C for 20 min with periodic shaking.
   c. Centrifuge at 800 × *g* for 30 min at 20°C.
   d. Remove the supernatant fraction and transfer to a clean centrifuge tube. At this point in the procedure the serum has been diluted 1:10 (0.1 ml serum, 0.4 ml borate saline, and 0.5 ml kaolin).
2. Procedure for removal of GRBC agglutinins.
   a. Add 0.025 ml of 50% GRBCs to each serum specimen, shake the tubes, cover with tape, and allow to stand for 20 min at 4°C. Shake the tubes occasionally to mix the contents.
   b. Centrifuge the sera at 800 × *g* for 20 min at 4°C to sediment the GRBCs.
   c. Dispense the sera into vials (3.5 ml screw-cap) and store at 4°C overnight or −20°C indefinitely.

Procedure:

1. To each, except the first, of seven consecutive wells (1:640 dilution) in each row of a round-bottomed microtiter plate, add 0.025 ml of BABS solution (p. 574).
2. For control wells, prepare the following:
   a. *Test serum GRBC agglutinin control.* Add 0.025 ml of BABS solution to the last well in each row.
   b. *GRBC.* Add 0.025 ml of BABS solution to three consecutive wells near the bottom of the plate.
   c. *Antigen.* Add 0.025 ml of BABS solution to three consecutive wells near the bottom of the plate.
   d. *Back titration of antigen.* Add 0.025 ml of BABS solution to the first six wells in a row at the bottom of the plate.

e. *Serum with and without St. Louis and Western equine virus antibody.* Add 0.025 ml of BABS solution to each of seven consecutive wells in one row as described in Step 1 above.
3. Add 0.05 ml of the kaolin-GRBC-treated test serum (p. 577) with a disposable dropper into the first well of the microtiter plate. The serum will be diluted 1:10 in the first well. Serially dilute the serum. Add a loopful of test serum to the last well in the row (for GRBC agglutinin controls). Repeat this step for each serum sample and the positive and negative control sera.
4. Add 0.025 ml of the diluted antigen (containing 4 to 8 HAU) to the serum dilutions. No antigen is added to the test serum-GRBC agglutinin or the GRBC control wells. For the back titration of antigen, add 0.025 ml of BABS solution to five consecutive wells in one row. Add 0.025 ml of antigen dilution [that dilution used in the HI test (p. 568)] to the first well. Serially dilute the antigen with the microtiter loop. Add 0.025 ml of BABS solution to each well. Then add 0.025 ml of the GRBC suspension to each well.
5. Tap the side of the plate to mix the contents. Allow the plates to incubate at 20°C for 4 hr.
6. Dilute the washed 50% suspension of GRBC 1:35 (prepared by adding 0.025 ml GRBCs and 0.85 ml PBS-BABS) in the appropriate buffer with the optimal pH determined by the HA test (p. 573).
7. Add 0.025 ml of GRBCs to each of the buffer-containing wells.
8. Tap the sides of the plates to mix the contents, cover the wells with plastic tape and incubate at 35°C for 1 hr.

Interpretation:

Positive test: The end point in the HAI test is the reciprocal of the highest dilution of serum which prevents the agglutination of GRBCs.

Negative test: Nonagglutinated GRBCs settle into a compact, sharply outlined button at the bottom of the microtiter plate well.

Controls:

Positive: Reference sera known to contain antibody to St. Louis or Western equine virus

Negative: Pooled human sera from normal individuals with no detectable antibody to St. Louis and Western equine viruses

Comment:

A fourfold rise in antibody titer between acute and convalescent phase sera is considered diagnostic of infection with that virus. Because inapparent infection is common in endemic areas, titers of $\leq$1:160 in single specimens probably do not indicate recent infection.[123]

Counterimmunoelectrophoresis (CIE) for Detection of Antibodies to California Virus

Purpose:
To detect antibodies to California virus in serum.

Principle:
A precipitin line is formed by the migration of antibodies into California virus antigen according to principles described elsewhere (p. 270). The first application of CIE in virology was for detection of hepatitis B surface antigen. Later, the test was used for the identification of rotavirus in stool specimens. In both of these instances, CIE was useful because of the high concentration of the antigenic components of these agents in clinical specimens and because they failed to grow in conventional cultures. CIE has also been used, following concentration of the viral antigens, to serotype echo and coxsackievirus (type B).[81] Practical applications of CIE to detect other viral antigens in clinical specimens from patients with CNS infection have been hampered by cross-reactions and lack of sensitivity.[11]

Balfour and Edelman[4] found that CIE was as sensitive as other serological tests and were able to identify 41% of patients with California virus encephalitis during their acute illness. Furthermore, they found that precipitating antibody decayed to undetectable levels in a few weeks after acute infection. Therefore, the precipitins seen in the CIE test are an important marker of active infection.

Specimen:
Approximately 20 μl of serum (quantity sufficient to fill agar well).

Reagents:
The reagents and the preparation of the agarose-coated glass slide are as described elsewhere (p. 270).

1. California virus antigen. LaCrosse strain of CV (initially obtained from H. H. Balfour, Jr., M.D., University of Minnesota) is subpassaged five times by intracerebral inoculation of infant mice.
   a. Prepare 10^{-1} dilution of the seed La Crosse virus strain in serum-free Eagle's minimal essential medium (MEM).
   b. Inoculate infant mice (1 to 4 days old) intracerebrally with the virus suspension (0.03 ml/mouse).
   c. Kill the mice when the majority of the animals exhibit signs of encephalitis, usually 2 to 3 days after inoculation.
   d. Remove the brains from the mice, grind with a chilled pestle and

mortar, and dilute to a 50% suspension (v/v) in cold tris (hydroxymethyl) aminomethane (Tris)-buffered saline, pH 8.

e. Clarify the brain suspension by centrifugation at 35,000 ×*g*.

f. Dispense the supernanant fraction, containing the antigen, into vials and store at −70°C.

2. California virus antiserum. Reference antiserum to the La Crosse strain of California virus, supplied through the courtesy of Dr. Henry Balfour, produces a 4+ precipitin reaction with the antigen in the CIE test.

Procedure:
The electrophoresis procedure and test conditions are identical to those described elsewhere in this volume for electrophoresis of bacterial antigens, including a power supply of 60V for a current of 12 to 15 mA, 60 min of electrophoresis, a cooling period of 15 min at 4°C, and slide storage at 4°C for at least 24 hr for possible reexamination.

Interpretation:

Positive test: A precipitin line forms between the antigen and corresponding antibody well. The line should be straight or only very slightly arced.

Negative test: No precipitin line forms between the antigen and the corresponding antibody well.

Controls:

Positive: Serum containing precipitating antibody from patient known to have had California virus encephalitis.

Negative: 1. Serum without precipitating antibody to California virus.
2. Normal mouse brain tissue extract, processed in exactly the same manner as the virus-infected preparation, should not produce a precipitin line after reaction with serum containing precipitating antibody to California virus.

Enzyme-Linked Immunoassay Test for Measuring Antibody to Rubella Virus (Rubazyme™, Abbott Laboratories, North Chicago, IL)

Purpose:
To assess immune status to rubella virus and to diagnose acute rubella virus infections.

Principle:
In the Rubazyme test, specific rubella virus antibody binds with antigen attached to a plastic-coated bead. After removing unreacted serum com-

ponents from the bead, antihuman IgG conjugated with horseradish peroxidase is added and binds to the antigen-antibody complex. The presence of the reaction is determined by incubating the washed bead with the substrate *o*-phenylenediamine·2HCl. A yellow-orange product develops, which is measured in a spectrophotometer adjusted to a wavelength of 492 nm.

Specimen:
0.5 ml of serum (2 ml sterile clotted blood).

Procedure:
Materials, reagents, and control sera are provided in the kit by the manufacturer. Washing and spectrophotometric equipment are obtained by lease through Abbott Laboratories. An insert is included with each kit describing the complete test procedure.

Interpretation:

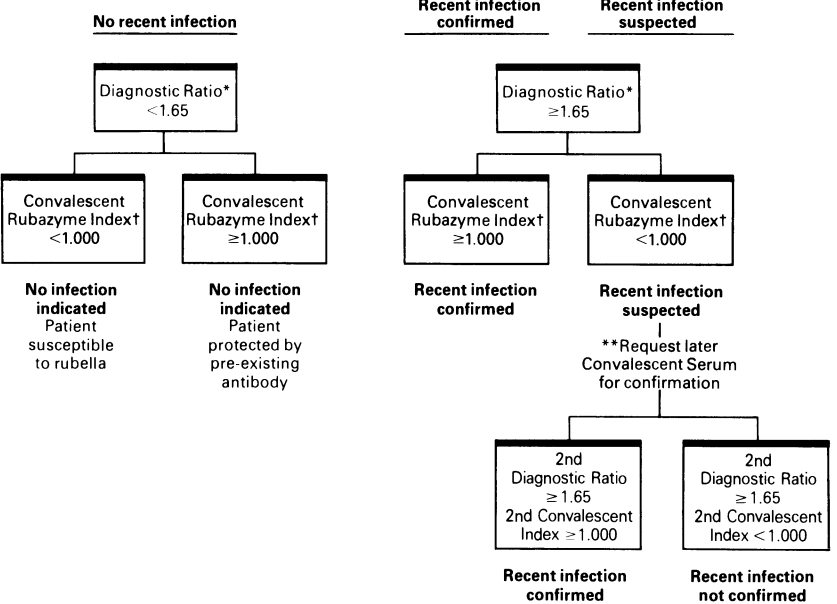
Immune Status
Antibody levels to rubella virus are calculated by determining a "Rubazyme Index." This is obtained by dividing the absorbance value of the test specimen by the mean value of two low-positive control (LPC) sera provided in the kit.

1. Specimens with an absorbance value equal to or greater than the LPCx are positive for rubella IgG antibody by the Rubazyme test (Figure 10–14).
2. Specimens with an absorbance value less than the LPC are negative for rubella IgG antibody by the Rubazyme test.

Recent Infection
The Rubazyme assay is performed in duplicate using acute (S1) and convalescent phase (S2) sera that have been obtained from an individual with suspected rubella virus infection. The "Rubazyme diagnostic ratio" for the acute and convalescent pair is obtained by dividing the mean absorbance values of the S2 by those values of S1. A Rubazyme diagnostic ratio of ≥1.65 indicates recent infection with rubella virus.

Comment:
Our laboratory also uses a Rubazyme-M procedure in kit form from Abbott Laboratories for the specific determination of IgM-class antibodies to rubella virus. By this test, a laboratory diagnosis of acute rubella virus infection can be obtained by testing only one serum specimen. The basic methodology of this assay is the same as that for the Rubazyme test for IgG-class antibodies. A Rubazyme-M index greater than 1.090 is positive and confirms the existence of primary rubella infection.

As in HAI, when testing for recent infection, Acute and Convalescent serums must be run in the same assay. There are three possible results:



The Rubazyme value of the convalescent serum is divided by the acute value. This gives us a Rubazyme Diagnostic Ratio. If the Diagnostic Ratio is less than 1.65, there was no recent infection indicated. In addition, if the convalescent Index is less than 1.000, we know that the patient is negative for IgG antibody, and should be considered susceptible to rubella. If the convalescent Index is equal to or greater than 1.000, we know the patient is protected by pre-existing antibody.

**In this case, the Rubazyme Diagnostic Ratio has detected a significant increase in antibody level but the patient has not yet seroconverted. A later convalescent sample should be taken and tested with the original acute sample. If the third specimen Index is equal to or greater than 1.000, the patient has seroconverted and recent infection is confirmed. If not, infection is still suspected but not confirmed by the Rubazyme assay.

An IgM or HAI test may be used for confirmation instead.

*Rubazyme Diagnostic Ratio = Convalescent Serum/Acute Serum
†Rubazyme Index = Patient O.D. Value/Low Positive Control Mean

Figure 10–14. Interpretation of Rubazyme™ results in serodiagnosis. (© 1981, Abbott Laboratories. Used with permission.)

The Rubazyme-M assay is used in our laboratory to determine acute phase rubella virus infection, especially if only one serum specimen from the patient is available. In addition, however, we routinely use the Rubazyme-M method for the determination of IgM-class antibodies in infants less than 6 months of age. Since these antibodies are not transferred across the placental barrier to the fetus, in contrast to antibodies of the IgG class, their presence indicates *de novo* synthesis by the infant and hence active infection with rubella virus.

Many kits have been produced for the determination of immune status and for the detection of acute rubella virus infection. The methods are as follows: hemagglutination-inhibition, enzyme immunoassay, passive

hemagglutination, immunofluorescence, radioimmunoassay, latex agglutination. The Centers for Disease Control has a premarket evaluation program designed to provide quality assurance of an acceptable product.[16] In addition, each individual laboratory should compare test results obtained with any new kit methodology with an established procedure known to yield accurate results for measuring rubella virus antibody before changing methodology.[129]

D. Rotaviruses

1. Description

These viruses (60 nm to 70 nm in diameter), also referred to as reovirus-like agents, duovirus, orbivirus, and infantile gastroenteritis virus, are classified within the Reoviridae and have a double-stranded RNA enclosed in a double-layered capsid with a clearly defined outer layer appearing like the rim of a wheel set upon an inner capsid of subunits projecting radially, like spokes from a central core. Up to four serotypes have been identified.[138] These viruses are antigenically and morphologically related to viruses causing diarrhea in many animals.

2. Source

Up to 10^9 to 10^{10} viral particles/g of feces can be detected during the first few days of illness. Particles are almost never seen in stool specimens obtained during convalescence from the infection.

3. Clinical Importance

Studies performed from 1964 through 1967 revealed that adeno and entero-viruses were found with equal frequency in children with diarrhea and from an asymptomatic matched control population.[148] Subsequently, rotaviruses were detected in duodenal biopsy tissue from children with nonbacterial gastroenteritis and these viruses have been found to be the cause of several other outbreaks.[6,50,137] Infection in infants and children may be subclinical, of varying degrees of severity, and at times fatal.[85,147] Symptoms include diarrhea, vomiting, fever, and abdominal pain. The diarrhea may last for up to 5 to 8 days. Rotaviruses were associated with approximately 34% of infections in children with gastroenteritis.[8] The peak prevalence occurs in children (6 months to 2 years of age) during the winter months. By the age of 6 years, 60% to 90% of children have specific antibody to these agents. Adults may have only mild symptoms.

4. Growth Characteristics

Rotaviruses from humans do not replicate efficiently except after adaptation in certain cell cultures and after treating the virus with trypsin.[5] A number of techniques have been described for their detection, including electron microscopy, counterimmunoelectrophoresis, radioimmunoassay, and enzyme-linked immunosorbent assay. Several serological assays have been developed which are based on the antigenic similarity between the RVLA in humans and those in animals which may be propagated in some cell cultures.

5. Tests for Identification

Enzyme-Linked Immunoassay Test for Detecting Rotavirus (Rotazyme™, Abbott Laboratories, North Chicago, IL)

Purpose:
To detect rotavirus.

Principle:
When specimens containing the virus are incubated with specific antibodies bound to a coated bead (anti-rotavirus), a complex is formed. This immunocomplex can be detected by reaction with an antibody-enzyme (peroxidase) conjugate followed by the addition of a substrate (*o*-phenyl enediamine · 2HCl) with the formation of a colored product that is measured spectrophotometrically.

Specimen:

Liquid stools: a 1:3 (v/v) dilution is made with the sample diluent supplied with the kit.
Formed stools: an applicator stick can be used to transfer material to 0.4 ml of the sample diluent supplied with the kit to give an approximate dilution of 1:3 (v/v).

Reagents, Procedure, and Interpretation:
Explicit directions are provided as an insert with each kit.

Comment:
Detection of rotavirus by enzyme immunoassay has been found to be as sensitive as electron microscopy (Figure 10–15), a technique previously used in our laboratory.[117,127,137] In addition, the Rotazyme test obviates

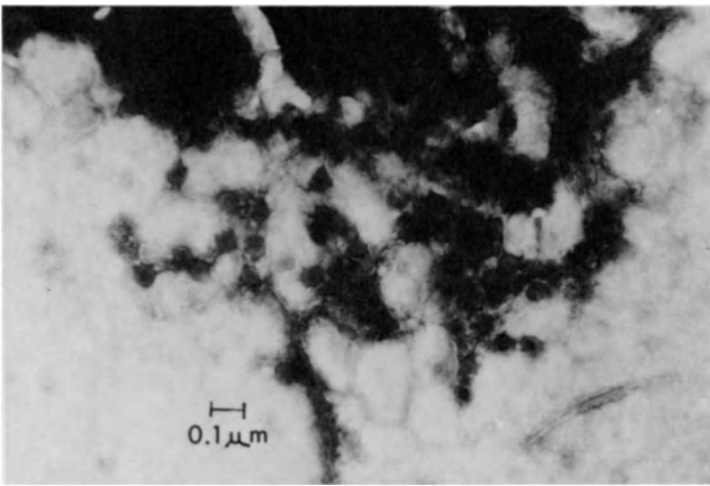


Figure 10-15. Electron micrograph of rotavirus-like particles in stool specimen of patient with gastroenteritis (×62,500). From Taraska et al.[137]

the use of expensive microscopic equipment and is amenable to rapid processing of several specimens each day.

Comment:
Another common virus causing gastroenteritis is the Norwalk agent which contains DNA and is classified as a parvovirus. Because of its small size (27 nm in diameter), the virus particles must be aggregated by homologous antiserum to be detected by EM techniques. Like the rotavirus, the Norwalk agent does not produce CPE in conventional cell cultures. Infections with the Norwalk agent tend to occur in family or community-wide outbreaks affecting adults, school-age children, and family contacts. These infections occur all during the year but most frequently from September to March. Related viruses are the Hawaii, Montgomery County, Ditchling, Marin County, Snow Mountain, and W viruses.

Other viruses, incapable of growth in cell cultures, may be etiological agents of gastroenteritis.[115,136]

V. Identification of DNA-Containing Viruses

A. Herpesviruses

1. Description

The major herpesviruses that infect man are herpes simplex (HSV), varicella-zoster (VZ), cytomegalovirus (CMV), and Epstein-Barr virus (EBV).

Their morphology is similar, and they range in size from 180 nm to 200 nm in diameter. The complete viral particles contain a DNA core surrounded by a protein capsid and an envelope which contains both host cell and viral components.

HSV can be divided into types 1 and 2 based on antigenic, biochemical, and biological differences. A single serotype causes varicella (chickenpox) and zoster (shingles). CMV has a widespread distribution in humans and numerous other mammals. Infection with these viruses are very species-specific and produce characteristic enlarged cells containing intranuclear and cytoplasmic inclusions. The older terminology of "salivary gland virus" results from the finding of cytomegalic cells in the salivary glands of infected children.[116] Strains may differ antigenically, suggesting the occurrence of more than one serotype in humans.

Although EBV resembles the herpesvirus group morphologically, it is antigenically distinct. The virus was initially detected by electron microscopy in lymphoma by Epstein et al. in 1964.[32]

Collectively, the herpesviruses comprise about 60% of the isolates in our laboratory. Most of these, 42% during one year, were HSV, but the number of isolates of VZ (8%) and CMV (6%) has steadily increased each year.

2. Source

HSV can be recovered from many anatomical sites: the oral cavity and lips, genitalia, eye, and skin. It has been recovered from tissue (brain, lung) but rarely from CSF and blood. In contrast, VZ is isolated almost exclusively from dermal vesicles, while CMV is most often recovered from urine and less often from the oropharynx. Although EBV is present in oral secretions, it is the only member of the herpesvirus group which cannot be recovered in conventional cell cultures used for viral diagnosis.

3. Clinical Importance

HSV causes several clinically significant infections, including a primary infection, gingivitis, in individuals without demonstratable antibodies to the virus. Although primary infection usually occurs in early childhood, it has been recognized in adults. The virus may remain in the body in a latent or inactive form and cause recrudescent infections following stressful situations, such as fever, emotional crises, and occasionally during menstruation. Recurrent infections usually present as lip lesions or herpes labialis; however, dissemination of the virus may occur, particularly in immunosuppressed hosts, which involvement of the trachea, esophagus, lungs, eyes, liver, and brain. Intact skin usually protects against HSV infection, but lesions may develop after exogenous exposure to the virus

in those individuals with skin diseases or in instances where the skin is abraded.

Genital infections due to HSV are common causes of sexually transmitted disease in both sexes[20]; however, the most important complication of this infection is its transmission to the newborn as it passes through the birth canal. Vary rarely, the fetus may be infected with the virus in utero. The prognosis with congenital or neonatal HSV infections is poor.

Chickenpox and zoster are manifestations of infection by the same virus. Primary infection with VZ virus results in chickenpox, approximately 85% of cases of which occur by the age of 9 years. Typically, vesicular lesions develop on the trunk of the body and then spread to the extremities. Complications are rare in normal hosts (0.3%) and usually involve the central nervous system.[26] The virus has been reported to cause severe congenital defects. VZ infections in children with neoplastic disease can be life-threatening, but those who are exposed and susceptible to the infection can be effectively protected with zoster-immune globulin.[96,143]

Herpes zoster, or shingles, occurs in those who have been previously infected with VZ virus. Apparently, the virus can remain latent after primary infection, perhaps in the sensory ganglia, and then be activated by predisposing factors, such as cancer chemotherapy, local irradiation, and immunosuppression of the host.[26,143] Patients with zoster usually experience localized pain in the infected area preceding the eruption of vesicles. Lesions occur most frequently in a unilateral distribution in the thoracic area and less commonly in the cervical and lumbosacral regions. Neurological complications of zoster include encephalitis, meningitis, myelitis, and peripheral neuropathies. Adenine arabinoside (vidarabine, ara-A) therapy is most effective in those patients with zoster having reticuloendothelial malignancies. Acyclovir is active against VZ virus in vitro and is being evaluated for clinical use.[57]

Like HSV, CMV may infect many organ systems and produce a wide variety of clinical problems. Clinically apparent infections occur most commonly in renal transplant patients receiving immunosuppressive therapy, although patients with neoplastic disease may also develop clinical evidence of infection with the virus. Symptoms usually appear within 2 months after renal transplantation and include fever, arthralgias, pneumonitis, and leukopenia, but hepatitis and retinitis can occur in later months.[19] Chronic viruria is common even in asymptomatic patients so that recovery of the virus from this source is not diagnostic of a disease process. Previous infection by CMV in a renal transplant recipient may determine whether the infection becomes symptomatic. Symptomatic CMV infection was likely to occur in renal transplant recipients who were seronegative before transplantation but received organs from seropositive donors.[58] The virus may also be transmitted by blood transfusion.[1]

CMV infection is not always limited to the immunologically compromised host, and CMV mononucleosis has been reported in adults.[103] Finally, CMV is the most common viral cause of congenital abnormalities, surpassing even rubella virus, and it is estimated that one of every 1000 infants is seriously retarded by congenital CMV disease.[134] In addition, it appears that newborns who excrete the virus, but are apparently asymptomatic, can manifest subtle mental defects later in childhood.[52]

EBV has been shown to cause infectious mononucleosis (IM) and has been associated with certain types of cancer (Burkitt's lymphoma and nasopharyngeal carcinoma) and proliferative disorders,[36,53,135] central nervous system disease,[59] cytomegalovirus-like disease in renal transplant recipients,[31] and the posttransfusion syndrome.[82]

4. Growth Characteristics

a. Herpes Simplex Virus

(1) MRC-5

Early CPE is marked by the presence of large, amorphous, globular cells most easily seen at the edges of the monolayer. With advanced infection, the cells shrivel into aggregates of various sizes (Figure 10–16). MRC-5 cell cultures are much more sensitive to infection and to the production of characteristic CPE by HSV than are CMK cell cultures.

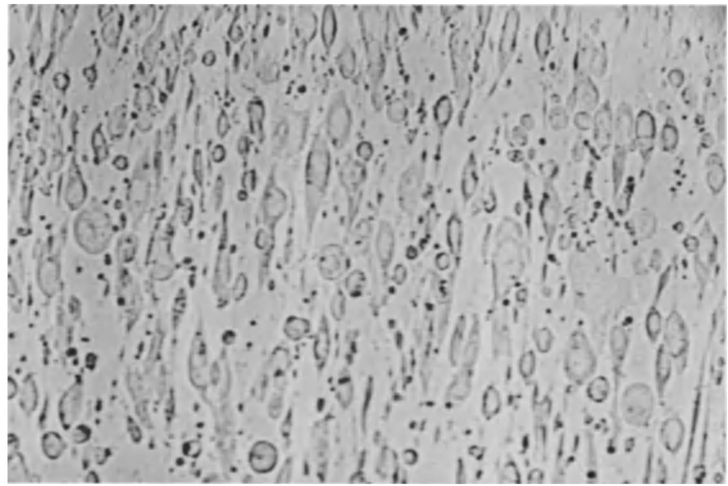


Figure 10–16. CPE produced by herpes simplex virus in MRC-5 cell culture (×100).

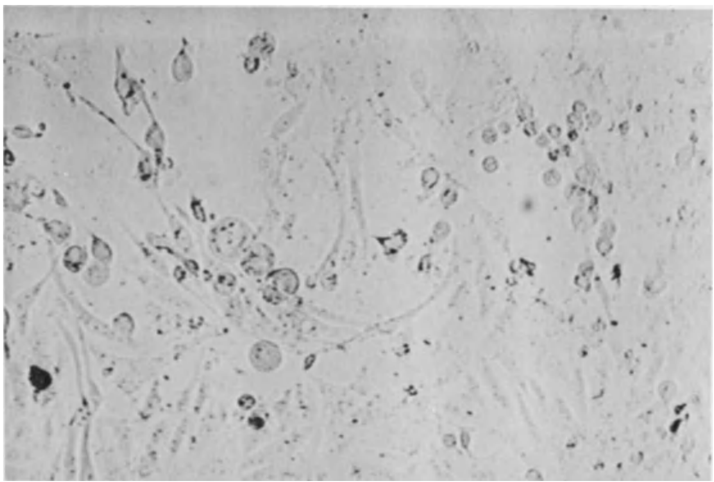


Figure 10–17. CPE produced by herpes simplex virus in monkey kidney cell culture (×100).

(2) Primary rabbit kidney (PRK)

Initial studies indicated that PRK detected HSV in 100% of 35 positive specimens, in contrast to 80% detected in MRC-5 cells.[77] However, two recent studies have indicated that either diploid fibroblasts or PRK may be used with equal efficiency for the recovery of HSV isolates.[13,89] CPE in PRK, characterized by round, swollen cells, especially at the edges of the monolayer, is easily detected and occurs earlier than in MRC5–5 cells.[13]

(3) CMK

Only 10 to 20% of HSV isolates produce CPE with swollen dense cells in clusters, especially at the edges of the monolayer (Figure 10–17).

(4) HEK

Infection proceeds rapidly producing enlarged, rounded cells (Figure 10–18). Frequently, syncytia can be seen. Ultimately, all cells demonstrate CPE, and many slough off the glass surface.

b. Varicella-Zoster Virus

(1) MRC-5

CPE develops slowly, or sometimes not at all, during the usual incubation period of 14 days and is characterized by granular, irregularly shaped,

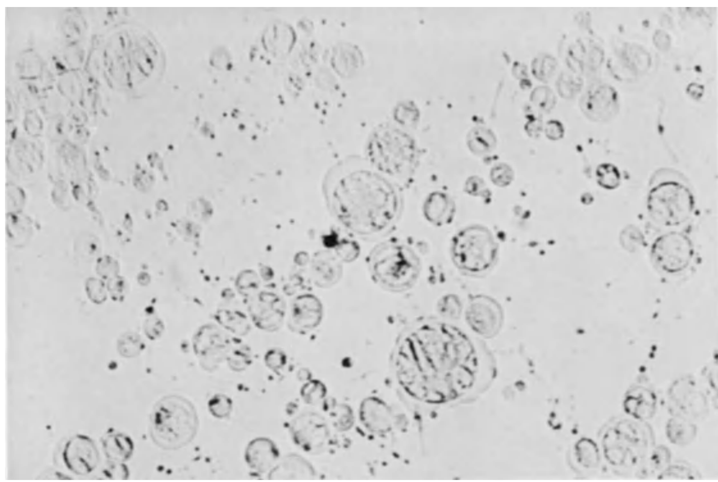


Figure 10–18. CPE produced by herpes simplex virus in HEK cell culture (×100).

sometimes shrunken, cells which appear in discrete plaques (Figure 10–19). Usually less than 10, and often only 1 or 2, such plaques will be seen surrounded by a field of normal fibroblasts. As the infection progresses, these infected cells slough off the glass. If no CPE is apparent after 14 days of incubation, the cells and fluid from the culture are subpassaged separately into fresh MRC-5 cultures.

(2) CMK
CMK cell cultures are resistant to infection with VZ virus.

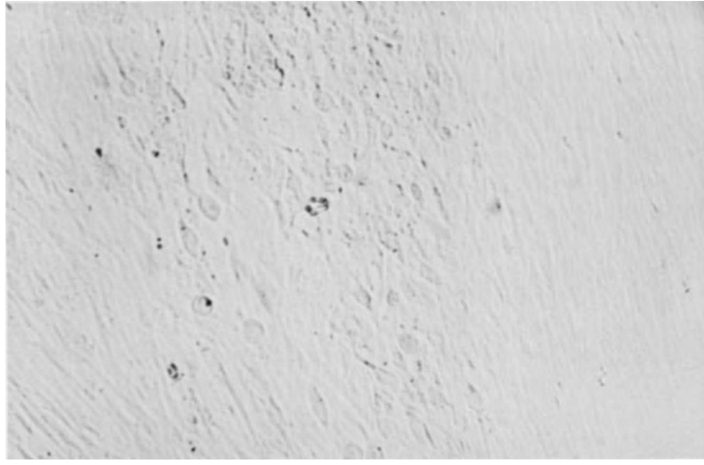


Figure 10–19. CPE produced by varicella-zoster virus in MRC-5 cell culture (×100).

c. Cytomegalovirus

(1) MRC-5

Infected cells become enlarged and frequently elliptical and appear to be lying in compact groups of six to eight cells on the cell sheet, forming characteristic plaques (Figure 10–20). Generally, less than ten plaques develop during the 14 days of incubation.

(2) CMK

RMK and CMK cell cultures are resistant to infection by cytomegalovirus.

HSV, VZ, and CMV all replicate (optimally, if not exclusively) in diploid fibroblast cells (MRC-5), however, the CPE produced by each virus is usually quite characteristic so that the virus can be immediately identified. Other information which can be helpful is that while HSV and VZ can be recovered from dermal lesions, VZ is rarely recovered from other sites. Also, the CPE of HSV is usually detected in 1 to 4 days, while at least 5 days are needed to detect the CPE of VZ.

Additional properties of the herpesviruses can be used for their identification. Herpesviruses are divided into two subgroups according to whether most of the infectious particles produced in cell cultures remain associated with the cells or are released into the fluid medium. Subgroup A, the particles of which are spontaneously released from the uninfected cell and are found in high titers in the extracellular fluid, includes HSV types 1 and 2. VZ and CMV are included in subgroup B in which mature virus is predominately cell-associated and can easily be differentiated from HSV by subpassage of the original cell culture as follows:

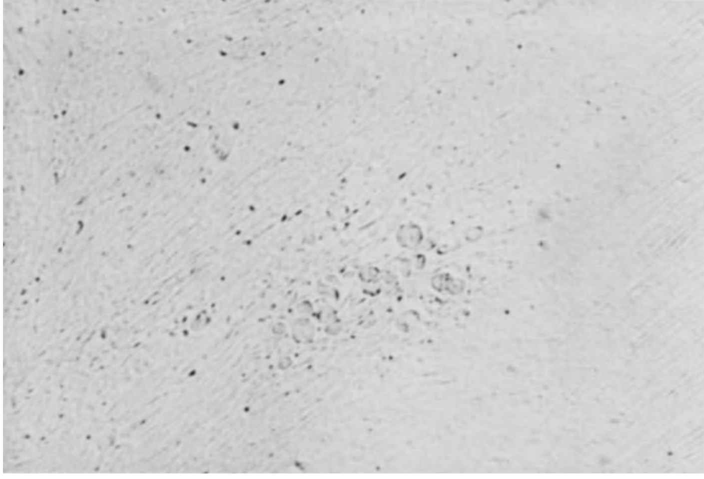


Figure 10–20. CPE produced by cytomegalovirus in MRC-5 cell culture (×100).

1. Remove the medium from the infected MRC-5 cell culture.
2. Add 0.15 ml of a solution of trypsin-EDTA to the culture and then remove.
3. Add 0.15 ml of fresh trypsin-EDTA solution to the tube and incubate at 35°C until the cells become detached from the glass.
4. Add 1 ml of medium (containing serum to stop the trypsin activity) to the tube and mix the cell suspension vigorously with a pipette.
5. Inoculate the original medium and the trypsinized cells into separate MRC-5 cultures (0.5 ml of medium or 0.5 ml of suspended cells per culture).

VZ and CMV can be recognized, particularly after subculture, by the presence of characteristic plaques in cultures inoculated with the trypsinized cells, since active virus characteristically remains associated with the cellular fraction. The MRC-5 cell culture, inoculated with the fluid phase, should be free of viral CPE.

Identification of HSV and CMV has been greatly facilitated by the recent availability of high-quality monoclonal antibodies specific for HSV types 1 and 2 (Syva Corp., Palo Alto, CA; Ortho Diagnostic Systems, Carpenteria, CA; Cappel Laboratories, West Chester, PA; Electro-Nucleonia Laboratories, Bethesda, MD) and for CMV (Biotech Research Laboratories, Rockville, MD).

5. Tests for Identification

Direct Immunofluorescence Test for CMV—Infected Cells by Centrifugation

Purpose:
To detect CMV infections rapidly.

Principle:
Conventional cell culture techniques generally require at least 8 days for recognition of characteristic CPE of CMV.[43] Low-speed centrifugation of the specimen onto cell culture monolayers has been shown to increase the efficiency of infection with several intracellular organisms, such as *Chlamydia*,[113] murine CMV, and the AD169 strain of human CMV.[65,104] Presumably, this step increases the number of active virus particles that come in contact with the cells. This technique combines centrifugation

with detection early nuclear protein of CMV by a monoclonal antibody using immunofluorescence.[38]

Specimens:

1. *Urine.* Obtain 5 ml in a screw-capped container.
2. *Throat swab* (Culturette™)
3. *Lung or liver tissues.* These specimens are first homogenized in a Stomacher.
4. *Blood.* Obtain 5 ml of heparinized blood. Lymphocytes and polymorphonuclear cells are separated for inoculation into cell culture vials.

Reagents:

1. MRC-5 cell cultures (Viromed Laboratories, Inc., Minneapolis, MN).
2. Monoclonal antibody to the early antigen (72,000 dalton) of CMV (Biotech Research Laboratories, Rockville, MD).
3. Conjugate (anti-mouse-FITC, IgG heavy and light-chain-specific (Cappell Laboratories, West Chester, PA).
4. Glass vials—1 dram shell vials (45 mm × 15 mm) (Arthur H. Thomas Co., Philadelphia, PA).
5. Coverslips—12-mm-round (Carolina Biological Supply Co., Burlington, NC).

Procedure:

1. Preparation of cell culture vials containing MRC-5 cells on coverslips.
   a. Seed 1 dram shell vials containing a 12-mm-round coverslip with MRC-5 cells (40,000 cells/vial) suspended in 1 ml Eagles Minimal Essential Medium (MEM) containing 10% fetal bovine serum.
   b. Close the vials with a rubber stopper and incubate at 35°C until a monolayer develops (2 to 3 days).
2. Inoculation of cell culture vials
   a. Remove the medium from each of the cell culture vials.
   b. Inoculate 0.2 ml of the specimen into two cell culture vials.
   c. Close the vials with a rubber stopper, place in carriers, and centrifuge at 700 × *g* for 1 hr (Sorvall RT6000, DuPont, Wilmington, DE).
   d. After centrifugation, add 1 ml of MEM back to each vial and incubate the cultures at 35°C.
   e. Controls
      i. *Positive:* Inoculate a known strain of CMV (AD169) into two vials to produce approximately 50–100 fluorescent foci/coverslip. Stain one coverslip after 16 hr and the other after 36 hr.

ii. *Negative:* Inoculate the vials with serum-free medium, centrifuge, and incubate, and examine the culture as for the positive control.

f. Remove one of the vials inoculated with each specimen from the incubator after 16 hr and the remaining vial after 36 hr. Aspirate the medium and remove the coverslips from the vials in preparation for the immunofluorescence assay.

3. Immunofluorescence Assay
   a. Wash the coverslips in the vials two times with PBS for 5 min each.
   b. Fix the cell on the coverslips with cold acetone for 20 min.
   c. Remove the coverslips from the vials and place them on a staining tray.
   d. Flood the coverslips with a 1:200 dilution of the monoclonal antibody (mouse) prepared to an early antigen of CMV.
   e. Incubate the coverslips at 35°C in a moist chamber for 30 min.
   f. Add PBS directly to the coverslips to wash away unattached CMV antibody. Aspirate the PBS immediately.
   g. Wash the coverslips two times with PBS (5 min each).
   h. Add the conjugate (anti-mouse-FITC) at a dilution of 1:70 to each coverslip and incubate at 35°C for 30 min in a moist chamber.
   i. After incubation, wash the unattached conjugate from the coverslips with PBS.
   j. Dry the coverslips with a paper towel and place them on slides (cell side down) using mounting fluid (phosphate buffered glycerol FA mounting media, Zeus Scientific, Inc., Raritan, NJ).

Examination and Interpretation:
Examine the coverslips at 200× magnification with a fluorescence microscope. Cell cultures positive for CMV have fluorescence localized to the nucleus of the cell—the site of initial replication of the virus. Distinct lobulated fluorescent inclusions are the hallmark of early CMV infection.

Direct Immunofluorescence Test for HSV-Infected Cells

Purpose:
To detect HSV infections rapidly.

Principle:
Fluorescein-labeled HSV antiserum reacts directly with HSV-specific antigens in infected cells. The immunofluorescence test is specific (with com-

mercial grade reagents) and we have noted no cross-reactions between HSV and VZ infected cells. The immunofluorescence test provides results within a few hours after receipt of the specimen; however, culture remains the more sensitive procedure for detecting the virus.[18,37,101] False negative results by immunofluorescence testing may occur when the number of HSV-infected cells on the slide is inadequate. Conversely, the immunofluorescence test may detect viral antigens, rather than active virus, in the cells.

Specimens:

1. *Skin vesicles.* Scrape the base of the lesion with a sterile scalpel blade to obtain infected epithelial cells. Place two drops of saline 5 to 10 mm apart on a glass microscope slide, suitable for use in the immunofluorescence test. Transfer the scrapings from the scalpel blade to the drops of saline and spread the cells over a small circular area (5 mm to 10 mm in diameter) in each of the two locations. Allow the slide to dry at room temperature, place in a petri dish, and send to the laboratory.
2. *Eye scrapings.* Spread scrapings from conjunctival lesions or from the cornea in *three* circular areas (5 mm to 10 mm in diameter) on a glass microscope slide as described above for skin vesicles.
3. *Tissue.* Prepare impression smears in two separate areas 5 mm to 10 mm in diameter as described above for skin and eye scrapings.

Reagents:

1. Lyophilized fluorescein isothiocynate (FITC)-labeled monoclonal antibody to HSV types 1 and 2 (anti-HSV-FITC), (Syva Corp., Palo Alto, CA) are reconstituted with 1 ml of sterile distilled water and allowed to stand at room temperature for 20 min. Conjugates are stored at 4°C. Rehydrated serum is stable for 1 month at 4°C.
2. Phosphate buffered saline (PBS)

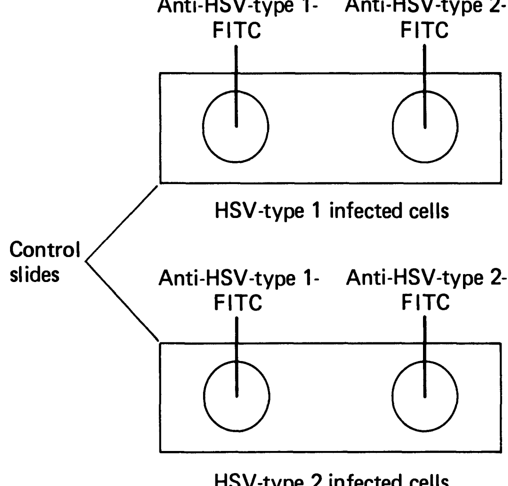
NaCl	8 g
$Na_2HPO_4 \cdot 7H_2O$	2.16 g
KH_2PO_4	0.2 g
KCl	0.2 g

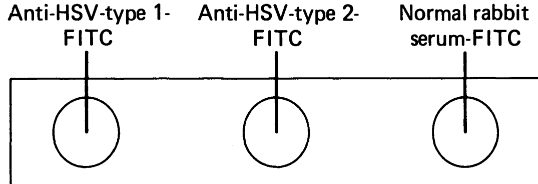
   Dissolve in 1,000 ml of distilled water. Adjust pH to 7.0 to 7.1 with HCl/NaOH. Store at 4°C. The solution is stable for 6 months.
3. Buffered glycerol. Add one part of PBS to nine parts of glycerol. Final pH should be 8.0–8.5 for optimal fluorescence.

4. Preparation of HSV-infected cell cultures
   a. Infection of cell cultures
      i. Inoculate HSV type 1 (Centers for Disease Control, Atlanta, GA) and type 2 viruses (American Type Culture Collection, Rockville, MD) into three to five MRC-5 cell culture tubes.
      ii. Incubate the cultures at 35°C until approximately one-half (2+) of the cells demonstrate CPE.
      iii. Remove and discard the fluid medium and add 0.3 ml of trypsin-EDTA solution to the cell culture tube.
      iv. After the cells have been removed from the glass surface, add 1 to 2 ml of PBS to each tube. The virus-infected cells are dispersed by agitation using a Vortex mixer.
      v. Combine the cell suspensions from all tubes and centrifuge at 400× *g* for 10 min.
      vi. Aspirate the supernatant fraction, resuspend the sedimented cells with 5 to 10 ml of PBS, and centrifuge at 400× *g*.
      vii. Resuspend the pellet with 0.5 ml of PBS.
   b. Preparation of slides with HSV-infected cells
      i. With an embroidery marker (Vogart Crafts Corporation) make two circles, each approximately 1 cm in diameter (each circle about 5 mm apart), on a glass microscope slide with a frosted end (1-mm thick, 25 × 75 mm, precision grade, precleaned, Curtin Scientific Co.)
      ii. With a capillary pipette, transfer one to two drops of the infected cell suspension to both of the circled areas on the microscope slide.
      iii. Allow the cell suspension to dry on the slide at room temperature.
      iv. Fix the cells by placing the slide in acetone (equilibrated at 4°C) for 10 min at 4°C.
      v. Store the slides at −70°C.

Procedure:

1. Circle the smear areas on the slide with an embroidery marker.
2. Fix the air dried smears of cells by immersing the slides in acetone (equilibrated at 4°C) for 10 min.
3. Apply the immunofluorescence reagents or store at −70°C.
4. Remove two control slides stored at −70°C—one containing HSV type I infected cells and the other HSV type 2 infected cells—and allow to dry at room temperature.
5. Place one to two drops of each anti-HSV-FITC on the control and specimen slides as follows:



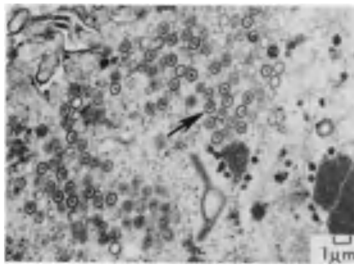


6. Incubate the slides in a moist chamber at 35°C for 45 min.
7. Immerse the slides in PBS, pH 7.6 for 10 min.
8. Transfer the slides to fresh PBS, pH 7.6 for 10 min.
9. Rinse the slides with distilled water.
10. Dry the slides at room temperature and then add a drop of buffered glycerol over the encircled cell smears. Cover the smears with a 24 × 50 mm (#1) or a 22 × 22 mm (#1) coverslip (Scientific Products).
11. Examine the slides at ×250 magnification with a fluorescence microscope.
12. Criteria for interpretation of the degree of fluorescence are as follows:

Glaring yellow-green fluorescence	4+
Bright yellow-green fluorescence	3+
Dull yellow-green fluorescence	2+
Dim yellow-green fluorescence	1+

Interpretation:

Positive test: The presence of HSV-infected cells is indicated by specific yellow-green fluorescence in the cells treated with either the anti-HSV type 1 or 2 conjugate. Sometimes an entire cell may exhibit a glaring yellow-green fluorescence, but most commonly the periphery of the virus-infected cells will be brighter (Figure 10–21, see



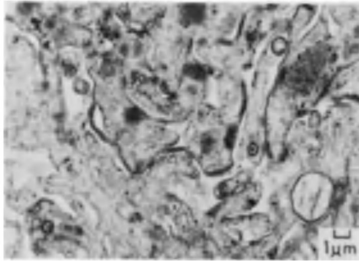


Figure 10-22. Electron micrograph of herpes simplex virus in brain tissue from patient with encephalitis.

color insert in the center of the book). Examine cells which are morphologically intact since cell debris and clumps of normal cells may exhibit a dull fluorescence which may be misinterpreted as a specific reaction.

Negative test: No fluorescence.

Controls:

Positive: HSV-1- and HSV-2-infected MRC-5 cells

Comment:
Electron microscopy can also be used to detect HSV in brain tissue. HSV particles have an inner core and two or three membranes. Many incomplete HSV particles (Figure 10-22) lacking nucleic acid and one or more membranes may be seen. Typical HSV particles are seen in brain cells from a biopsy yielding the virus in cell cultures.

Immunofluorescence Test for the Measurement of Antibodies to Epstein-Barr Virus

Purpose:
To diagnose heterophile-negative cases of Epstein-Barr virus infections.

Principle:
EBV infections are difficult to diagnose in the laboratory since the virus does not grow in standard cell cultures. The majority of infections can be recognized, however, by testing the patient's serum for heterophile antibodies which usually appear within the first 3 weeks of illness but then decline rapidly within a few weeks. The heterophile antibody, however, fails to materialize in about 10% of adults, more frequently in children, and almost uniformly in infants with primary EBV infections. Most

of these heterophile-antibody negative cases of IM-like infections are due to cytomegalovirus, but in one series of 43 cases EBV was the cause in 7.[61]

During primary IM infection, antibodies develop to several EBV-associated antigens. Antibodies to VCA antigen are not present prior to EBV infection, develop rapidly, are almost always detectable in the acute phase sera of patients with IM, and peak around the second week of illness. Anti-VCA IgG titers gradually decline, but persist for life and appear to be associated with permanent immunity (Figure 10–23).[25]

IgM antibodies to VCA, on the other hand, appear early in the course of infection and then decline to undetectable levels after a few weeks. As with other viral infections, IgM antibodies suggest a recent, primary infection.

While antibodies to VCA reach peak titers early in the course of IM, antibody to EBNA is usually delayed. Once acquired, usually in 2 to 6 months after the onset of infection, antibody to EBNA persists for life.

IgA antibodies to VCA and early antigen (EA) have not been useful in the diagnosis of EBV infections in our laboratory because these antibodies fail to appear in cases which have had severe and prolonged illness and low or absent heterophile response.[102] Most specimens tested in our laboratory come from such severely ill patients.

Specimen:
0.2 ml of serum.

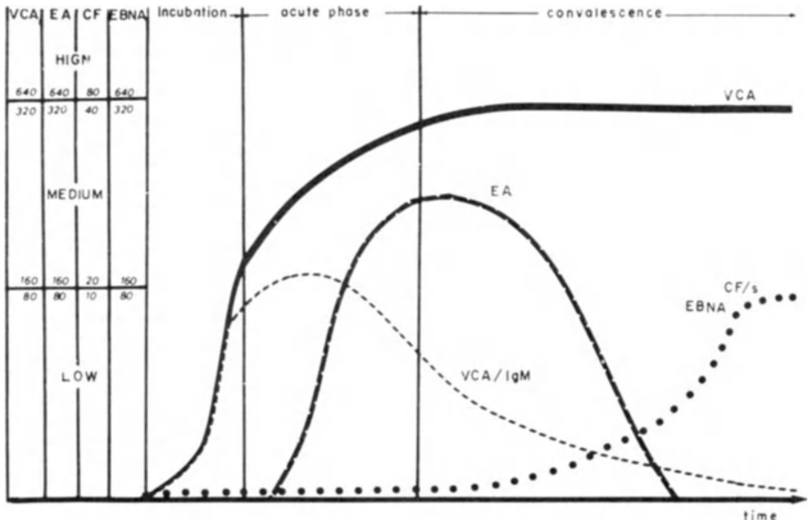


Figure 10–23. Schematic illustration of pattern of immune response to EBV infection.

Reagents:

1. Fluorescein conjugates
   a. anti-IgG (Hyland Laboratories)
   b. anti-IgM (Cappel Laboratories)
   c. antihuman complement (Bio-Rad Laboratories)
   The lyophilized conjugates are reconstituted according to the manufacturer's directions and allowed to stand at room temperature for 20 min. Conjugates are stored at 4°C. Rehydrated serum is stable for 1 month at 4°C.
2. Human complement. Blood (40 to 50 ml) is obtained from a patient (pretested to determine the absence of antibodies to EBV antigens) and allowed to clot for about 2 hr at room temperature. The serum fraction is separated by centrifugation at 300 × *g* for 10 min. The serum is dispensed into 1-dram vials in aliquots of 0.1 ml and frozen at −70°C.
3. Phosphate-buffered saline (PBS)

$NaCl$	80 g
$Na_2HPO_4 \cdot 7H_2O$	21.6 g
$MgCl_2 \cdot 6\,H_2O$	1 g
KH_2PO_4	2 g
KCl	2 g
$CaCl_2$	1 g

   Add to 4000 ml of distilled water in a flask. Mix and pour into a carboy with 5.5 liters of water. With vigorous stirring, add $CaCl_2$ solution (1.0 g $CaCl_2$ in 500 ml *cold* water) slowly to the carboy. Remove 500 ml of the PBS solution and measure the pH. Adjust the pH to 7.0 to 7.1 with HCl/NaOH.
4. Antigens.
   a. Viral capsid antigen (VCA). This antigen is made in spontaneously producing cell lines (HR1K) as a result of production of specific antigenic components of the virus by a small percentage of cells containing the EBV genome. Since only a small fraction (1 to 3%) of the cells produce viral particles, the fraction is increased to 5 to 10% by maintaining the culture for 3 to 4 days in arginine-free medium or for 10 days at a temperature of 32° to 33°C.
      (1) Maintenance of HRIK cultures
         (a) Grow the cells in suspension in either 1000 ml or 250 ml plastic flasks containing 300 ml to 400 ml of RPMI medium with 10% fetal calf serum.
         (b) Incubate at 35°C.
         (c) Twice weekly, remove one-third of the total volume of cell suspension and replace with fresh serum-containing RPMI

medium. Alternatively, add a volume of fresh medium equal to one-third of the total volume for large batches of antigen.

(d) Perform cell counts at least once a week. Mix 1 ml of cell suspension with 1 ml of trypan blue. Count the cells in a hemocytometer. Since trypan blue is a vital stain, only viable cells exclude the dye. The percentage of dead cells should be maintained between 25% to 50% of the total count.

(2) Preparation of EBV-VCA slides

(a) Prepare stock slides (Roboz Surgical Instrument Co., Inc., W-800-8 circles, frosted end, 5 mm i.d. each) with HR1K cultures containing approximately 10% VCA-positive cells.

(b) Wash the cells three times with PBS (Ca^{2+}- and Mg^{2+}-free).

(c) Count the viable cells in a hemocytometer by the trypan blue exclusion method and adjust the cell suspension with PBS to contain 2 to 3×10^6 cells/ml.

(d) Place a drop of the cell suspension in each of eight wells on a glass slide.

(e) Place the slides at 35°C and allow them to dry completely.

(f) Fix the dried smears in acetone for 10 min at room temperature.

(g) Store the slides at −70°C until use. Once thawed, do not reuse.

b. Epstein-Barr nuclear antigen (EBNA). The EBNA, present in all EBV genome-carrying cells, is not present in sufficient amount within each cell to be detected by the classic indirect immunofluorescence test. It can, however, be detected by the anticomplement immunofluorescence test which has much higher sensitivity.

(1) Wash cells from NC-37 or Raji lymphoblastoid cell lines twice with PBS. Dilute cells in PBS to a concentration of approximately 2×10^6 cells/ml and place a drop of suspension in each well of a Roboz eight-well slide.

(2) Dry quickly and fix for five minutes in cold alcohol-methanol mixture. Slides can be stored at −70°C approximately three months.

Procedure:

1. VCA (IgG)

a. Heat-inactivate the serum specimen at 56°C for 35 min.

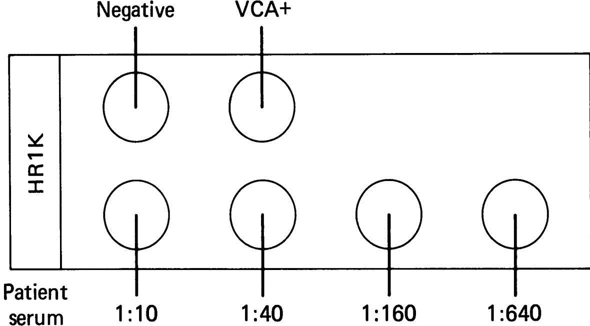
b. Remove slide (marked VCA) from −70°C freezer and place in 35°C incubator for a few minutes until dry.

c. Prepare dilutions of the test serum in 12 × 75 mm tubes by using Eppendorf pipettes (Curtin Scientific Co.) as follows:

Test mixtures		Final dilution
50 μl serum	+ 200 μl PBS[a]	1:5
100 μl of 1:5	+ 100 μl PBS	1:10
50 μl of 1:10	+ 150 μl PBS	1:40
50 μl of 1:40	+ 150 μl PBS	1:160
50 μl of 1:160	+ 150 μl PBS	1:640

[a] Use Ca^{2+}-and Mg^{2+}-free PBS.

d. With a 5¾ in. capillary pipette (Curtin Scientific), place one drop each of control and patient sera on slides prepared with VCA antigen as shown below. Fill each well completely.



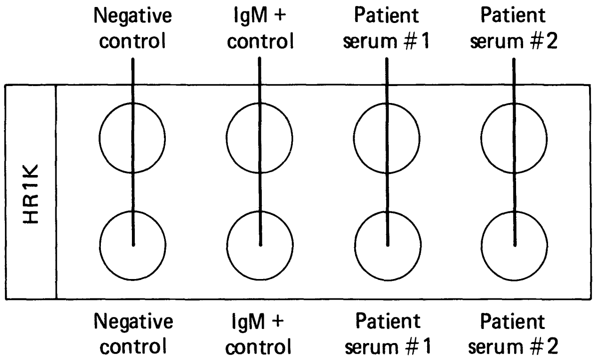
e. Incubate the slide in a moist chamber at 35°C for 30 min.
f. Rinse the slide gently by squirting PBS (with Ca^{2+} and Mg^{2+}) from a wash bottle over the top of the slide and then down the middle. The PBS rinse should not be squirted directly on the specimen wells.
g. Wash the slide gently in PBS containing Ca^{2+} and Mg^{2+} for 5 min in a Coplin or a slide jar (allowing the PBS to flow into the Coplin jar toward the back side of the slide) and repeat.
h. Rinse three times with deionized water.
i. Allow the slide to dry completely in a 35°C incubator (about 5 min).
j. Add one drop of fluorescein-labeled-anti-IgG (Hyland Laboratories) which has been diluted 1:20 with Ca^{2+}- and Mg^{2+}-free PBS.
k. Incubate the slide in a moist chamber at 35°C for 30 min.
l. Wash twice with PBS (with Ca^{2+} and Mg^{2+}) for 5 min each time.
m. Rinse three times with deionized water.
n. Dry the slide in the incubator at 35°C.
o. Add a thin film of a 50% glycerol/H_2O (v/v) solution down the center of the long axis of the slide. Place a 24 × 50 mm #1 coverslip (Scientific Products Co.) on the slide.
p. Examine the slide with a fluorescence microscope.

2. VCA (IgM)
   a. Remove slide (marked VCA) from −70°C freezer and place in incubator at 35°C for a few minutes until dry.
   b. Prepare a 1:5 dilution of the inactivated serum specimen as follows:

Test mixture	Final dilution
50 μl serum + 200 μl PBS[a]	1:5

[a] Ca^{2+}- and Mg^{2+}-free.

   c. Proceed as described in 1,d-p except that the slide is incubated in a moist chamber at 35°C for 3 hr and a drop of fluorescein-labeled anti-IgM is added to the appropriate wells (see below).

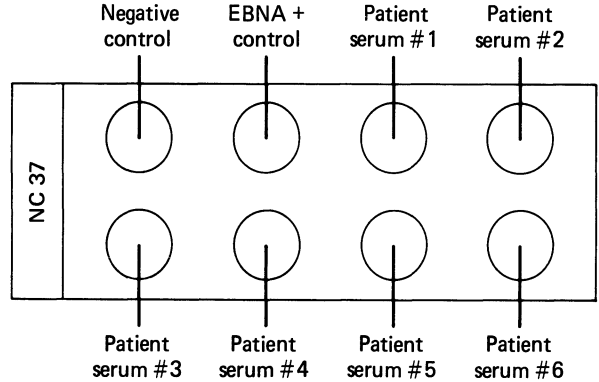


3. EBNA
   a. Remove slide (marked NC 37, Raji) from −70°C freezer and place in incubator at 35°C for a few minutes until dry.
   b. Prepare a 1:5 dilution of the inactivated serum specimen as follows:

Test mixture	Final dilution
50 μl serum + 200 μl PBS[a]	1:5

[a] Ca^{2+}- and Mg^{2+}-free.

   c. Proceed as described in 1,d-i (see below).
   d. Add one drop of human complement (C′) to all wells. (Dilute the reagent with PBS containing Ca^{2+} and Mg^{2+}.)
   e. Incubate in a moist chamber at 35°C for 30 min.
   f. Wash twice with PBS (with Ca^{2+} and Mg^{2+}) for 5 min each time.
   g. Rinse three times with deionized H_2O.



h. Add one drop of fluorescein-conjugated anti-human C′ to all wells. (Dilute the reagent with PBS containing Ca^{2+} and Mg^{2+}.)
i. Proceed as in 1,k-p.

Interpretation:

Positive test: VCA: IgG: 10% to 25% of the cells with fluorescence (Figure 10–24, see color insert in the center of the book). IgM: 10% to 25% of the cells with fluorescence (Figure 10–25, see color insert in the center of the book).
EBNA: 75% to 100% of the cells with fluorescence (Figure 10–26, see color insert in the center of the book). The interpretation of several possible test results are listed (Table 10–8).
Negative test: No fluorescence.

Controls:

Positive: Serum specimens from patient known to have had EBV infection in past
Negative: Serum specimens from patient with no detectable antibodies to EBV antigens

---

B. Adenovirus

1. Description

Adenoviruses are medium-sized (70 to 80 nm) DNA-containing particles with icosahedral symmetry. Thirty-three antigenically distinct adenoviruses have been recognized; all strains have a common group antigen.

2. Source

Adenovirus replicates in susceptible cells of the pharynx or conjunctiva, but can seed the bloodstream. Like enteroviruses, they can survive pas-

Table 10–8. Interpretation of the Results of Antibody Responses to Epstein-Barr Virus Infection

Result	Interpretation
VCA-IgG + IgM + EBNA −	Results indicate a recent, primary infection with EBV.
IgG + IgM − EBNA +	Results indicate infection with EBV in past (at least 2 to 6 months ago).
IgG + IgM + EBNA +	Results indicate infection with EBV in past (at least 2 to 6 months ago). Presence of IgM antibody suggests recent primary infection.
IgG − IgM − EBNA −	Results indicate no evidence of infection with EBV.
IgG ≥ 1:640 IgM − EBNA +	Results indicate infection with EBV in past (at least 2 months ago). Titer of IgG ≥ 1:640 suggests recent infection.
IgG low + IgM − EBNA −	Results indicate a recent infection with EBV. EBNA and IgM titers should be present 2 months after onset of symptoms.
IgG + IgM − EBNA −	Results indicate infection with EBV within the last 2 months.

sage through the acid environment of the stomach and be excreted in the feces.

3. Clinical Importance

Adenoviruses are responsible for 2 to 3% of acute respiratory tract infections in ambulatory children and for 5 to 25% of acute respiratory infections in hospitalized children. The patient often presents with pharyngoconjunctival fever characterized by the triad of fever, pharyngitis, and follicular conjunctivitis, frequently associated with regional lymphadenopathy. Adenovirus type 3 seems most often involved, although types 1, 4, 5, 6, 7, and 14 are known to produce a similar clinical condition.[133] Systemic symptoms, such as malaise, myalgia, headache, and diarrhea, may also be present. In many cases, there is a follicular conjunctivitis with preauricular lymphadenopathy. Keratitis, if present, is usually mild. Membranous conjunctivitis has been reported.[12] Adenovirus types 4 and 7 commonly cause epidemic acute respiratory disease and pneumonia in military populations, but other serotypes are also involved in pulmonary infections.

Epidemic keratoconjunctivitis occurred in epidemic form in the United States in the 1940s in association with the wartime construction of ships and was referred to as "shipyard eye." The infection is usually due to adenovirus type 8, although types 1, 2, 3, 7, 9, 11, and, most recently, 19 have caused sporadic outbreaks.[133] The clinical course is characterized by an initial stage (lasting approximately 48 hr) of hyperemia and edema of the conjunctiva along with photophobia and lacrimation. Thereafter, a follicular conjunctivitis appears, which can lead to membrane formation. Corneal infiltrates appear on the seventh or eighth day. The keratitis usually persists for 2 to 3 months and occasionally for as long as 3 years. Severe complications of epidemic keratoconjunctivitis are corneal erosions with conjunctival scarring after membrane formation.[12]

Adenovirus types 11 and 21 have been associated with acute hemorrhagic cystitis.[99]

4. Growth Characteristics

a. MRC-5

Adenovirus infection produces rounded, granular, and sometimes clumped cells at the edge of the cell sheet (Figure 10–28). Although a few elliptic cells are present within the cell sheet, CPE does not usually progress to involve the entire cell monolayer. Usually, MRC-5 cells are of limited value in detecting adenoviruses because CMK cells are more sensitive to viruses in this group. Nevertheless, adenoviruses can be detected as frequently but not as rapidly in MRC-5 cells as in CMK cells.

Figure 10–28. CPE produced by adenovirus in MRC-5 cell culture (×100).

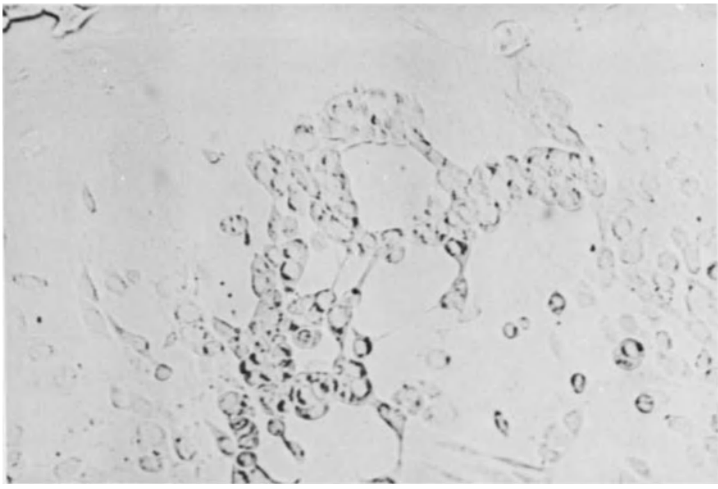


Figure 10–29. CPE produced by adenovirus in monkey kidney cell culture (×100).

b. CMK

The CPE for adenovirus types 1, 2, 3, and 5 (types most frequently isolated) in CMK cells differs from that produced by other adenovirus serotypes. The cells become increasingly granular and thickened and tend to clump together (Figure 10–29). Some of the cells retract from the cell clusters, producing a weblike appearance in the cell sheet.

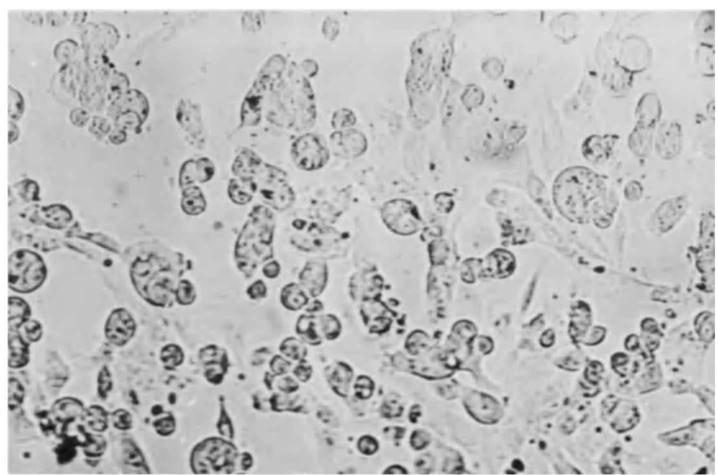


Figure 10–30. CPE produced by adenovirus in HEK cell culture (×100).

c. HEK

Adenovirus-infected cells are enlarged and grouped together in plaques forming grapelike clusters (Figure 10–30). These cells are more sensitive than CMK for the recovery of adenovirus.

5. Tests for Identification

Neutralization to Infectivity

Viral isolates are identified by the neutralization of infectivity in cell cultures by specific antiserum. A 10^{-1} dilution of the virus suspension harvested with CPE grade 3+ or 4+ in cell cultures is used in the test described on p. 547.

Comment:
Because CMK cells can be contaminated with an adenovirus indigenous to monkeys, such as SV40,[63] the virus pools used for neutralization tests should be derived from MRC-5 cells. Adenovirus is released from the infected MRC-5 cells by one freeze-thaw cycle. Individual antisera to adenovirus serotypes may be combined and used as a polyvalent pool in the neutralization test.

Direct Immunofluorescence Test for Adenovirus-Infected Cells

Purpose:
To detect adenovirus infection rapidly.

Principle:
Fluorescein-labeled adenovirus antiserum reacts directly with adenovirus-specific antigens in infected cells.

Specimen:
Scrapings from conjunctival lesions or the cornea are spread on two circular areas (5 to 10 mm in diameter) on a glass slide, as described on p. 595.

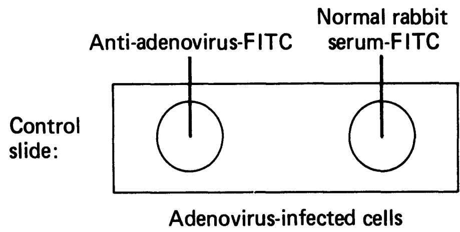
Reagents:

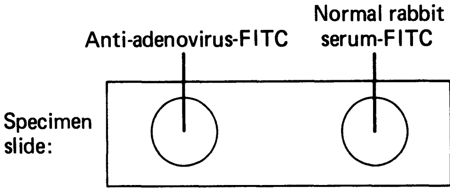
1. Fluorescein isothiocynate (FITC)-labeled antiserum to adenovirus (Microbiological Associates). Rehydration of the lyophilized conjugate and storage are as described for the HSV reagents (p. 595).

2. Preparation of adenovirus-infected cell cultures:
   a. Infection of cell cultures
      i. Inoculate MRC-5 cell cultures (three to five tubes) with a suspension of adenovirus (type 7, Centers for Disease Control, Atlanta, GA).
      ii. Incubate the adenovirus-infected cells and harvest as described for HSV (p. 596).
   b. Preparation of slides with adenovirus-infected cells:
      Prepare as described for HSV (p. 596) except that only two encircled areas of the cell smears are made.

Procedure:

1. Remove a slide with adenovirus-infected cells from storage at −70°C and allow to dry at room temperature.
2. Apply anti-adenovirus-FITC conjugate on the control and specimen slide as follows:





3. Incubate and wash the slides as described on p. 597.

Interpretation:

Positive test: The presence of adenovirus-infected cells is indicated by specific yellow-green fluorescence in the cells treated with anti-adenovirus–FITC conjugate.

Negative test: No fluorescence should be observed in the cells treated with the FITC-labeled normal rabbit serum.

Controls:

Positive: Adenovirus-infected MRC-5 cells

Negative: Adenovirus-infected MRC-5 cells treated with FITC-labeled normal rabbit serum

---

C. Poxvirus

1. Description

Viruses in this group possess DNA, a protein coat, and a lipid envelope. The nucleocapsids of these viruses are brick-shaped and have a complex symmetry since they do not conform to either helical or cubical geometric forms. The agents which cause smallpox (variola major and variola minor), milkers nodules (paravaccinia), contagious pustular dermatitis (Orf), and vaccinia are medically important.

2. Source

Although infection is usually initiated in the respiratory tract, the skin is the principle site of clinical disease. The viruses can thus be detected in specimens from the vesicular lesions.

3. Clinical Importance

The world's last known case of smallpox resulting from human-to-human transmission in an endemic focus occurred in Somalia in October 1977.[10] In 1971 the Public Health Advisory Committee on Immunization Practices in the United States recommended that since the risk of smallpox in the United States was so unlikely, routine smallpox vaccination was no longer indicated. The committee thereby sought to reduce complications of vaccination (live vaccinia virus) which include nonspecific vascular reactions (erythema multiforme, allergic purpura, and urticaria) or eruptions of vaccinial vesicles (accidental vaccinia, eczema vaccinatum, or generalized vaccinia). Though most complications are benign, approximately seven or eight vaccine-related deaths occurred annually in the United States.[72]

Orf is a viral disease that is endemic in sheep and can be transmitted to humans. The disease is common among sheepherders in all parts of the world, and most do not seek medical advice.

4. Growth Characteristics

Vaccinia virus rapidly infects human diploid fibroblast and monkey kidney cell cultures. Infected cells become detached from the glass surface within 48 to 72 hr after inoculation with the specimen.

VI. Reporting of Viral Isolates

All information regarding cultures with CPE—such as specimen source, age of patient, physician's clinical impression, season of the year, and the specific type of CPE—is considered in preparing a report to the physician. For example, the frequency of isolation of certain viruses varies seasonally (Table 10–9); therefore, it is often possible to predict tentatively which of two viruses producing similar CPE may be present.

Once CPE has progressed to involve approximately 25% of the cells, it is usually possible to identify the virus by the specific cellular changes present in the monolayer. Thus, HSV, VZ, CMV, adenovirus, enterovirus, and rhinovirus can usually be reported when the CPE is first recognized. Further identification procedures, requiring subpassage of the viral isolates, are generally needed (especially early in an outbreak) to distinguish between parinfluenza and influenza viral isolates. The paramyxoviruses, however, may be initially distinguished from one another and from influenza virus by the elution pattern of red blood cells from infected cells. For instance, an isolate from a patient with "croup" that shows less HAd at 20°C that at 4°C can be reported as "parainfluenza virus" (Table 10–7). If syncytial cells are observed in the CMK cell culture of a specimen from a patient with parotitis or the amount of HAd does not change or is increased at 20°C, relative to that at 4°C, the virus is presumptively reported as "mumps virus." Influenza virus will cause hemagglutination on the infected CMK cells as well as in the liquid medium. Definitive identification is then made with the HAd or HAI tests; however, in the interim, these isolates can be reported as "hemadsorbing viruses." The CPE of HSV can be easily recognized except when so much virus is present that the MRC-5 monolayer is completely destroyed when initially observed. In the latter instance, unless CPE was simultaneously present in CMK cells, the isolate is simply reported as "virus" because the CPE and infectivity pattern can be confused with those produced by echovirus and poliovirus.

When one or perhaps only a few areas of the monolayer suggest CPE or when the total cell sheet is destroyed, it is important to know the relative sensitivity of each of the cell cultures to each virus (Table 10–10).

A scheme for arriving at a presumptive and the definitive identifications of viruses is shown in Table 10–11.

VII. Viral Serology (Table 10–12)

In the past, the diagnosis of viral infections by serologic methods has required that blood specimens be taken early in the course of the disease

Table 10–9. Frequency of Virus Isolations at Mayo Clinic

Virus	Total (no.)	Total % of	% of group by month											
			Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Adenovirus	405	11.8	6.9	7.2	4.2	8.4	7.4	10.8	6.2	10.1	6.4	8.6	11.9	11.9
Coxsackievirus A	98	2.8	0	0	0	0	0	0	17.2	55.2	19.4	8.2	0	0
Coxsackievirus B	265	7.7	0	1.1	0	0	0	4.2	11.7	39.2	22.6	15.5	4.9	0.8
Echovirus	238	6.9	0	0	0	0.4	0	1.7	14.7	38.7	33.6	8.0	2.1	0.8
Rhinovirus	358	10.4	2.5	5.0	8.7	15.1	7.8	8.4	11.2	10.6	10.3	12.9	5.0	2.5
Herpes simplex virus	748	21.7	5.8	5.8	9.1	9.6	7.2	9.2	8.3	11.4	7.6	7.7	9.6	8.7
Respiratory syncytial virus	160	4.6	16.9	14.4	5.6	5.0	8.1	4.4	1.9	0	0	1.2	13.1	29.4
Mumps virus	131	3.8	9.2	5.3	7.6	10.7	8.4	11.5	8.4	5.3	6.9	7.6	6.9	12.2
Parainfluenza virus	576	16.7	10.1	10.2	11.5	12.0	5.9	7.1	3.1	2.1	3.1	9.7	13.9	11.3
Influenza virus	469	13.6	15.6	27.5	6.4	0.4	0	0	0	0	0	1.1	4.3	44.7
Total	3448	100.0												

Table 10–10. Sensitivity of Cell Cultures to Viruses when CPE is Initially Detected in any Cell Culture

Virus	% of Virus Isolates[a]	
	In diploid fibroblasts	In monkey kidney
Parainfluenza	—	~100
Influenza	—	~100
Mumps	—	~100
Herpes simplex	~100	—
Varicella-zoster	~100	—
Cytomegalovirus	~100	—
Adenovirus	<25	~100
Respiratory syncytial	~100	<50
Echovirus	~100	<75
Coxsackievirus B	—	~75
Coxsackievirus A9 and A16	~100	~100
Poliovirus	~25	~100
Rhinovirus	~100	<25

[a] Approximate percentage of virus isolates which produce CPE in a cell culture.

(acute phase) followed by a second specimen two to three weeks later (convalescent phase). Serologic procedures have very little practical significance for the self-limited viral infections in outpatients, but they can provide valuable diagnostic information when the virus disseminates, causing systemic disease in the patient.

Serologic tests of single serum specimens have become increasingly important in the management of pregnant patients (rubella), renal transplant recipients, neonates requiring transfusions (cytomegalovirus), patients with neoplastic disease requiring immune globulin after exposure to a viral infection (varicella-zoster virus). Also clinically important are serologic tests with single serum specimens for detecting recent or chronic Epstein-Barr virus infections in patients who do not produce heterophile antibodies but do have typical lymphocytes suggestive of leukemia (Table 10–12).[128,129]

IgM-class antibody may also be detected in the acute-phase sample in susceptible individuals because they lack humoral antibodies to a particular virus. IgM-class antibody to a particular virus is the first to develop after infection, so that its qualitative detection is indicative of a primary, acute phase infection (Table 10–12). Assay of viral-specific IgM antibodies requires the proper controls and separation methods to obtain reliable results. These considerations are presented in detail elsewhere.[118,128,129]

Table 10–11. Scheme for Presumptive Identification of Viruses Based on CPE Pattern in Cell Culture on Primary Isolation

If CPE present in				
MRC	CMK	Virus could be	Clinical or laboratory features	Try
No	Yes	Parainfluenza	Fine granular lacy appearance to cell sheet; sometimes syncytial cells present. Pharyngitis, tonsillitis, croup; mostly in children.	Hemadsorption; hemadsorption-inhibition
		Influenza	Prominent enlarged cells of various shapes; debris from disintegrated CMK cells floating in medium. Myalgia, headache, cough, and tightness in chest prominent symptoms.	Hemadsorption; hemagglutination-inhibition
		Mumps	Large granular syncytial cells frequently present. Parotitis, orchitis, and CNS involvement.	Hemadsorption; hemadsorption-inhibition
Yes	No	Herpes simplex	Large globular cells easily seen at edge of cell sheet; CPE develops and advances very rapidly. Specimen source is throat (other oral sites too), genitalia, eye, skin.	Report as HSV
		Varicella-zoster	Diffuse plaques usually after 5 days. Specimen source is dermal vesicle.	Pass fluid and cells of infected culture separately to fresh MRC-5 cell cultures
		Cytomegalovirus	Focal, compact plaques usually after 5 days. Specimen source is throat, urine, autopsy tissue, or cervix	Pass fluid and cells of infected culture separately to fresh MRC-5 cell cultures

Rarely	Yes	Adenovirus	Clusters of enlarged granular cells in CMK. Pharyngitis and tonsillitis, mostly in children.	Transfer of virus to MRC-5 produces characteristic CPE; adenovirus neutralization
		Coxsackievirus B	Initially rounded tear-shaped cells slough rapidly off glass surface. Specimen usually from throat, rectum, and CNS.	Transfer of virus to MRC-5 rarely produces CPE; coxsackievirus B neutralization
		Poliovirus	CPE-like coxsackievirus B. Frequently isolated from children with recent oral poliovirus vaccination.	Transfer of virus to MRC-5 causes rapid lysis and detachment of the cells from the glass surface; poliovirus neutralization
Yes	Maybe	Respiratory syncytial	Syncytial cells develop usually after 5 days. Isolated frequently from children with cough, bronchitis, and bronchiolitis. Specimen from respiratory tract.	Pass fluid to MRC-5 to observe characteristic syncytia; RSV neutralization
		Echovirus	MRC-5 cells usually lysed and shriveled; infected CMK cells are tear-shaped, refractile, and randomly scattered over cell sheet. Specimen from throat, rectum, or CNS.	Passage in CMK cells reveals characteristic tear-shaped refractile cells; echovirus neutralization by intersecting serum scheme
		Rhinovirus	Discrete foci of rounded cells of various sizes. Specimens from respiratory tract.	pH 3 and $CHCl_3$ tests
		Coxsackievirus A9 and A16	CPE-like echovirus. Associated with herpangina and hand, foot and mouth disease.	Passage in CMK cells reveals characteristic tear-shaped refractile cells; A9 and A16 neutralization; mouse inoculation

Table 10–12. Serologic Tests for Diagnosis of Viral Infections

Virus	Method	IgM determination
RNA-Containing		
Influenza		
type A	IF[a]	Yes
type B	IF	Yes
California	CIE[b]	No
Respiratory syncytial	IF	Yes
Mumps	IF	Yes
Rubella	Rubazyme	Yes
Rubeola	IF	Yes
St Louis encephalitis	HAI[c]	No
Western equine encephalitis	HAI	No
DNA-Containing		
Adeno	CF[d]	No
Cytomegalo	ACIF[e]	Yes
Epstein-Barr		
capsid antigen (VCA)	IF	Yes
nuclear antigen (EBNA)	ACIF	No
Herpes simplex	ACIF	Yes
Varicella-zoster	ACIF	Yes

[a] Indirect immunofluorescence.
[b] Counterimmunoelectrophoresis.
[c] Hemagglutination-inhibition.
[d] Complement fixation.
[e] Anticomplement immunofluorescence.

References

1. Adler, S. P. Transfusion-associated cytomegalovirus infections. *Rev. Infect. Dis.* **5**:977, 1983.
2. Amber, R. W., Block, A. B., Orenstein, W. A., Bart, K. J., Turner, P. M., and Hinman, A. R. Imported measles in the United States. *JAMA* **248**:2129, 1982.
3. Austin, T. W., and Ray, C. G. Coxsackievirus group B infections and the hemolytic-uremic syndrome. *J. Infect. Dis.* **127**:698, 1973.
4. Balfour, H. H., Jr., and Edelman, C. K. Diagnosis of California (La Crosse) encephalitis by precipitin techniques. A prospective study. *Appl. Microbiol.* **28**:807, 1974.
5. Birch, C. J., Rodger, S. M., Marshall, J. A., and Gust, I.D. Replication of human rotavirus in cell culture. *J. Med. Virol.* **11**:241, 1983.
6. Bishop, R. F., Davidson, G. P., Holmes, I. H., and Ruck, B. J. Virus particles in epithelial cells of duodenal mucosa from children with acute nonbacterial gastroenteritis. *Lancet* **2**:1281, 1973.

7. Blair, H. T., Greenberg, S. B., Stevens, P. M., Bilunos, P. A., and Couch, R. B. Effects of rhinovirus infection on pulmonary function of healthy human volunteers. *Am. Rev. Respir. Dis.* **114**:95, 1976.
8. Brandt, C. D., Kim, H. W., Rodriguez, W. J., Arrobio, J. O., Jeffries, B. C., Stallings, E. P., Lewis, C., Miles, A. J., Chanock, R. M., Kapikian, A. Z., and Parrott, R. H. Pediatric viral gastroenteritis during eight years of study. *J. Clin. Microbiol.* **18**:71, 1983.
9. Brill, S. J., and Gilfillan, R. F. Acute parotitis associated with influenza type A. *N. Engl. J. Med.* **296**:1391, 1977.
10. Brilliant, L. B., and Hodakevic, L. N. Certification of smallpox eradication. *Bull. WHO* **56**:723, 1978.
11. Brown, R. L., Zinner, S. H., Meglio, F. D., and Garrity, F. L. Countercurrent immunoelectrophoresis in the diagnosis of viral infections of the central nervous system. *J. Infect. Dis.* **138**:911, 1978.
12. Buchta, R. M. Membranous conjunctivitis due to adenovirus type 7 infection. *Clin. Pediatr.* **13**:233, 1974.
13. Calligan, D. R., and Menegus, M. A. Rapid detection of herpes simplex virus in clinical specimens with human embryonic lung fibroblast and primary rabbit kidney cell cultures. *J. Clin. Microbiol.* **19**:563, 1984.
14. Carlsen, K.-H., Orstavik, I., and Halvorsen, K. Viral infections of the respiratory tract in hospitalized children. *Acta Paeiatr. Scand.* **72**:53, 1983.
15. Center for Disease Control. *Control of Western Equine Encephalitis.* Issued October 1978.
16. Centers for Disease Control. *Status of Commercial Rubella Antibody Test Kits. Reagents Evaluation Program.* Supplement No. 23, October 1, 1983.
17. Chernesky, M. A., Ray, C. G., and Smith, T. F.: *Cumitech 15. Laboratory diagnosis of viral infections.* (Drew, W. L., coordinating ed.) Washington, D.C., American Society of Microbiology, pp. 1–17, 1982.
18. Cho, C. T., and Feng, K. K. Sensitivity of the virus isolation and immunofluorescent staining methods in diagnosis of infections with herpes simplex virus. *J. Infect. Dis.* **138**:536, 1978.
19. Chumbley, L. C., Robertson, D. M., Smith, T. F., and Campbell, R. J. Adult cytomegalovirus inclusion-uveitis. *Am J. Ophthalmol.* **80**:807, 1975.
20. Corey, L., Adams, H. G., Brown, Z. A., and Holmes, K. K. Genital herpes simplex virus infections: clinical manifestations, course, and complications. *Ann Intern. Med.* **98**:958, 1983.
21. Couch, R. B. An assessment of amantadine for influenza. *Infect. Dis.* **7**:4, 16, 32, 1977.
22. Creech, W. B. St. Louis encephalitis in the United States, 1975. *J. Infect. Dis.* **135**:1014, 1977.
23. Davis, L. E., Bodian, D., Price, D., Butler, I. J., and Vickers, J. H. Chronic progressive poliomyelitis secondary to vaccination of an immunodeficient child. *N. Engl. J. Med.* **297**:241, 1977.
24. Denny, F. W., Murphy, T. F., Clyde, W. A., Jr., Collier, A. M., and Henderson, F. W. Croup: An 11-year study in a pediatric practice. *Pediatrics* **71**:871, 1983.
25. de Thé, G., Lenoir, G.: Comparative diagnosis of Epstein-Barr virus-related diseases. *In* Kurstak, E., and Kurstak, C. (eds.), *Infectious Mononucleosis, Burkitt's Lymphoma, and Nasopharyngeal Carcinoma.* New York, Academic Press, 1977, p. 195.

26. Dolin, R., Reichman, R. C., Mazur, M. H., and Whitley, R. J. Herpes zoster-varicella infections in immunosuppressed patients. *Ann. Intern. Med.* **89**:375, 1978.
27. Donat, J. F., Rhodes, K. Hable, Groover, R. V., and Smith, R. F. A study of etiology and outcome in 42 children with acute nonbacterial disorders of the CNS. *Mayo Clin. Proc.* **55**:156, 1980.
28. Dowdle, W. R., and Coleman, M. T. Influenza virus. *In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 678.
29. Dowdle, W. R., and Hattwick, M. A. W. Swine influenza virus infections in humans. *J. Infect. Dis.* **113**:S386, 1977.
30. Dowdle, W. A., Kendal, A. P., and Noble, G. R. Influenza viruses. *In* Lennette, E. H., and Schmidt, N. J. (eds.), *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections,* 5th ed. Washington, D.C., American Public Health Association, 1979, p. 585.
31. Epstein, M. A., and Achong, B. G.: Recent progress in Epstein-Barr virus research. *Annu. Rev. Microbiol.* **31**:421, 1977.
32. Epstein, M. A., Barr, Y. M., and Achong, B. G. Studies with Burkitt's lymphoma. *Wistar Inst. Symp. Monogr.* **4**:69, 1965.
33. Farmer, K., MacArthur, B. A., and Clay, M. M. A follow-up study of 15 cases of neonatal meningoencephalitis due to coxsackie virus B5. *J. Pediatr.* **87**:568, 1975.
34. Frank, A. L., Taber, L. H., and Wells, J. M. Individuals infected with two subtypes of influenza A virus in the same season. *J. Infect. Dis.* **147**:120, 1983.
35. Friedman, H. M., and Koropchak, C. Comparison of WI-38, MRC-5, and IMR-90 cell strains for isolation of viruses from clinical specimens. *J. Clin. Microbiol.* **7**:368, 1978.
36. Gartner, J. G., and Seemayer, T. A. New oncologic associations for the Epstein-Barr virus. *Am. J. Surg. Pathol.* **6**:471, 1982.
37. Gittzus, J. G., and Rubin, S. J. Clinical evaluation of commercial conjugates for direct immunofluorescence of herpes simplex virus. *J. Clin. Microbiol.* **6**:574, 1977.
38. Gleaves, C. A., Smith, T. F., Shuster, E. A., and Pearson, G. R. Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens using monoclonal antibody to an early antigen and low-speed centrifugation. *J. Clin. Microbiol.* **19**:917, 1984.
39. Glezen, W. P. Serious morbidity and mortality associated with influenza epidemics. *Epidemiol. Rev.* **4**:25, 1982.
40. Glezen, W. P. Viral pneumonia as a cause of result of hospitalization. *J. Infect. Dis.* **147**:765, 1983.
41. Glezen, W. P., Loda, F. A., and Denny, F. W. The parainfluenza viruses. *In* Evans, A. S. (ed.), *Viral Infections of Humans.* New York, Plenum, 1976.
42. Goodman, R. A., Orenstein, W. A., Munro, T. F., Smith, S. C., and Sikes, K. Impact of influenza A in a nursing home. *JAMA* **247**:1451, 1982.
43. Gregory, W. W., and Menegus, M. A. Effect of incubation temperature on isolation of cytomegalovirus from fresh clinical specimens. *J. Clin. Microbiol.* **18**:1003, 1983.
44. Grist, N. R. Coxsackie virus infections of the heart. *In* Waterson, A. P. (ed.),

Recent Advances in Clinical Virology. New York, Churchill Livingstone, 1977, p. 141.

45. Hable, K. A., Washington, J. A., II, and Herrmann, E. C., Jr. Bacterial and viral throat flora. *Clin. Pediatr.* **10**:199, 1971.
46. Halfon, N., and Spector, S. A. Fatal Echovirus Type II infections. *Am. J. Dis. Child.* **135**:1017, 1981.
47. Hall, C. B., Douglas, R. G., Jr., Geiman, J. M., and Messner, M. K. Nosocomial respiratory syncytial virus infection. *N. Engl. J. Med.* **293**:1343, 1975.
48. Hall, C. B., Geiman, J. M., Biggar, R., Kotok, D. I., Hogan, P. M., Douglas, R. G., Jr. Respiratory syncytial virus infections within families. *N. Engl. J. Med.* **294**:414, 1976.
49. Hall, C. B., Geiman, J. M., Breese, B. B., and Douglas, R. G., Jr. Parainfluenza viral infections in children: Correlation of shedding with clinical manifestations. *J. Pediatr.* **91**:194, 1977.
50. Halvorsrud, J., and Orstavik, I. An epidemic of rotavirus-associated gastroenteritis in a nursing home for the elderly. *Scand. J. Infect. Dis.* **12**:161, 1980.
51. Hamparian, V. V., Ketter, A., and Hilleman, M. R. Recovery of new viruses (coryzavirus) from cases of common cold in human adults. *Proc. Soc. Exp. Biol. Med.* **108**:444, 1961.
52. Hanshaw, J. B., Scheiner, A. P., Moxley, A. W., Gaev, L., Abel, V., and Scheiner, B. School failure and deafness after "silent" congenital cytomegalovirus infection. *N. Engl. J. Med.* **295**:468, 1976.
53. Hanto, D. W., Gajl-Peczalska, K. J., Frizzera, G., Arthur, D. C., Balfour, H. H., Jr., McClain, K., Simmons, R. L., and Najarian, J. S. Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occurring after renal transplantation. *Ann. Surg.* **198**:356, 1983.
54. Herrmann, E. C., Jr., and Hable, K. A. Experiences in laboratory diagnosis of parainfluenza viruses in routine medical practice. *Mayo Clin. Proc.* **45**:177, 1970.
55. Herrmann, E. C., Jr., Person, D. A., and Smith, T. F. Experience in laboratory diagnosis of enterovirus infections in routine medical practice. *Mayo Clin. Proc.* **47**:577, 1972.
56. Hinman, A. R., Orenstein, W. A., Block, A. B., Bart, K. J., Eddins, D. L., Amler, R. W., and Kirby, C. D. Impact of measles in the United States. *Rev. Infect. Dis.* **5**:439, 1983.
57. Hirsch, M. S., and Schooley, R. T. Treatment of herpesvirus infections. *N. Engl. J. Med.* **309**:963;1034; 1983.
58. Ho, M., Suwansirikul, S., Dowling, J. N., Youngblood, L. A. and Armstrong, J. A. The transplanted kidney as a source of cytomegalovirus infection. *N. Engl. J. Med.* **293**:1109, 1975.
59. Hochberg, F. H., Miller, G., Schooley, R. T., Hirsch, M. S., Feorino, P., and Henle, W. Central-nervous system lymphoma related to Epstein-Barr virus. *N. Engl. J. Med.* **309**:745, 1983.
60. Hollick, G. E., Reichrath, L., and Smith, T. F. Comparison of primary rhesus and cynomolgus monkey kidney cell cultures for viral isolation from clinical specimens. *Am J. Clin. Pathol.* **68**:276, 1977.
61. Horwitz, C. A., Henle, W. Henle, G., Polesky, H., Balfour, H. H., Jr., Siem, R. A., Borken, S., and Ward, P. C. J. Heterophile-negative infectious mononucleosis and mononucleosis-like illness. *Am. J. Med.* **63**:947, 1977.

62. Hotchin, J. E., Cohen, S. M., Ruska, H., and Ruska, C. Electron microscopical aspects of hemadsorption in tissue cultures infected with influenza virus. *Virology* **6**:689, 1958.
63. Hsiung, G. D. Latent virus infections in primate tissues with special reference to simian viruses. *Bacteriol. Rev.* **32**:185, 1968.
64. Hsiung, G. D. Parainfluenza-5 virus: Infection of man and animal. *Prog. Med. Virol.* **14**:241, 1972.
65. Hudson, J. B., Misra, V., and Mosmann, T. R. Cytomegalovirus infectivity: analysis of the phenomenon of centrifugal enhancement of infectivity. *Virology* **72**:235, 1976.
66. Jacobs, J. P., Jones, C. M., and Baille, J. P.: Characteristics of a human diploid cell designated MRC-5. *Nature (London)* **227**:168, 1970.
67. Jackson, G. G., and Muldoon, R. L. Viruses causing common respiratory infections. *J. Infect. Dis.* **127**:328, 1973.
68. Johnson, K. M., Bloom, H. H., Chanock, R. M., Mufson, M. A., and Knight, V. VI. The newer enteroviruses. *Am. J. Public Health* **52**:933, 1962.
69. Kapikian, A., Greenberg, H. B., Wyatt, R. G., Kalica, A. R., and Chanock, R. M. *The Norwalk Group of Viruses—Agents Associated with Epidemic Viral Gastroenteritis.* Tyrrell, D. A. J., and Kapikian, A. Z. (eds.), New York, Marcel Dekker, Inc. 1982, p. 147.
70. Kapila, R., Lintz, D. I., Tecson, F. T., Ziskin, L., and Louria, D. B. A. nosocomial outbreak of influenza A. *Chest* **71**:576, 1977.
71. Kappius, K. D., Sather, G. E., Kaplan, J. E., Schonberger, L. B., Morath, T. P., and Calisher, C. H. Human arboviral infections in the United States in 1980. *J. Infect. Dis.* **145**:283, 1982.
72. Kempe, C. H. The end of routine smallpox vaccination in the United States. *Pediatrics* **49**:489, 1972.
73. Kepfer, P. D., Hable, K. A., and Smith, T. F. Viral isolation rates during summer from children with acute upper respiratory tract diseases and healthy children. *Am. J. Clin. Pathol.* **61**:1, 1974.
74. Ketter, A., Hamparian, V. V., and Hilleman, M. R. Characterization and classification of ECHO 28-rhinovirus-coryzavirus agents. *Proc. Soc. Exp. Biol. Med.* **110**:821, 1962.
75. Kipps, A., Dick, G., and Moodie, J. W. Measles and the central nervous system. *Lancet* **2**:1406, 1983.
76. Krous, H. F., Dietzman, D., and Ray, C. G. Fatal infections with echovirus types 6 and 11 in early infancy. *Am. J. Dis. Child* **126**:842, 1973.
77. Landry, M. L., Mayo, D. R., and Hsiung, G. D. Comparison of guinea pig embryo cells, rabbit kidney cells, and human embryonic lung fibroblast cell strains for isolation of herpes simplex virus. *J. Clin. Microbiol.* **15**:842, 1982.
78. Larke, R. P. B., Preiksaitis, J. K., Devine, R. D., and Harley, F. L. Haemolytic uraemic syndrome: evidence of multiple viral infections in a cluster of ten cases. *J. Med. Virol.* **12**:51, 1983.
79. Lerner, M., Silverman, S. H., Rausen, A. R., Haughton, P., and Winter, J. W. Viral meningitis. *N.Y. State J. Med.* **78**:746, 1978.
80. Levy, J. S., Carver, H. D., Moseby, I. K., Calisher, C. H., Francy, B., Monath, T. P. St. Louis encephalitis in Memphis—Shelby County, Tennessee, 1975: Epidemiologic aspects of human cases. *South. Med. J.* **71**.633, 1978.
81. MacWilliam, K. M., and Cook, K. M. Counter-electrophoresis as a possible

method for typing ECHO and coxsackie B viruses. *J. Hyg (Camb.)* **74**:239, 1975.

82. McMonigal, K., Horwitz, C. A., Henle, W., Henle, G., Lawton, J., Polesky, H., and Peterson, L. Post-perfusion syndrome due to Epstein-Barr virus. *Transfusion* **23**:331, 1983.
83. Mausbach, T. W., and Cho, C. T. Pneumonia and pleural effusion. *Am. J. Dis. Child.* **130**:1005, 1976.
84. Melnick, J. L. Taxonomy of viruses, 1980. *Prog. Med. Virol.* **26**:214, 1980.
85. Middleton, P. J. Pathogenesis of rotaviral infection. *J. Am. Vet. Asso.* **173**:544, 1978.
86. Minor, T. E., Dick, E. C., Baker, J. W., Ouellette, J. J., Cohen, M., and Reed, C. E. Rhinovirus and influenza type A infections as precipitants of asthma. *Am. Rev. Respir. Dis.* **113**:149, 1976.
87. Mintz, L., and Drew, L. Relation of culture site to the recovery of nonpolio enteroviruses. *Am. J. Clin. Pathol.* **74**:324, 1980.
88. Mirkovic, R. R., Kono, R., Yin-Murphy, M., Sohier, R., Schmidt, N. J., and Melnick, J. L. Enterovirus type 70: The etiologic agent of pandemic acute haemorrhagic conjunctivitis. *Bull. WHO* **49**:341, 1973.
89. Moore, D. F. Comparison of human fibroblast cells and primary rabbit kidney cells for isolation of herpes simplex virus. *J. Clin. Microbiol.* **19**:548, 1984.
90. Moore, M. Enteroviral disease in the United States, 1970–1979. *J. Infect. Dis.* **146**:103, 1982.
91. *Morbidity and Mortality Weekly Report.* Arboviral encephalitis—United States, 1983. Centers for Disease Control **32**:557, 1983.
92. *Morbidity and Mortality Weekly Report.* Influenza surveillance summary—United States, 1982–1983 season. Centers for Disease Control **32**:373, 1983.
93. *Morbidity and Mortality Weekly Report.* Influenza vaccinia, 1983–1984. Centers for Disease Control **32**:333, 1983.
94. *Morbidity and Mortality Weekly Report.* Mumps—United States, 1980–1983. Centers for Disease Control **32**:545, 1983.
95. *Morbidity and Mortality Weekly Report.* Mumps vaccine. Centers for Disease Control **31**:617, 1982.
96. *Morbidity and Mortality Weekly Report.* Varicella-zoster immune globulin distribution—United States and other countries. Centers for Disease Control **33**:81, 1984.
97. Morgan, C., Hsu, K. C., Rifkind, R. A., Knox, A. W., and Rose, H. M. The application of ferritin-conjugated antibody to electron microscopic studies of influenza virus in infected cells. I. The cellular surface. *J. Exp. Med.* **114**:825, 1961.
98. Morgan, E. M., and Rapp, F. Measles virus and its associated diseases. *Bacteriol. Rev.* **41**:636, 1977.
99. Mufson, M. A., and Belshe, R. B. A review of adenoviruses in the etiology of acute hemorrhagic cystitis. *J. Urol.* **115**:191, 1976.
100. Muldoon, R. L., Stanley, E. D., and Jackson, G. G. Use and withdrawal of amantadine chemoprophylaxis during epidemic influenza A. *Am. Rev. Resp. Dis.* **113**:487, 1976.
101. Nerurkar, L. S., Namba, M., and Sever, J. L. Comparison of standard tissue culture, tissue culture plus staining and direct staining for detection of genital herpes simplex virus infection. *J. Clin. Microbiol.* **19**:631, 1984.

102. Nikoskelainen, J., Neel, E. U., and Stevens, D. A. Epstein-Barr virus-specific serum immunoglobulin A as an acute-phase antibody in infectious mononucleosis. *J. Clin. Microbiol.* **10**:55, 1979.
103. Oill, P. A., Fiala, M., Schofferman, J., Byfield, P. E., and Guze, L. B. Cytomegalovirus mononucleosis in a healthy adult. *Am. J. Med.* **62**:413, 1977.
104. Osborn, J. E., and Walker, D. L. 1968. Enhancement of infectivity of murine cytomegalovirus *in vitro* by centrifugal inoculation. *J. Virol.* **2**:853, 1968.
105. Parsons, R., and Tyrrell, D. A. J. A plaque method for assaying some viruses isolated from common colds. *Nature (London)*, **189**:640, 1961.
106. Patriorca, P. A., Onorato, I. M., Sklar, V. E. F., Schonberger, L. B., Kaminski, R. M., Hatch, M. H., Morens, D. M., and Forster, R. K. Acute hemorrhagic conjunctivitis. *JAMA* **249**:1283, 1983.
107. Person, D. A., and Herrmann, E. C., Jr. Experiences in laboratory diagnosis of rhinovirus infections in routine medical practice. *Mayo Clin. Proc.* **45**:517, 1970.
108. Person, D. A., Smith, T. F., and Herrmann, E. C., Jr. Experiences in laboratory diagnosis of mumps virus infection in routine medical practice. *Mayo Clin. Proc.* **46**:544, 1971.
109. Peters, A. H., O'Grady, J. E., Milanovich, R. A. Aseptic meningitis associated with echovirus type 3 in very young children. *Am. J. Dis. Child.* **123**:452, 1972.
110. Pike, R. M., Laboratory-associated infections: Incidence, fatalities, causes, and prevention. *Annu. Rev. Microbiol.* **33**:41, 1979.
111. Price, D. A., Postlethwaite, R. J., and Longson, M. Influenza A_2 infections presenting with febrile convulsions and gastrointestinal symptoms in young children. *Clin. Pediatr.* **15**:361, 1976.
112. Reed, L. J., and Muench, H. A. simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493, 1938.
113. Reeve, P., Owen, J., and Oriel, J. D. Laboratory procedures for the isolation of *Chlamydia trachomatis* from the human genital tract. *J. Clin. Pathol.* **28**:910, 1975.
114. Reichman, R. C., and Dolin, R. Viral pneumonias. *Med. Clin. North Am.* **64**:491, 1980.
115. Retter, M., Middleton, P. J., Tam, J. S., and Petric, M. Enteric adenoviruses: Detection, replication, and significance. *J. Clin. Microbiol.* **10**:574, 1979.
116. Reynolds, D. W., Stagno, S., and Alford, C. A. Laboratory diagnosis of cytomegalovirus infections. *In* Lennette, E. H., and Schmidt, N. J. (eds.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial infections,* 5th ed. Washington, D.C. American Public Health Association, 1979, p. 399.
117. Rubenstein, A. S., and Miller, M. F. Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus. *J. Clin. Microbiol.* **15**:938, 1982.
118. Schmidt, N. J. Laboratory Diagnosis of Viral Infections. *In* Galasso, G. L., Merigan, T. C., and Buchanan, R. A. (eds.), *Antiviral Agents and Viral Diseases of Man.* New York, Raven Press, 1979, p. 209.
119. Schmidt, N. J., Guenther, R. W., and Lennette, E. H. Typing of ECHO virus isolates by immune serum pools: The "intersecting serum scheme." *J. Immunol.* **87**:623, 1961.
120. Sells, C. J., Carpenter, R. L., and Ray, C. G. Sequelae of central nervous system enterovirus infections. *N. Engl. J. Med.* **293**:1, 1975.

121. Sever, J. L. Persistent measles infection of the central nervous system: subacute sclerosing panencephalitis. *Rev. Infect. Dis.* **5**:467, 1983.
122. Shelokov, A., Vogel, J. E., and Chi, L. Hemadsorption (adsorption-hemagglutination) test for viral agents in tissue culture with special reference to influenza. *Proc. Soc. Exp. Biol. Med.* **97**:802, 1958.
123. Shope, R. E. Arboviruses. *In* Lennette, E. H., Spaulding, E. H., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 2nd ed. Washington, D.C., American Society for Microbiology, 1974, p. 740.
124. Shope, R. E., and Sather, G. E. Arboviruses. *In* Lennette, E. H., and Schmidt, N. J. (eds.), *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections,* 5th ed. Washington, D.C., American Public health Association, 1979, p. 585.
125. Smith, T. F., and Reichrath, L. Comparative recovery of 1972–1973 influenza virus isolates in embryonated eggs and primary rhesus monkey kidney cell cultures after one freeze-thaw cycle. *Am. J. Clin. Pathol.* **61**:579, 1974.
126. Smith, T. F. Specimen requirements, transport, and recovery of viruses in cell cultures. *In* Lennette, D. A., Specter, S., and Thompson, K. D. (eds.), *Diagnosis of Viral Infections.* Baltimore, University Park Press, 1979, p. 33.
127. Smith T. F. Viruses. *In* Washington, J. A. II (ed), *Laboratory Procedures in Clinical Microbiology.* New York, Springer-Verlag, 1981, p. 525.
128. Smith, T. F. Clinical uses of the diagnostic virology laboratory. *Med. Clin. North Am.* **67**:935, 1983.
129. Smith, T. F. Viral serology in clinical medicine. *In* Homburger, H. A. and Betsakis, J. G. (eds), *Clinical Laboratory Annual,* Vol. 2. New York, Appleton-Century-Crofts, 1983, p. 31.
130. Smith, T. F., Burgert, E. O., Dowdle, W. R., Noble, G. R., Campbell, R. J., and Van Scoy, R. E. Isolation of swine influenza virus from autopsy lung tissue of man. *N. Engl. J. Med.* **294**:708, 1976.
131. Smith, T. F., Holley, K. E., Keys, T. F., and Macasaet, F. F. Cytomegalovirus studies of autopsy tissue. I. Virus isolation. *Am. J. Clin. Pathol.* **63**:854, 1975.
132. Smith, T. F., Person, D. A., and Herrmann, E. C., Jr. Experiences in laboratory diagnosis of respiratory syncytial virus infections in routine medical practice. *Mayo Clin. Proc.* **46**:611, 1971.
133. Sprague, J. B., Hierholzer, J. C., Currier, R. W., II, Hattwick, M. A. W., and Smith, M. D. Epidemic keratoconjunctivitis. *N. Engl. J. Med.* **289**:1341, 1973.
134. Stagno, S., Pass, R. F., Dworsky, M. E., Henderson, R. E., Moore, E. G., Walton, P. D., and Alford, C. A. Congenital cytomegalovirus infection. *N. Engl. J. Med.* **306**:945, 1982.
135. Sugden, B. Epstein-Barr virus: a human pathogen inducing lymphoproliferation in vivo and in vitro. *Rev. Infect. Dis.* **4**:1048, 1982.
136. Taniguchi, K., Urasawa, S., and Urasawa, T. Virus-like particle, 35 to 40 nm, associated with an institutional outbreak of acute gastroenteritis in adults. *J. Clin. Microbiol.* **10**:730, 1979.
137. Taraska, S. P., Hable-Rhodes, K., Smith, T. F., and Washington, J. A., II. Etiology of pediatric gastroenteritis in Rochester, Minnesota. *Mayo Clin. Proc.* **54**:151, 1979.
138. Thouless, M. E., Bryden, A. S., and Flewett, T. H. Serotypes of human rotavirus. *Lancet* **1**:39, 1978.

139. Valenti, W. M., Hall, C. B., Douglas, R. G. Jr., Menegus, M. A., and Pincus, P. H. Nosocomial viral infections: 1. Epidemiology and significance. *Infect. Control* **1**:33, 1980.
140. Vogel, J., and Shelokov, A. Adsorption-hemagglutination test for influenza virus in monkey kidney tissue culture. *Science* **126**:358, 1957.
141. Wilfert, C. M., Buckley, R. H., Mohanakumar, T., Griffith, J. F., Katz, S. L., Whisnant, J. K., Eggleston, P. A., Moore, M., Treadwell, E., Oxman, M. N., and Rosen, F. S. Persistent and fatal central nervous system echovirus infections in patients with agammaglobulinemia. *N. Engl. J. Med.* **296**:1485, 1977.
142. Wilfert, C. M., Nusinoff Lehrman, S., and Katz, S. L. Enterovirus and meningitis. *Pediatr. Infect. Dis.* **2**:333, 1983.
143. Weller, T. H. Varicella and herpes zoster. *N. Engl. J. Med.* **309**:1362,1433, 1983.
144. Welliver, R. C., Cherry, J. D., and Holtzman, A. E. Typical, modified, and atypical measles. *Arch. Intern. Med.* **137**:39, 1977.
145. Wenner, H. A. The enteroviruses. *Am. J. Clin. Pathol.* **57**:751, 1972.
146. Yeager, A. S. Transmission of cytomegalovirus to mothers by infected infants: another reason to prevent transfusion-acquired infections. *Pediatr. Infect. Dis.* **2**:295, 1983.
147. Yolken, R. H., Bishop, C. A., Townsend, T. R., Bolyard, E. A., Bartlett, J., Santos, G. W., and Seral, R. Infectious gastroenteritis in bone marrow transplant recipients. *N. Engl. J. Med.* **306**:109, 1982.
148. Yow, M. D., Melnick, J. L., Blattner, R. J., Stephenson, W. B., Robinson, N. M., and Burkhardt, M. A. The association of viruses and bacteria with infantile diarrhea. *Am. J. Epidemiol.* **92**:33, 1970.

11

Parasites

John H. Thompson, Jr., Ph.D.

I. General Considerations

The detection and accurate identification of parasitic organisms depends in most instances on appropriate methods of visualization (gross or microscopic) of these organisms and in certain instances on serological procedures. Methods for visualizing parasitic organisms are only accurate and productive when used by trained, competent personnel.

A. Ocular Micrometer

One prerequisite is thorough knowledge of how to use the microscope, magnifications of the fields, and an ocular micrometer. A rough estimation of the size of a parasite may be made by comparing the parasite with a red blood cell; however, accurate measurement of objects in a microscopic field is one of the important criteria in the identification of parasites. A reliable and simple procedure for calibration is presented in the following:

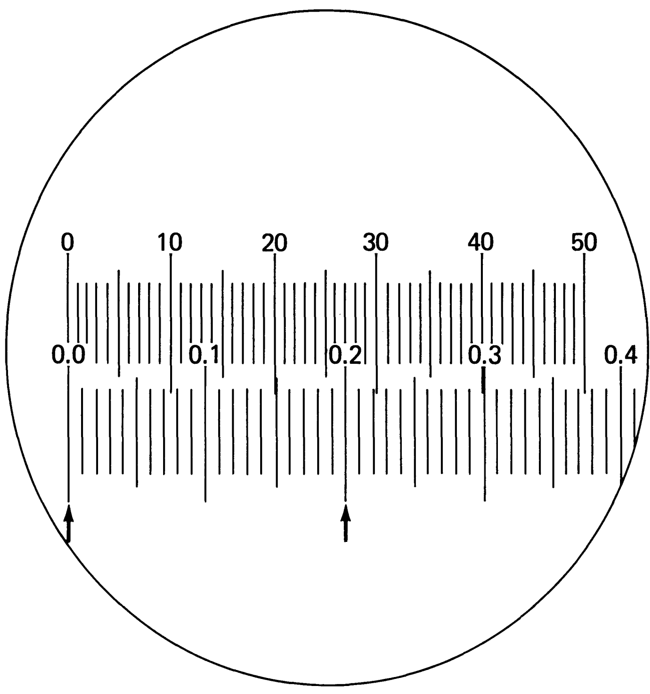
1. Calibration

It is advisable to use a calibrated micrometer disk that is placed within the ocular of the microscope. The type most often used is a disk with a line scale divided into 50 spaces of ocular micrometer units.

Inasmuch as the units on the ocular micrometer are arbitrary and the exact value of each varies with different objectives and microscopes,

it is necessary to calculate the value with each combination of lenses—low, high dry, and oil immersion. To do this, the ocular micrometer units are compared with those on a scale of known dimensions by superimposing the image of the unknown ocular scale on the known scale of a stage micrometer (slide with a carefully etched and calibrated scale, divided into 0.1-mm and 0.01-mm divisions) as demonstrated in Figure 11–1. The procedure for calibration is as follows:

a. Remove the 10× ocular from the microscope and unscrew the top eye-lens. Place the micrometer disk on the diaphragm within the ocular so that *the engraved side* is underneath. Screw back the eye-lens and insert the ocular in the microscope. Care must be taken to keep both micrometer and lens clean and free of dust which might interfere with observation.
b. Place the stage micrometer on the stage and focus on some portion of the scale.



Ocular micrometer—top scale
Stage micrometer—bottom scale

Figure 11–1. Example of calibrated ocular micrometer. Top scale, ocular micrometer; bottom scale, stage micrometer. (From Melvin, D. M., and Brooke, M. M. *Laboratory Procedures for the Diagnosis of Intestinal Parasites.* Atlanta, U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, 1974.)

c. By looking through the microscope, examine the ruling of the stage micrometer to distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.
d. Adjust the field so that the 0 lines on the ocular and stage micrometers are *exactly* superimposed.
e. Without moving the stage micrometer, find another point as far to the right as possible from the 0 lines where two other lines are exactly superimposed. The distance will vary with the objective and microscope.
f. Knowing that each of the large divisions of the stage micrometer equals 0.1 mm, determine the total distance (in millimeters) between the two sets of superimposed lines and the number of small ocular units in the same distance.
g. Calculate the number of millimeters that is measured by one small ocular unit.

   Example: 49 ocular units (small) = 8 stage units (large) or 0.8 mm

$$1 \text{ ocular unit} = \frac{0.8 \text{ mm}}{49} \text{ or } 0.0163 \text{ mm or } 16.3 \ \mu\text{m}$$

   (Measurement of protozoa and other microscopic structures are usually given in microns rather than millimeters.).
h. Record the calibrations of the ocular micrometer obtained with the 10× oculars when used with each of the three objectives. The lines of the stage micrometer will increase in magnification while those of the ocular micrometer will remain the same. With high dry and oil magnifications, it will be necessary to center the thinner ocular micrometer line on the broader stage micrometer line for more accurate measurement.

   Thus, the size of any microscopic object can be quickly determined by measuring it with the ocular micrometer, noting the particular combination of ocular and objective lenses, and referring to the record of the ocular micrometer calibrations.

b. Training

One very basic approach is to examine *negative* specimens to establish familiarity with normal objects (Table 11–1).

1. Stool Specimens[28,30]

One must learn to recognize immediately gross objects, such as mucus casts, food fibers, etc., and microscopic objects, such as yeasts, starch granules, vegetable cells, spores, meat fibers, *Blastocystis,* and pus and

Table 11–1. Nonparasitic Objects[a]

Artifact[b]	Resemblance	Saline mount	Differential characteristics of artifact in permanent stain	
			Cytoplasm	Nucleus
Polymorphonuclear leukocytes (seen in dysentery and other inflammatory bowel diseases)	*E. histolytica* cyst	Usually not a problem. Granules in cytoplasm. Cell border irregular.	Less dense, often frothy. Border less clearly demarcated than that of ameba.	More coarse. Large, relative to size of organism. Irregular shape and size. Chromatin unevenly distributed. Chromatin strands may link nuclei.
Macrophages (seen in dysentery and other inflammatory bowel diseases; may be present in purged specimens)	Amebic trophozoite, especially *E. histolytica*	Nuclei larger and of irregular shape, with irregular chromatin distribution. Cytoplasm granular; may contain ingested debris. Cell border irregular and indistinct. Movement irregular and pseudopodia indistinct.	Coarse. May contain inclusions.	Large and often irregular in shape. Chromatin irregularly distributed.
Squamous epithelial cells (from anal mucosa)	Amebic trophozoite	Nucleus refractile and large. Cytoplasm smooth. Cell border distinct.	Stains poorly.	Large and single. Large chromatin mass may resemble karyosome.

Columnar epithelial cells (from intestinal mucosa)	Amebic trophozoite	Nucleus refractile and large. Cytoplasm smooth. Cell border distinct.	Stains poorly.	Large with heavy chromatin on nuclear membrane. Often large central chromatin mass resembling karyosome.
Blastocystis hominis (yeastlike organism that frequently grows in feces; ruptures in water)	Protozoan cyst	Spherical to oval. 6–15 μm in length. Central clear area. Peripheral refractile granules (3–7) may resemble nuclei.	Central mass may stain light or dark. Prominent wall.	Peripheral granules may resemble nuclei. Granules vary in size and appearance. True nuclear structure not present.
Yeasts (normal constituent of feces)	Protozoan cyst	Oval. Thick wall. No internal structure. Budding forms may be seen.	Oval. Little internal structure. Refractile cell wall. Budding forms may be seen.	None.
Starch granules	Protozoan cyst	Rounded or angular. Very refractile. No internal structure. Stain pink to purple in iodine mounts.	Not a problem in permanently stained slides.	

[a] From Smith, J. W., McQuay, R. M., Ash, L. R., Melvin, D. M., Orihel, T. C., and Thompson, J. H. *Intestinal Protozoa.* Chicago, American Society of Clinical Pathologists, 1976.

[b] Other artifacts, such as contaminating plant cells and pollen grains, are occasionally seen. These should not be difficult to differentiate.

AMEBAE						
	Entamoeba histolytica	*Entamoeba hartmanni*	*Entamoeba coli*	*Entamoeba polecki**	*Endolimax nana*	*Iodamoeba butschlii*
Trophozoite						
Cyst						

Figure 11–2. Protozoa found in stool specimens of man. (Adapted from Brooke, M. M., and Melvin, D. M. *Common Intestinal Protozoa of Man—Life Cycle Charts.* DHEW Publication No. (CDC) 76–8311, 1964, reprinted 1978.) Note that *Dientamoeba fragilis* has been removed from the amebas and transferred to the flagellates (Camp, R. R., Mattern, C. F. T., and Honigberg, B. M. *Protozoology* **21**:69, 1974.) (See Figure 11–4.)

* Rare, probably of animal origin.

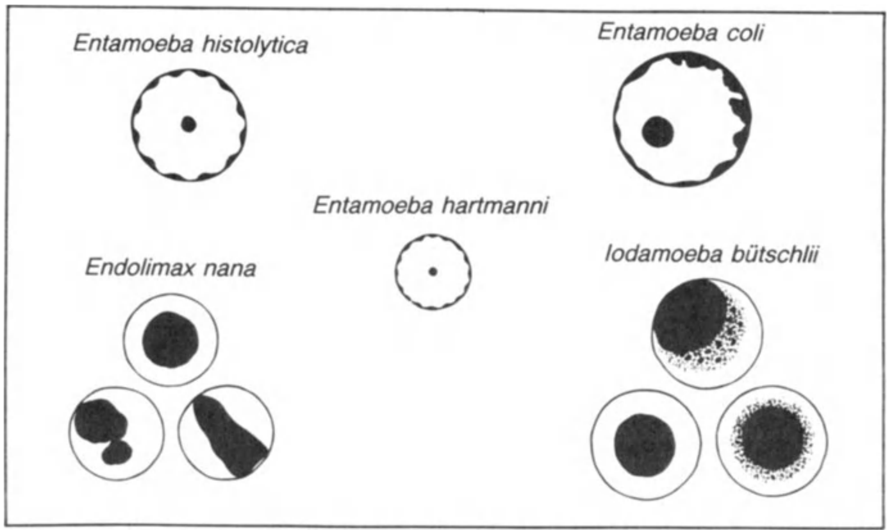


Figure 11–3. Nuclei of amebas. Note that *Iodamoeba* cysts often have eccentric karyosomes against the nuclear membrane, as in the upper *Iodamoeba* nucleus, whereas the trophozoite nuclei are usually not against the nuclear membrane. Achromatic granules are not always visible (lower left) in *Iodamoeba* nuclei. (Adapted from Smith, J. W., McQuay, R. M., Ash, L. R., Melvin, D. M., Orihel, T. C., and Thompson, J. H. *Intestinal Protozoa.* Chicago, American Society of Clinical Pathologists, 1976.)

epithelial cells. Pus and epithelial cells probably account more than any other form for false positive reports of *Entamoeba histolytica.* If stools with blood and pus are not readily available from patients, such specimens can be prepared by simply mixing a sample of blood from the Hematology Laboratory with emulsified feces. A fresh, wet preparation and Trichrome-stained smear of this specimen should be examined microscopically to acquire familiarity with common objects in normal stool. One can then introduce organisms found primarily in the gastrointestinal tract (Figures 11–2 to 11–5). This can be done by mixing cultures of *E. histolytica,* helminth eggs, and larval worms into a stool specimen and studying their forms (Figure 11–5), morphologies, and sizes.

Commercially prepared material is available for purchase (Ward's Natural Science Establishment, Inc., P. O. Box 1712, Rochester, NY 14603; P. O. Box 1749, Monterey, CA 93940; Turtox/Cambosco, 8200 South Hoyne Avenue, Chicago, IL 60620; Ann Arbor Biological Center, Inc., 6780 Jackson Road, Ann Arbor, MI 48103; Dr. R. M. McQuay, Mt. Sinai Hospital of Chicago, Ogden at California Avenue, Chicago, IL 60608), and excellent material can be obtained from animal rooms if the hospital or clinic has any or from veterinary clinics, meat packing houses, and

	FLAGELLATES				CILIATE	COCCIDIA*
	Dientamoeba fragilis	*Trichomonas hominis*	*Chilomastix mesnili*	*Giardia lamblia*	*Balantidium coli*	*Isospora spp.*
Trophozoite						immature oöcyst; mature oöcyst
Cyst	No cyst	No cyst				single sporocyst; double sporocysts

Figure 11–4. Protozoa found in stool specimens of man. (Adapted from Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man.* USDHEW PHS Publication No. 1966, 1969.)

* *Cryptosporidium* sp. belongs with this group of intestinal parasites. A new phylum, Apicomplexa, in the subkingdom Protozoa, in addition to these intestinal types also includes the blood and tissue organisms *Toxoplasma, Plasmodium, Sarcocystis,* and *Babesia.*

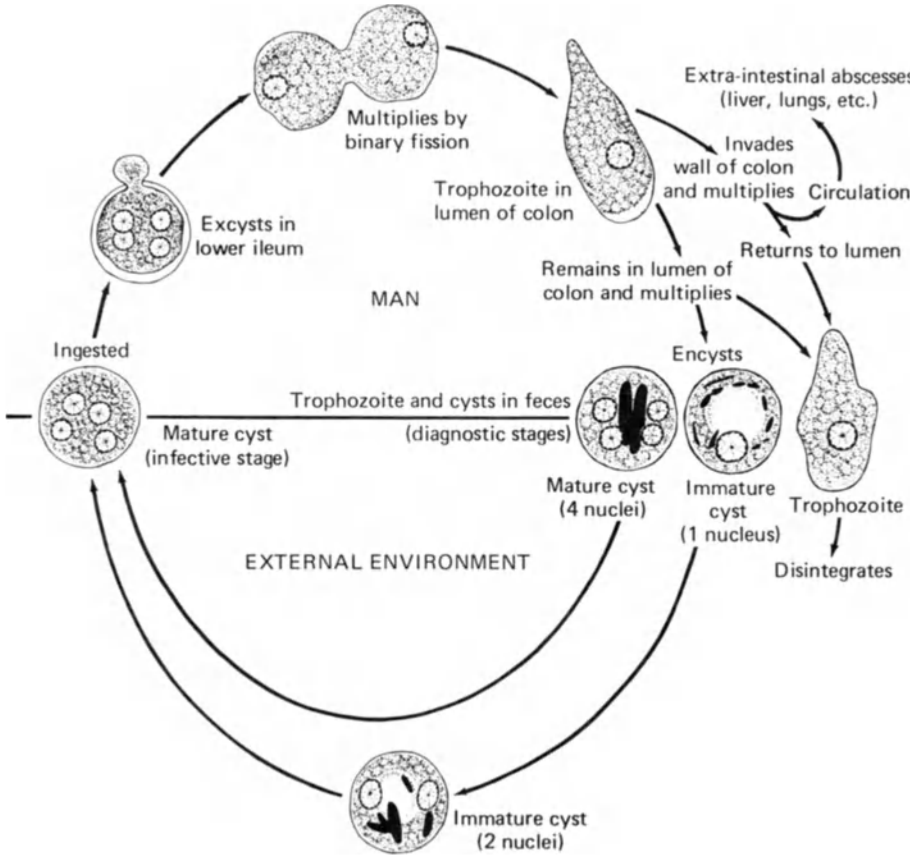


Figure 11-5. Life cycle of *Entamoeba histolytica.* (From Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man.* USDHEW PHS Publication No. 1966, 1969.)

common farm animals. The live ciliate, *Balantidium caviae,* and common flagellates may be seen in guinea pig feces, allowing one to study the very characteristic motilities of these organisms. A comprehensive *Atlas of Diagnostic Medical Parasitology* is available from the American Society of Clinical Pathologists.[21-23]

2. Blood Films[27,30]

Competence in examining blood films, especially the thick films, is initially acquired by examining smears of normal blood. A properly prepared Giemsa-stained thick film has only a few white cells, platelets, occasionally some cellular debris such as granules from white cells, and a blue-gray background of hemolyzed ("laked") red blood cells. The red blood cells

may occasionalliy not be completely laked so that the ghosts of the cells will be present. Also, some stain may precipitate and remain on the slide. Platelets may occur singly or in clumps and are frequently fused into crescent shapes. The difference between these fused platelet cresents and the crescent-shaped gametocyte of *Plasmodium falciparum* is the differential staining or pigment seen in the gametocyte of *P. falciparum* and not in platelets. Platelets are probably responsible more than any other objects for false positive reports of malaria. The errors are directly related to incomplete study and lack of familiarity. Other artifacts that may appear on slides include molds, clumps of bacteria, spores, and various fibers. The fibers can occasionally mimic the appearance of microfilariae and require careful study to recognize them as artifacts. The calibrated ocular micrometer is extremely valuable comparing the size of the artifact to that of microfilariae.

II. Procedures Suggested for Use in Examination of Clinical Specimens for Parasitic Infection*

A. Collection of Stool Specimens

Collection of satisfactory specimens is essential for reliable stool examination. The following procedures are suggested.

1. Fresh, unpreserved feces should be obtained, if feasible, and transported to the laboratory immediately. Fresh specimens are preferred for examinations for trophozoites, and are necessary when concentrations for *Strongyloides* larvae are to be performed.
2. Unpreserved feces should be examined *within 1 hr* after passage, especially if the stool is loose or watery and might contain protozoan trophozoites. Examination of formed feces may be delayed for a short time, but must be completed on the day on which the specimen is received in the laboratory. If prompt examination or proper fixation cannot be carried out, formed specimens may be refrigerated (do not incubate) for 1 or 2 days.
3. If specimens are delayed in reaching the laboratory or if they cannot be examined promptly (such as those received at night, on weekends, or when no parasitologist is available), portions should be preserved

* Based on a statement made by the Subcommittee on Laboratory Standards (Lawrence R. Ash, Ph.D., Mae Melvin, Ph.D., Donald V. Moore, Ph.D., Franklin Sogandares-Bernal, Ph.D., Marietta Voge, Ph.D., and Edward K. Markell, Ph.D., Chairman), Committee on Education, American Society of Parasitologists, and approved by the Council of the American Society for Medical Technologists, the Board of Scientific Advisors of the American Association of Bioanalysts, and the Board of Directors of the International Society for Clinical Laboratory Technology.

in fixatives such as 5% or 10% aqueous formalin or formol-saline and polyvinyl alcohol (PVA)-fixative.* Formalin preserves cysts, eggs, and larvae for subsequent wet-mount examination or for concentration; PVA-fixative preserves trophozoites, cysts, and eggs for subsequent permanent staining. A ratio of one part of feces to three parts of fixative is recommended. The merthiolate-iodine-formaldehyde (MIF) stain-preservative technique is more complicated than the above methods, and only recommended for use by those familiar with it. The specimen may be placed in fixatives in the laboratory, or the patient may be provided with fixatives and instructions for collection and preservation of his own specimens.

B. Methods of Stool Examination and Related Procedures

Stool specimens may be examined by the three complementary methods listed below. The advantages and limitations of each technique must be recognized.

1. Saline mounts are of value primarily for demonstrating the characteristic motility of certain amebae and flagellates. These may be found in fresh, unformed stools or at times in bloody mucus adhering to the surface of formed stools. Material should be obtained from several different parts of the specimen. An iodine stain (a drop of 1% iodine in 2% potassium iodide), mixed with a saline stool suspension, will stain and facilitate identification of protozoan cysts, but will kill and distort trophozoites.
2. Concentration techniques, which are useful in detecting small numbers of cysts and helminth eggs, are of less value for liquid stools. They may be used on unpreserved stool specimens, those preserved in 5% or 10% aqueous formalin or formol-saline, or on PVA-fixed material. The Ritchie formalin-ether sedimentation technique[19] or the zinc sulfate flotation method of Faust et al.[8] may be used. An iodine stain may be used with both techniques. As *Strongyloides* larvae do not concentrate well, the Baermann technique or culture methods should be utilized in cases in which this parasite is suspected, and for posttreatment checks.
3. Stained fecal films should be made if possible on all fresh or PVA-fixed specimens. *If properly prepared,* they are the single most productive means of stool examination for protozoa. Films may be stained

* PVA-fixative and PVA powder (pretested for use in the fixative solution) may be purchased from Delkote, Inc., 76 South Virginia Avenue, Penns Grove, NJ 08069 or Med Chem (Medical Chemical Corporation), P. O. Box 445, Santa Monica, CA 90404.

with such stains as Wheatley's modification of the Trichrome stain (p. 655) or the Tompkins-Miller iron hematoxylin phosphotungstic acid technique. Stained slides of positive specimens should be placed in a permanent accession file, analogous to those used for surgical and cytological specimens.

4. If egg counts are desired, the Beaver method[2] of egg counting on direct smears is recommended. Commercial modifications of the Scotch tape swab for pinworm and *Taenia* eggs are available, and are a satisfactory substitute for those made up in the laboratory. Specific identification of taeniid tapeworms is based on the morphology of the uterus in gravid segments. Unpreserved tapeworm proglottids (brought to the laboratory in water or normal saline) lend themselves best to such identification.

C. Number of Specimens to be Examined, and Appropriate Intervals

1. For the detection of amebas, a minimum of three specimens should be examined; if these (obtained preferably at intervals of 2 to 3 days) are negative and amebic infection remains a diagnostic consideration, additional specimens should be examined.
2. In suspected cases of giardiasis, an initial three specimens should be examined and if negative, three additional specimens should be obtained at weekly intervals. Duodenal aspiration or the enteric string-containing capsule* may also be of value in occult infections.
3. A single concentrate from one stool specimen is frequently sufficient to detect intestinal helminth infections of clinical importance. In very light schistosomal infections, few or no eggs may be found in the feces or urine. *Strongyloides* is best diagnosed by means of the Baermann concentrate. Various fecal culture methods or the enteric capsule may also be used to demonstrate the larvae of *Strongyloides,* which concentrate poorly by the usual techniques.
4. Posttreatment checks should under most circumstances be delayed until 1 month after completion of therapy, or 3 months after treatment for schistosomiasis.

D. Examination of Blood

1. Smears for malaria should consist of both thick and thin films. It is important that all responsible laboratory personnel be aware of the technique for making thick films. Giemsa-stained smears should be

* Entero Test, available from Hedeco, 2411 Pulgas Avenue, Palo Alto, CA 94303.

prepared and a minimum of 100 microscopic fields examined before reporting a specimen as negative. If the first specimen is negative, additional thick and thin films should be taken every 6 hr for 24 hr.

2. In suspected cases of filarial infection, the possibility of diurnal or nocturnal periodicity of microfilariae in the peripheral blood must be considered in securing specimens. Thick smears or blood concentration methods are most likely to demonstrate infection.

E. Serological Methods

A large variety of immunodiagnostic methods may serve as useful adjuncts to the clinical diagnosis of parasitic infections. In some cases, serological methods may be the only laboratory recourse in making a diagnosis. Certain serological tests provide a high degree of diagnostic accuracy; however, mixed infections, antigen sharing by related and unrelated parasites, and other diseases or physiological conditions in man may interfere with this diagnostic accuracy.

Most serum specimens may be shipped frozen, or preserved with thiomerosal to a final concentration of 1:10,000, to a state public health laboratory for forwarding to the Centers for Disease Control in Atlanta, GA. The vial, containing at least 2 ml of serum, should indicate the preservative used.

III. Processing of Stool Specimens

A. Stool Examination for Intestinal Parasites—Specimen Immediately Delivered to Laboratory

1. Gross Examination

When fresh stool specimens are received in the laboratory, they should be examined under a strong light to detect the presence of tapeworm segments, adult worms (*Ascaris,* or pinworm), blood, mucus, and other abnormal conditions. If the stool has a fatty appearance or if it is very liquid with little fecal material, this is also noted and reported to the physician.

Whether or not the patient has taken Epsom salts should be noted on the report form accompanying the specimen by personnel in the collection area. If the patient has not taken Epsom salts and has passed a diarrheal stool, a permanent stained slide must be made.

2. Microscopic Examination

Microscopic techniques include examination of an eosin-saline wet preparation, a formalin-ether (Ritchie) concentration, and a permanent stain. Trichrome is the preferred permanent stain for routine laboratory use, but iron hematoxylin is preferred for slides stored for teaching purposes. In certain instances, a differential count of leukocytes in diarrheal stool is helpful in distinguishing between an invasive or toxigenic etiological agent.

a. Eosin-Saline Wet Preparation[28,30]

(1) General Considerations

Eosin-saline is preferred to saline alone for a wet preparation because the solution is nontoxic to living organisms and does not stain them but does impart a pink to red background against which to scan for the presence, nuclear structure, and motility of light green amebas, flagellates and ciliates (Tables 11–2 to 11–5), various colorless eggs and larvae, or certain golden, bile-stained worm eggs.[28,30] The viability of organisms provides an indication of the age of the specimen since only dead pus cells and yeasts are stained. Through experience with this preparation, the examiner learns to distinguish between parasitic forms and fecal debris more quickly than with the frequently recommended saline or iodine preparations.

With a wet preparation, it is possible to detect the presence of barium, mineral oil or other oily compounds, or other particulate matter which

Table 11–2. Nuclear Visibility in Unfixed Material[a,b]

	Saline mount		Iodine mount	
	Tropho-zoites	Cysts	Tropho-zoites	Cysts
Entamoeba histolytica	−	−	+	+
Entamoeba hartmanni	−	−	+	+
Entamoeba coli	±	±	+	+
Endolimax nana	−	−	±	+
Iodamoeba bütschlii	−	−	−	−
Dientamoeba fragilis	−	None	±	None
Trichomonas hominis	−	None	−	None
Chilomastix mesnili	−	−	−	+
Giardia lamblia	−	−	−	+

[a] From Smith, J. W., McQuay, R. M., Ash, L. R., Melvin, D. M., Orihel, T. C., and Thompson, J. H. *Intestinal Protozoa*. Chicago, American Society of Clinical Pathologists, 1976.

[b] Nuclei not visible in unfixed material may be seen in unstained mounts of formalin-fixed material.

preclude adequate examination of stool and to request that another specimen be submitted in 2 or 3 days following passage of the interfering compounds.

The trained technologist can confirm and report waxy and glistening specimens as "fatty" stools by demonstrating clumps of needlelike, yellowish crystals in the eosin-saline preparation. In a double-blind study we found that the presence of "occasional" or "few" correlated well with low and nonsignificant total fat determinations and that the gross appearance of the stool was not indicative of excess fat. The presence of many fatty acid crystals in the eosin-saline observation correlated well with the gross appearance of a typical "fatty stool" and is generally an indication for the clinician to obtain 48 to 96 hr stool collections to determine fat levels quantitatively to rule out a metabolic disorder. A simple test to confirm that crystals seen are actually fatty acid type is to remove the coverslip from the eosin-saline preparation, place a drop of glacial acetic acid on the slide, warm it slightly over a Bunsen burner, replace the coverslip and reexamine the slide microscopically. If the crystals are truly fatty acid, they will have resolved in the warmed acid to globules of fat and their crystalline appearance will no longer be evident. The experienced worker will seldom need to use this test as few other crystalline forms appear quite like the fatty acid types in eosin-saline.

(2) Preparation

Reagents:
Eosin 0.25 g
Saline 250 ml

Procedure:

1. Mix a small quantity of feces with a drop or two of eosin-saline on a glass microscope slide.
2. Adjust the density of the preparation so that it is not too heavy to prevent penetration of light or too light to prevent adequate examination. As a general rule, one should be able to read newsprint through the preparation.
3. Examine the preparation immediately under low power (100×) and, when organisms are seen, under high power (400×).

Results:
In addition to reporting the presence or absence of parasitic organisms and fatty acid crystals, a qualitative (i.e., many, few, or occasional) report should be made of the presence of leukocytes, epithelial cells, red blood cells, and Charcot-Leyden crystals.

Table 11–3. Morphology of Trophozoites of Intestinal Amebas[a]

Species	Size (diameter or length)	Motility	Nucleus			Cytoplasm	
			Number	Peripheral chromatin	Karyosomal chromatin	Appearance	Inclusions
Entamoeba histolytica	10–60 μm. Usual range, 15–20 μm—commensal form.[b] Over 20 μm—invasive form.[c]	Progressive, with hyaline, fingerlike pseudopods.	1 Not visible in unstained preparations.	Fine granules. Usually evenly distributed and uniform in size.	Small, discrete. Usually centrally located, but occasionally is eccentric.	Finely granular.	Erythrocytes occasionally. Noninvasive organisms may contain bacteria.
Entamoeba hartmanni	5–12 μm. Usual range, 8–10 μm.	Usually nonprogressive, but may be progressive occasionally.	1 Not visible in unstained preparations.	Similar to *E. histolytica.*	Small, discrete, often eccentrically located.	Finely granular.	Bacteria.
Entamoeba coli	15–50 μm. Usual range, 20–25 μm.	Sluggish, nonprogressive, with blunt pseudopods.	1 Often visible in unstained preparations.	Coarse granules, irregular in size and distribution.	Large, discrete, usually eccentrically located.	Coarse, often vacuolated.	Bacteria, yeasts, other materials.

Entamoeba polecki	10–25 μm. Usual range, 15–20 μm.	Usually sluggish, similar to *E. coli.* Occasionally in diarrheic specimens, motility may be progressive.	1 May be slightly visible in unstained preparations. Occasionally distorted by pressure from vacuoles in cytoplasm.	Usually, fine granules evenly distributed. Occasionally, granules may be irregularly arranged. Chromatin sometimes in plaques or crescents.	Small, discrete, eccentrically located. Occasionally large, diffuse, or irregular.	Coarsely granular, may resemble *E. coli.* Contains numerous vacuoles.	Bacteria, yeasts.
Endolimax nana	6–12 μm. Usual range, 8–10 μm.	Sluggish, usually nonprogressive, with blunt pseudopods.	1 Visible occasionally in unstained preparations.	None.	Large, irregularly shaped, blotlike.	Granular, vacuolated.	Bacteria.
Iodamoeba bütschlii	8–20 μm. Usual range, 12–15 μm.	Sluggish, usually nonprogressive.	1 Not usually visible in unstained preparations.	None.	Large, usually centrally located. Surrounded by refractile, achromatic granules. These granules are often not distinct even in stained slides.	Coarsely granular, vacuolated.	Bacteria, yeasts, or other material.

[a] Adapted with permission from Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man,* USDHEW PHS Publication No. 1966, 1969.

[b] Usually found in asymptomatic or chronic cases; may contain bacteria.

[c] Usually found in acute cases; often contain red blood cells.

Table 11–4. Morphology of Trophozoites of Intestinal Flagellates[a]

Species	Size (length)	Shape	Motility	Number of nuclei	Number of flagella[b]	Other features		
						Nucleus		
						Number	Peripheral chromatin	Karyosomal chromatin
Dientamoeba fragilis	5–15 μm. Usual range, 9–12 μm.	Ameboid	Pseudopodia are angular, serrated, or broad lobed and hyaline, almost transparent	2 (In approximately 20% or organisms only 1 nucleus is present.) Nuclei invisible in unstained preparations.	None	(See Nuclei)	None	Large cluster of 4–8 Granules
						Cytoplasm		
						Appearance		Inclusion
						Finely granular, vacuolated		Bacteria
Trichomonas hominis	8–20 μm. Usual range, 11–12 μm.	Pear-shaped	Rapid, jerking	1 Not visible in unstained mounts	3–5 anterior 1 posterior	Undulating membrane extending length of body.		

Chilomastix mesnili	6–24 μm. Usual range, 10–15 μm.	Pear-shaped	Stiff, rotary	1 Not visible in unstained mounts	3 anterior 1 in cytostome	Prominent cytostome extending ⅓–½ length of body. Spiral groove across ventral surface.
Giardia lamblia	10–20 μm. Usual range, 12–15 μm.	Pear-shaped	"Falling leaf"	2 Not visible in unstained mounts	4 lateral 2 ventral 2 caudal	Sucking disk occupying ½–¾ of ventral surface.
Enteromonas hominis	4–10 μm. Usual range 8–9 μm.	Oval	Jerking	1 Not visible in unstained mounts	3 anterior 1 posterior	One side of body flattened. Posterior flagellum extending free, posteriorly or laterally.
Retortamonas intestinalis	4–9 μm. Usual range, 6–7 μm.	Pear-shaped or oval	Jerking	1 Not visible in unstained mounts	1 anterior 1 posterior	Prominent cytostome extending approximately ½ length of body.

[a] Adapted with permission from Brooke, M, M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man,* USDHEW PHS Publication No. 1966, 1969.
[b] Not a practical feature for identification of species in routine fecal examinations.

Table 11–5. Morphology of Intestinal Ciliate and Coccidia[a]

Species	Size (length)	Shape	Motility	Number of nuclei	Other features
Balantidium coli					
Trophozoite	50–70 μm or more. Usual range, 40–50 μm.	Ovoid with tapering anterior end.	Rotary, boring.	1 large, kidney-shaped macronucleus. 1 small, subspherical micronucleus immediately adjacent to macronucleus. Macronucleus occasionally visible in unstained preparation as hyaline mass.	Body surface covered by spiral, longitudinal rows of cilia. Contractile vacuoles are present.
Cyst	45–65 μm. Usual range, 50–55 μm.	Spherical or oval		1 large macronucleus visible in unstained preparations as hyaline mass.	Macronucleus and contractile vacuole are visible in young cysts. In older cysts, internal structure appears granular.

Isospora species (*I. belli* and *I. hominis*)	Oocyst: 25–30 μm. Usual range, 28–30 μm. Immature oocyst not usually seen in *I. hominis*.	Ellipsoidal.	Nonmotile.		Mature oocyst contains 2 sporocysts with 4 sporozoites each. *I. belli:* Usual diagnostic stage is immature oocyst with single granular mass (zygote) within. *I. hominis:* Mature sporocysts, singly or in pairs, are usually passed in feces. Oocyst wall not apparent.
	Sporocyst: *I. belli*— 12–14 μm. *I. hominis*— 14–16 μm.	Round or oval.			
Cryptosporidium sp.	Oocyst: 3–6 μm usual range, 4–5 μm.	Sperical.	Nonmotile.		Mature oocyst contains four sporozoites. There is no sporocyst stage. Oocysts are passed in feces.

[a] Adapted with permission from Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man,* USDHEW PHS Publication No. 1966, 1969.

b. Methylene Blue Stain for Leukocytes

(1) General Considerations

The enumeration and differentiation of leukocytes in feces may help to distinguish between bacteria causing diarrhea primarily by invasive (e.g., *Shigella, Salmonella*) and toxigenic (e.g., *Vibrio cholerae,* toxigenic *Escherichia coli*) mechanisms.[11]

(2) Procedure

(a) Scan the eosin-saline wet preparation for the presence of ≥10 leukocytes/lpf.
(b) If present, place a small fleck of mucus or feces on a clean, glass microscope slide.
(c) Mix with one drop of methylene blue stain (p. 89).
(d) Scan under low power magnification (100×) to determine the approximate number of cells and report ≥10 leukocytes/lpf as "many."
(e) Perform differential count under high power magnification (400×) and report percentages of polymorphonuclear and mononuclear leukocytes/hpf. The presence of many leukocytes suggests that the diarrhea is due to an invasive organism. Monocytes are likely to be seen in enteric fever due to *Salmonella typhi.* When a request is specifically made to examine feces for eosinophils, it is suggested that Giemsa stain be used to stain an air-dried, methanol-fixed smear that is examined under high power (400×) or oil immersion (1000×) magnification.

c. Concentration

Concentration of the stool specimen to detect protozoan cysts, eggs, and/or larvae of worms is best performed by the Ritchie formalin-ether[19] or formalin-ethyl acetate[34] method and is a routine procedure for examining at least one of the three specimens submitted from each patient.

This technique has the advantage of simplicity in preparation, and will provide the examiner with a slide showing both protozoan and helminth forms in adequate numbers and excellent morphology (Tables 11–6 and 11–7) with less distortion of eggs, cysts, and larvae than other methods. The Ritchie technique also permits staining with iodine for further identification of cyst forms, makes excellent slides for semipermanent mounting for study material, and permits concentration of large volumes of specimens that may contain valuable teaching material. The type of stool collection (purged or formed stool) will govern the amounts of stool specimen and diluent to be used.

(1) Formalin-Ether Concentration

(a) Mix a portion of stool about the size of a walnut with sufficient water (saline can be used) in a 50 ml centrifuge tube so that 10 ml of suspension will yield about 1 ml of sediment upon centrifugation.

(b) Strain about 10 ml of the suspension through three thicknesses of wet gauze and pour into a 15 ml conical centrifuge tube.
(c) Centrifuge at 1500 rpm for 2 min. Decant the supernatant. About 1 to 1.5 ml of sediment should be present. It is important to have approximately this amount of sediment because too much or too little result in ineffective concentration.
(d) Resuspend the sediment in 10 ml of fresh water by first loosening it with an applicator stick after adding the water and then vigorously shaking it using a fingercot. Centrifuge again at 1500 rpm for 2 min.
(e) Repeat this washing step until the supernatant becomes clear. For a normal stool specimen, adequately mixed in water at the beginning, two washings are generally required. *Note:* Plastic squeeze bottles for water and formalin facilitate dispensing these solutions into the tubes.
(f) Add about 9 ml of 10% formalin to the sediment, mix thoroughly and allow to stand for 5 min or longer. The formalin acts as a preservative for protozoan cysts.
(g) Add 3 ml of ether* to the tube and shake vigorously using a fingercot. The ether dissolves neutral fats and free fatty acids.
(h) Centrifuge at 1500 rpm for 2 min. Four layers should result as follows: a small layer of sediment, a layer of formalin, a plug of fecal debris, and a layer of ether.
(i) Free the plug of debris by ringing with an applicator stick and carefully decant the top three layers.
(j) Let some of the excess liquid on the sides of the tube drain to the bottom. Mix this liquid and the sediment with a pipette.
(k) The sediment is usually too thick to examine without dilution using either a drop of saline or iodine. The drop of diluting fluid should always be placed on the slide first, then add the drop of sediment from the pipette to avoid contamination of the saline or iodine dropper bottle. This is particularly a problem with cysts of *Giardia lamblia,* which may be present in the sediment in great numbers and readily contaminate the tip of the dropper if contact is made with the sediment. One can suspect that contamination has occurred if every sediment examined shows a few *Giardia* cysts following a sediment that showed great numbers of cysts. All examinations should stop until the fluid in the dropper bottle is centrifuged and the sediment then examined for organisms. If this sediment shows cysts, the fluid should be discarded, the bottle thoroughly rinsed and refilled, and stools rechecked.

* Release of C.D.C. Current Item #264, regarding ethyl acetate as a substitute solvent for ether, prompted our laboratory to perform comparative studies which indicated that ethyl acetate was as good as or better than ether in this concentration technique. Ethyl acetate is less flammable, less of a hazard to use, and is less expensive. There is no change in the procedure in using ethyl acetate and the amount used is identical.

Table 11–6. Morphology of Cysts of Intestinal Amebas[a]

			Nucleus			Cytoplasm	
Species	Size	Shape	Number	Peripheral chromatin	Karyosomal chromatin	Chromatoid bodies	Glycogen
Entamoeba histolytica	10–20 μm. Usual range, 12–15 μm.	Usually spherical.	4 in mature cyst. Immature cysts with 1 or 2 occasionally seen.	Peripheral chromatin present. Fine, uniform granules, evenly distributed.	Small, discrete, usually centrally located.	Present. Elongated bars with bluntly rounded ends.	Usually diffuse. Concentrated mass often present in young cysts. Stains reddish brown with iodine.
Entamoeba hartmanni	5–10 μm. Usual range, 6–8 μm.	Usually spherical.	4 in mature cyst. Immature cysts with 1 or 2 often seen.	Similar to *E. histolytica.*	Similar to *E. histolytica.*	Present. Elongated bars with bluntly rounded ends.	Similar to *E. histolytica.*
Entamoeba coli	10–35 μm. Usual range, 15–25 μm.	Usually spherical. Occasionally oval, triangular, or of another shape.	8 in mature cyst. Occasionally, supernucleate cysts with 16 or more are seen. Immature cysts with 2 or more occasionally seen.	Peripheral chromatin present. Coarse granules irregular in size and distribution, but often appear more uniform than in trophozoites.	Large, discrete, usually eccentrically, but occasionally centrally located.	Present, but less frequently seen than in *E. histolytica.* Usually splinterlike with pointed ends.	Usually diffuse, but occasionally well-defined mass in immature cysts. Stains reddish brown with iodine.

Entamoeba polecki	9–18 μm. Usual range, 11–15 μm.	Spherical or oval.	1. Rarely 2. Occasionally visible in unstained preparations.	Usually, fine granules evenly distributed.	Usually small and eccentrically located.	Present. Many small bodies with angular or pointed ends, or few large ones. May be oval, rodlike or irregular.	Usually small, diffuse masses. Stains reddish brown with iodine. A dark area called inclusion mass (possibly concentrated cytoplasm) is often also present. Mass does not stain with iodine.
Endolimax nana	5–10 μm. Usual range, 6–8 μm.	Spherical, ovoid, or ellipsoidal.	4 in mature cysts. Immature cysts with less than 4 rarely seen.	None.	Large (blotlike), usually centrally located.	Occasionally, granules or small oval masses seen, but bodies as seen in *Entamoeba* species are not present.	Usually diffuse. Concentrated mass seen occasionally in young cysts. Stains reddish brown with iodine.
Iodamoeba bütschlii	5–20 μm. Usual range, 10–12 μm.	Ovoid, ellipsoidal, triangular, or of another shape.	1 in mature cyst.	None.	Large, usually eccentrically located. Refractile, achromatic granules on one side of karyosome. Indistinct in iodine preparations.	Granules occasionally present, but chromatoid bodies as seen in *Entamoeba* species are not present.	Compact, well-defined mass. Stains dark brown with iodine.

[a] Adapted with permission from Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man,* USDHEW PHS Publication No. 1966, 1969.

Table 11–7. Morphology of Cysts of Intestinal Flagellates[a]

Species	Size	Shape	Number of nuclei	Other features
Dientamoeba fragilis	No cyst			
Trichomonas hominis	No cyst			
Chilomastix mesnili	6–10 μm. Usual range. 8–9 μm.	Lemon-shaped, with anterior hyaline knob or "nipple"	1 Not visible in unstained preparations.	Cytostome with supporting fibrils. Usually visible in stained preparation.
Giardia lamblia	8–19 μm. Usual range, 11–12 μm.	Oval or ellipsoidal	Usually 4. Not distinct in unstained preparations. Usually located at one end.	Fibrils or flagella longitudinally in cyst. Occasionally may be slightly visible in unstained cysts. Deep-staining fibers or fibrils may be seen lying laterally or obliquely across fibrils in lower part of cyst. Cytoplasm often retracts from portion of cell wall.
Enteromonas hominis	4–10 μm. Usual range, 6–8 μm.	Elongated or oval	1–4, usually lying at opposite ends of cyst. Not visible in unstained mounts.	Resembles *E. nana* cyst. Fibrils or flagella are usually not seen.
Retortamonas intestinalis	4–9 μm. Usual range, 4–7 μm.	Pear-shaped or slightly lemon-shaped	1 Not visible in unstained mounts.	Resembles *Chilomastix* cyst. Shadow outline of cytostome with supporting fibrils extends above nucleus.

[a] Adapted with permission from Brooke, M. M., and Melvin, D. M. *Morphology of Diagnostic Stages of Intestinal Parasites of Man,* USDHEW PHS Publication No. 1966, 1969.

(l) Examine as a wet mount, scanning with 10× objective and using 40× for specific identification.

The formalin-ethyl acetate procedure will demonstrate all helminth eggs and larvae (Figure 11–6) and provides excellent concentration of all protozoan cysts (Figures 11–2 and 11–4). A drop of iodine may be used to stain protozoan cysts and is helpful in demonstrating the internal structures in cysts of *Giardia lamblia*. The concentration technique can also be used on stools preserved in PVA fixative.[3]

The sediment from this concentration procedure is especially valuable in the detection and identification of *Cryptosporidium* sp., and a skilled and experienced microscopist may detect the tiny (3–6 μm), highly refractile bodies in a routine scan of the sediment. The suspicion that oocysts of *Cryptosporidium* are present can be confirmed by examination of a carbol fuchsin stained smear.[6]

Carbol-Fuchsin Stain

(1) Rapid Screening

1. Mix thoroughly a drop of the sediment from the formalin-ethyl acetate concentrate with a drop of Kinyoun's carbol-fuchsin stain on a glass microscope slide, smear into a film about the size of a nickel, and air dry.*
2. Add immersion oil directly to the dried film, cover with a coverslip.
3. Examine with the 40× bright field objective.
4. Everything on the smear will stain darkly except the oocysts of *Cryptosporidium.*
5. The *Cryptosporidium* oocysts will be bright and refractile because they contain water and everything else is in an oil medium.
6. This negative-stain preparation must be examined within 10 to 15 min before the oocysts begin to collapse.
7. If this rapid oil immersion screening procedure is negative, no further preparations are required.
8. If the oil immersion preparation is positive or if there is any question or doubt, a permanently stained slide should be prepared from the formalin-ethyl acetate sediment.

---

(2) Permanently Mounted Carbol-Fuchsin Stain

1. Smear sediment from formalin-ethyl acetate concentrate to about the size of a nickel and allow to air dry.

* Both the unknown and a control slide should be prepared. *Cryptosporidium* oocysts for control slides are available from Black Cat Laboratories, Rt. 6, Box 6589, Nampa, ID 83651.

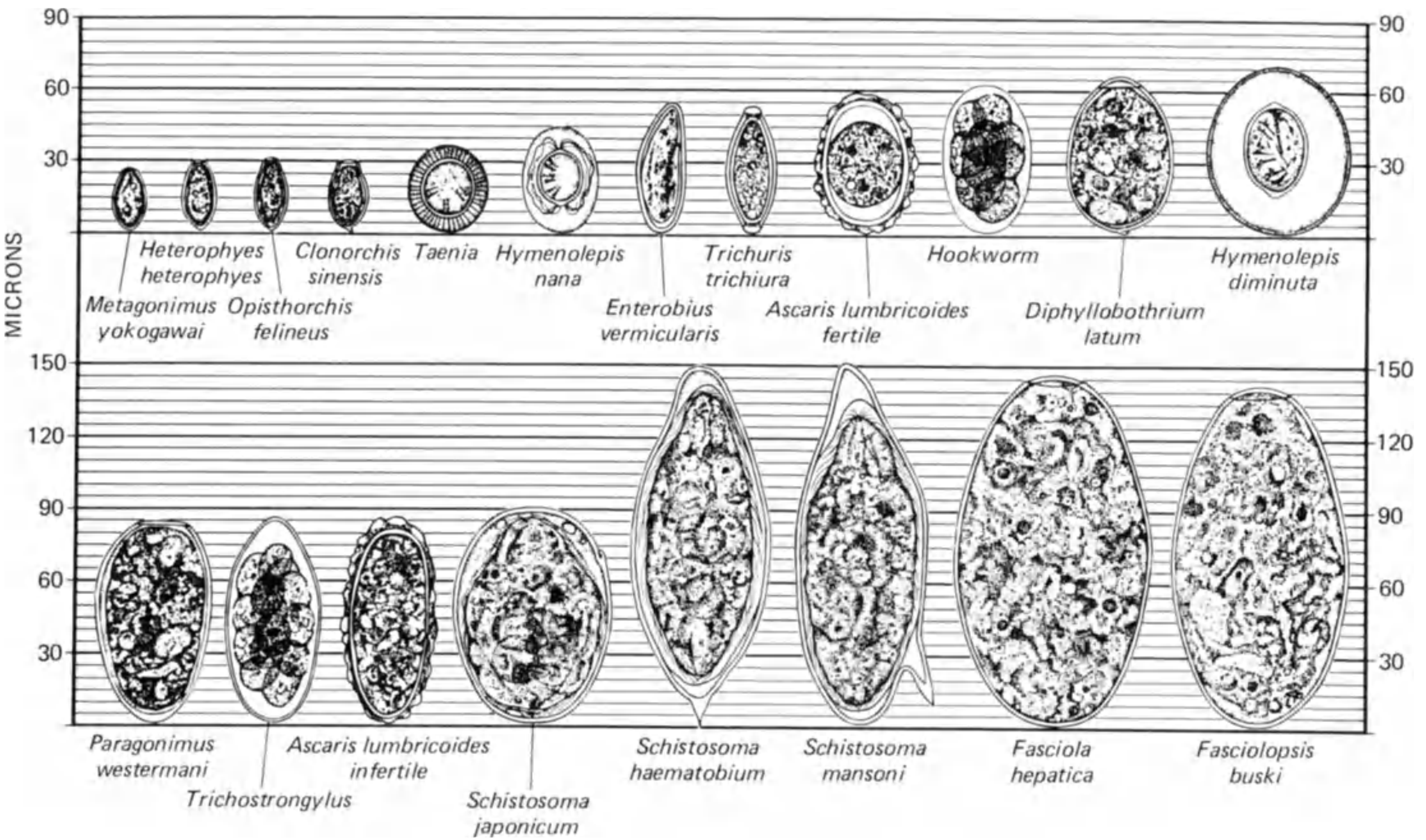


Figure 11–6. Relative sizes of helminth eggs. (From Smith, J. W., Ash, L. R., Thompson, J. H., McQuay, R. M., Melvin, D. M., and Orihel, T. C. *Intestinal Helminths.* Chicago, American Society of Clinical Pathologists, 1976.)

2. Fix dried smear in 95% methanol for 2 to 5 min, and dry at room temperature.
3. Stain with carbol-fuchsin for 20 to 30 min without heating.
4. Rinse in tap water.
5. Decolorize with H_2SO_4 (0.25 to 10% may be used) for 20–60 sec, and rinse thoroughly with tap water.
6. Counterstain with 5% malachite green for 5 min.
7. Rinse thoroughly with tap water, dry at room temperature, coverslip with mounting medium and examine with 40× objective.
8. Cryptosporidia will appear (3–6 μm in size) as densely stained reddish purple bodies clearly distinguishable against a green background. The cryptosporidia will contain a varying number of dark blue or brown internal bodies.

Reagents:

1. *Carbol Fuchsin*

Basic fuchsin	4 g
Phenol (melted)	8 ml
95% Ethyl alcohol	20 ml
Distilled water	to 100 ml

2. *10% Sulfuric Acid*

Sulfuric acid (Concentrated)	20 ml
Distilled water	180 ml

Add acid to water and mix well.

3. *Malachite Green*

Malachite green	5 g
Distilled H_2O	100 ml

Dissolve by shaking.

As noted previously regarding concentration of stools preserved in PVA fixative, these procedures for detection and identification of *Cryptosporidium* sp. can be performed using the sediment without any additional processing.

Before the advent of the formalin-ether (Ritchie) concentration, the acid-ether concentration was used to detect helminth ova and the zinc sulfate flotation technique was used for protozoan cysts. Now that the Ritchie is being used, these other two techniques are used mainly for research. A brief description of each is included below:

(3) Zinc Sulfate Centrifugal Flotation[8]

The zinc sulfate centrifugal flotation method is described in detail in texts on parasitology. A small sample of the feces is mixed with a 33% solution of zinc sulfate ($ZnSO_4$) causing all protozoan cysts and certain of the helminth eggs to float. Exceptions are larger eggs, such as those of some of the trematodes and certain *Ascaris,* for which acid-ether centrifugal sedimentation, described below, is recommended.

After the preparation has undergone repeated centrifugation and resuspension, a final suspension is lightly centrifuged to produce a top layer of meniscus containing concentrated protozoan cysts and lighter worm eggs. This layer can be removed either with a coverslip, a wire loop, or a droper pipette.

A drop of the concentrated material is then mixed with a drop of iodine solution (Lugol's solution or a dilute tincture) on a microscope slide, covered with a coverslip, and examined directly under a low power objective.

(4) Acid-Ether Centrifugal Sedimentation[15]

Acid-ether centrifugal sedimentation is another procedure for detecting worm eggs and larvae but not of protozoan cysts due to their distortion or destruction.)

(a) Mix a portion of feces approximately the size of a pecan with 10 ml of 15% HCl in a 50 ml centrifuge tube; stir vigorously to break up particulate matter.
(b) Pour the suspension through four thicknesses of gauze into another 50 ml centrifuge tube to remove food fibers.
(c) Transfer approximately 5 to 10 ml into a 15 ml conical centrifuge tube.
(d) Add ether to the conical tube, almost to the top, but allowing sufficient space for expansion of gases to avoid overflow.
(e) Cover the tube with the thumb, protected by a disposable fingercot; invert two or three times; then shake vigorously to mix its contents and further break up particulate matter; and remove fatty material from the final concentration.
(f) Centrifuge the mixture for 3 to 5 min at 1500 rpm, after which the tube presents the following appearance: a top, light layer of ether and fat, a layer of fat and fecal material, a layer of water and fecal material, and, at the very bottom of the tube, a button of material containing the worm eggs and larvae.
(g) Free the sticky layers from the walls of the tube by means of a wooden applicator and then carefully decant.
(h) Place drop of sediment on a slide and examine.

d. Permanent Stain

(1) Trichrome Staining Technique[33]

Reagents:

Schaudinn's Fixative

Stock solution	
Mercuric chloride	70 g
Distilled water	1000 ml
Ethyl alcohol, 95%	500 ml

Heat to dissolve mercuric chloride in water; allow to cool. Add the ethyl alcohol and mix thoroughly.

Working solution	
Stock solution	95 ml
Acetic acid, glacial	5 ml

Prepare solution fresh as required.

Iodine-alcohol

Alcohol, 70%	25 ml
Iodine solution	12 ml
I_2 crystals	1.5 g
KI powder	1 g
Distilled water	100 ml

Alcohol, 70%

Trichrome Stain

Chromotrope 2R	6 g
Light green SF	1.5 g
Fast green FLF	1.5 g
Phosphotungstic acid	7 g
Acetic acid, glacial	10 ml
Distilled water	1000 ml

Alcohol, 90%, Acidified

Ethyl alcohol, 90%	99.55 ml
Acetic acid, glacial	0.45 ml

Alcohol, 95%

Carbol-Xylene

Phenol	1 part
Xylene	3 parts

Transfer the dry stains into a flask. Add the acetic acid, mix, and allow to stand for 30 min. Add the water and mix thoroughly. The stain is deep purple to black and is stable in solution.

Procedure:

(a) With an applicator stick, make a thin, even smear of the feces on a clean, grease-free, glass microscope slide. If necessary, dilute with normal saline.

(b) Immediately after preparation, immerse slide, face down, in Schaudinn's fixative (p. 655) in a petri dish equipped with a rectangular glass rod support. The smear *must not be allowed to dry* from the time it is made until it is mounted.

(c) Stain by immersing the slides successively in Coplin jars containing reagents for each step. (Drain the excess liquid on the slides between steps by briefly touching the edge of the slide to an absorbent paper towel.)

(i) Schaudinn's fixative (p. 655)—30 min at room temperature or 5 min at 50°C.

(ii) 70% alcohol plus iodine (p. 655)—1 min.

(iii) 70% alcohol—1 min.

(iv) 70% alcohol—1 min.

(v) Trichrome stain—5 min.

(vi) 90% alcohol, acidified (p. 655)—10 sec (swish back and forth). *Note:* If several slides are being stained simultaneously, they should be destained separately. Remove one slide at a time from the stain, destain it (vi, above), rinse in the 95% alcohols (vii and viii, below) and place in the 100% alcohol (xi, below).

(vii) 95% alcohol—rinse briefly (swish back and forth).

(viii) 95% alcohol—rinse twice.

(ix) 100% alcohol or carbol-xylene—1 min.

(x) Xylene—2 min.

(xi) Mount with coverslip in Pro-Texx or other mounting medium.

Stain Reactions:

The cytoplasm of fixed and stained cysts and trophozoites should be blue-green and tinged with purple. The nuclear chromatin, chromatoid bodies, as well as ingested red cells and bacteria, stain red or purplish red, while other ingested particles, such as yeasts or molds, usually stain green; however, variations in the color reaction of ingested particles occur fre-

quently. Background material usually stains green in contrast to the protozoa. Incompletely fixed cysts either are not stained or stain predominantly red. Some red forms may be seen when staining Epsom salts purged specimens; however, the percentage of red forms in these instances is usually quite low. The 70% alcohol plus iodine can also influence the staining reaction. While the exact concentration of iodine in the solution is not important, it should be a port wine color, since too dark a solution may stain the protozoa and interfere with the Trichrome stain. If the solution is too light, the mercuric chloride crystals in the fixative will not be removed and will interfere with microscopic examination.. Generally, two parts of 70% alcohol to one part iodine will give good results.

It should also be noted that the different solutions in the staining process should be changed regularly as follows:

70% alcohol plus iodine—every 20 slides or sooner if iodine becomes lighter in color.
70% alcohol—every 20 slides.
Trichrome stain—every 2 months: between changes, add fresh stain, as needed, to offset loss due to use and evaporation.
90% alcohol, acidified—every 8 slides.
95% alcohol—every 8 slides.
100% alcohol—every 2 weeks.
Xylene—every month.

---

(2) Iron Hematoxylin Staining Technique (Regaud's)[5]

Reagents:

Schaudinn's Fixative (*p. 655*)

Mordant
Ferric ammonium sulfate, 4% solution

Decolorizer
Ferric ammonium sulfate, 2% solution

Stains

Hematoxylin	1 g
Alcohol, absolute	10 ml
Glycerin	10 ml
Distilled water	80 ml

Filter stain prior to storage and monthly.

Procedure:

(a) With an applicator stick, make a smear on clean, grease-free glass microscope slide.
(b) As edges of the smear are just beginning to dry, place the slide face down in Schaudinn's fixative in a petri dish equipped with rectangular glass rod support. Fix for 15 to 30 min.
(c) Wash in running tap water for 15 to 30 min. Rinse in several changes of distilled water.
(d) Transfer successively to 50%, 70%, and 95% alcohols for 3 to 5 min each.
(e) Rinse in several changes of distilled water.
(f) Transfer to mordant (4% ferric ammonium sulfate solution) for 15 to 30 min. Rinse as before in distilled water.
(g) Transfer to hematoxylin stain for 30 to 45 min. Rinse off excess stain in distilled water.
(h) Decolorize in 2% ferric ammonium sulfate solution by microscopic observation, or by precise timing by using 2 to 3 min as an average for most smears in fresh decolorizer. Time will increase as decolorizer is reused.
(i) Stop decolorizing by rinsing in distilled water. Wash in running tap water 15 to 30 min.
(j) Dehydrate successively in 50%, 70%, and 95% alcohols for 5 to 10 min each. Mount with Diaphane or other resin soluble in 95% alcohol. Permanent mounts should go from 95% to acetone to xylol. Mount in balsam or Permount.
(k) Rectal biopsy or other tissue slides are hydrated by transferring them from xylol to acetone and then successively to 95%, 70%, 50% alcohols, and, finally, water. The staining procedure is then the same as above. Before final dehydration in xylol and prior to mounting in balsam or Permount, tissue slides are lightly counterstained by briefly dipping them in acetone containing a few grains of eosin.

B. Stool Examination for Intestinal Parasites—Delayed Delivery, or Mailed In

Specimens received on a delayed basis in the laboratory should be in containers with polyvinyl alcohol (PVA) fixative. A fresh wet preparation of these specimens is not examined. The Ritchie formalin-ether concentration is performed (as described on p. 646, except that more specimen is used to counteract dilution by PVA), and Trichrome-stained smears of the specimen are examined microscopically.

1. Trichrome Staining Technique

The staining of PVA fixed specimens differs from that of fresh specimens in omitting the step in Schaudinn's fixative (p. 655) and prolonging many of the other steps.

Procedure:

(1) Spread specimen on clean, grease-free microscope slide and let stand until it has completely dried (usually 1 hr).
(2) Stain by immersing slide successively in Coplin jars containing the following:
  (a) 70% alcohol plus iodine—15 min.
  (b) 70% alcohol—4 min.
  (c) 70% alcohol—4 min.
  (d) Trichrome stain—8 min.
  (e) 90% alcohol, acidified—10 sec.
  (f) 95% alcohol—rinse to remove acid-alcohol.
  (g) 95% alcohol—5 min.
  (h) 100% alcohol or carbol-xylene—7.5 min.
  (i) Xylene—10 min.
  (j) Mount with coverslip in Pro-Texx or other mounting media. Unsatisfactorily stained organisms in stained smears of specimens submitted in PVA fixative usually indicates incomplete fixation associated with poor emulsification. Also, the expiration date of the PVA should be checked.

IV. Examination for Blood and Tissue Parasites[27,30]

A. Malaria, Babesia, Trypanosomes, Microfilariae—Thick and Thin Films (Tables 11–8 and 11–9, Figure 11–7)

Procedure:

1. The slides with blood smears (two thick and one thin blood film) should be prepared for routine examination [p. 32] and are properly labeled upon arrival in the laboratory for identification.
2. The thin film is placed in a Coplin jar of absolute methyl alcohol for 1 min of fixation. (The thick films are *not* fixed and should not be allowed to come in contact with even the vapors of the alcohol.)
3. Add 1 ml of stock Giemsa stain to 50 ml of buffer solution in a Coplin jar (p. 87). Mix well by stirring with the pipette used to deliver the

Table 11–8. Blood and Tissue Flagellates Found in Man[a]

Species	Developmental stages				Transmission	Vectors
	Amastigote	Promastigote	Epimastigote	Trypomastigote		
Leishmania						
L. donovani	Intracellular, in reticuloendothelial system, lymph nodes, liver, spleen, bone marrow, etc. Culture.	Midgut and pharynx of vector. Culture.	—	—	Bite	Sand flies (*Phlebotomus*, *Lutzomyia*)
L. tropica and *braziliensis*	Intra- and extracellular in skin and mucous membranes of man.	Midgut and pharynx of vector. Culture.	—	—	Bite	Sand flies (*Phlebotomus*, *Lutzomyia*)
Trypanosoma						
T. brucei gambiense	—	—	Salivary glands of vector. Culture.	Blood, lymph nodes, cerebrospinal fluid of final host. Intestine and salivary glands of vector.	Bite	Tsetse fly (*Glossina*)
T. brucei rhodesiense	—	—	Salivary glands of vector. Culture.	Blood, lymph nodes, cerebrospinal fluid of final host. Intestine and salivary glands of vector.	Bite	Tsetse fly (*Glossina*)
T. cruzi	Intracellular in viscera, myocardium, brain of man. Tissue culture.	Intracellular in man but transitional.	Midgut of vector. Culture.	Blood (temporary) of man. Intestine and rectum, feces of vector. Culture.	Feces of vector into wound.	Reduvid bugs (Triatominae)
T. rangeli	—	Transitional only.	Midgut of vector. Culture.	Blood of man. In hemolymph, salivary glands, and proboscis of vector. Culture.	Bite.	Reduvid bugs (Triatominae)

[a] From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.

Table 11–9. Filariae That Parasitize Man[a]

	Wuchereria bancrofti	*Brugia malayi*	*Loa loa*	*Mansonella ozzardi*	*Dipetalonema perstans*	*Dipetalonema streptocerca*	*Onchocerca volvulus*
Geographic distribution	Cosmopolitan; tropics and subtropics	Asia	West and Central Africa	South and Central America	Africa, South and Central America	West Africa	Africa, Central and South America
Adult habitat	Lymphatic system	Lymphatic system	Subcutaneous tissues	Mesenteries, body cavities	Mesenteries, perirenal, retroperitoneal tissues	Subcutaneous tissues	Subcutaneous tissues
Vector	Mosquitoes	Mosquitoes	*Chrysops* (deer fly)	*Culicoides; Simulium*	*Culicoides* (midge)	*Culicoides* (midge)	*Simulium* (black fly)
Location of microfilariae	Blood	Blood	Blood	Blood	Blood	Skin	Skin
Periodicity	Nocturnal[b]	Nocturnal[c]	Diurnal	None	None	None	None
Morphology of microfilariae							
Sheath	Present	Present	Present	Absent	Absent	Absent	Absent
Length (μm)	230–300	175–260	250–300	175–240	190–200	180–240	Two Sizes 285–370 150–290
Width (μm)	7.5–10	5–6	6–8.5	4–5	4–5	5–6	5–9
Tail and tail nuclei	Tapered to point; no nuclei in end of tail.	Tapered; terminal and subterminal nuclei.	Tapered; nuclei irregularly spaced to end of tail.	Long, slender tail; no nuclei in end of tail.	Tapered, bluntly rounded; nuclei to end of tail.	Tapered, bluntly rounded nuclei to end of tail. Tail bent in hook shape.	Tapered to point; no nuclei in end of tail.

[a] From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.
[b] Subperiodic in Pacific Islands.
[c] Subperiodic form as well.

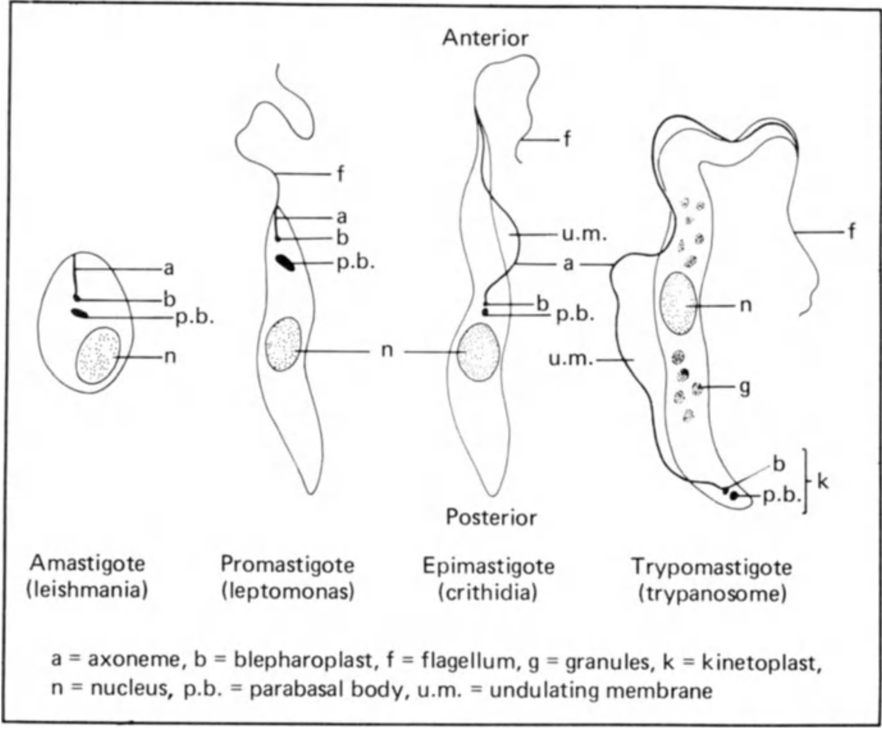


Figure 11-7. Morphological stages of blood and tissue flagellates. (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites*. Chicago, American Society of Clinical Pathologists, 1976.)

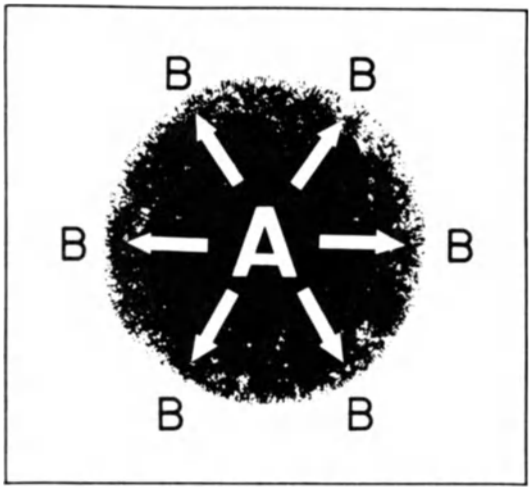


Figure 11-8. Method of examining a thick film. (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites*. Chicago, American Society of Clinical Pathologists, 1976.)

stain. Place sufficient buffer in a second Coplin jar to wash slides following staining.

4. After the thin film is dry from alcohol fixation, place it and the two thick smears in the stain for 30 min.
5. Wash excess stain from the slides by gently dipping them in the jar of buffer solution.
6. Allow the slides to drain by touching one end to a towel.
7. Allow to air dry.
8. Examine the slides under oil immersion (×1000) by using the two thick films for screening purposes and the thin film for species identification when the thick films are positive (Figures 11–8 to 11–10 and

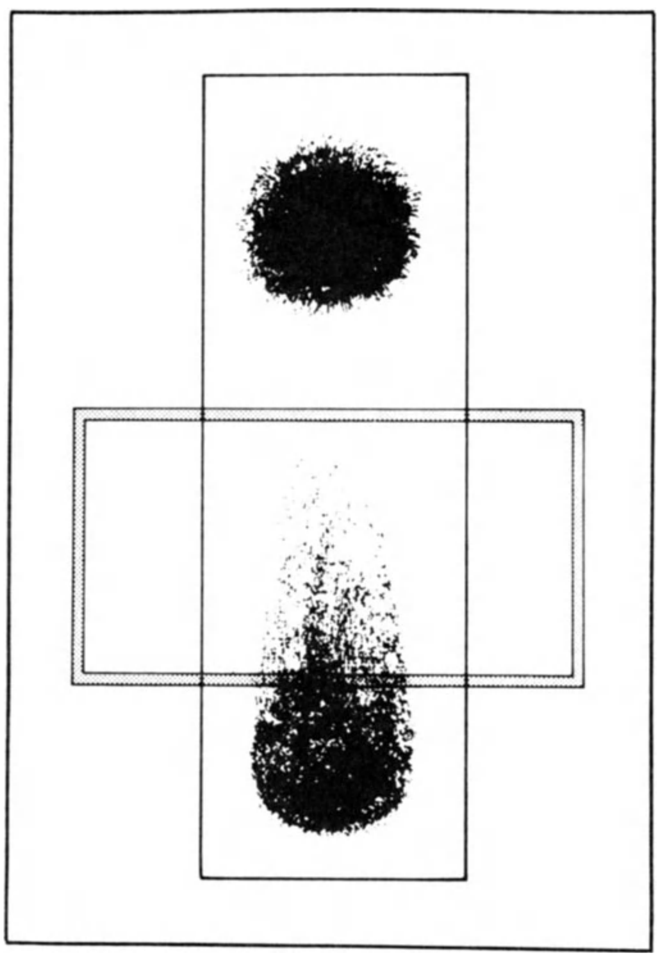


Figure 11–9. Area of thin film to examine. (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.)

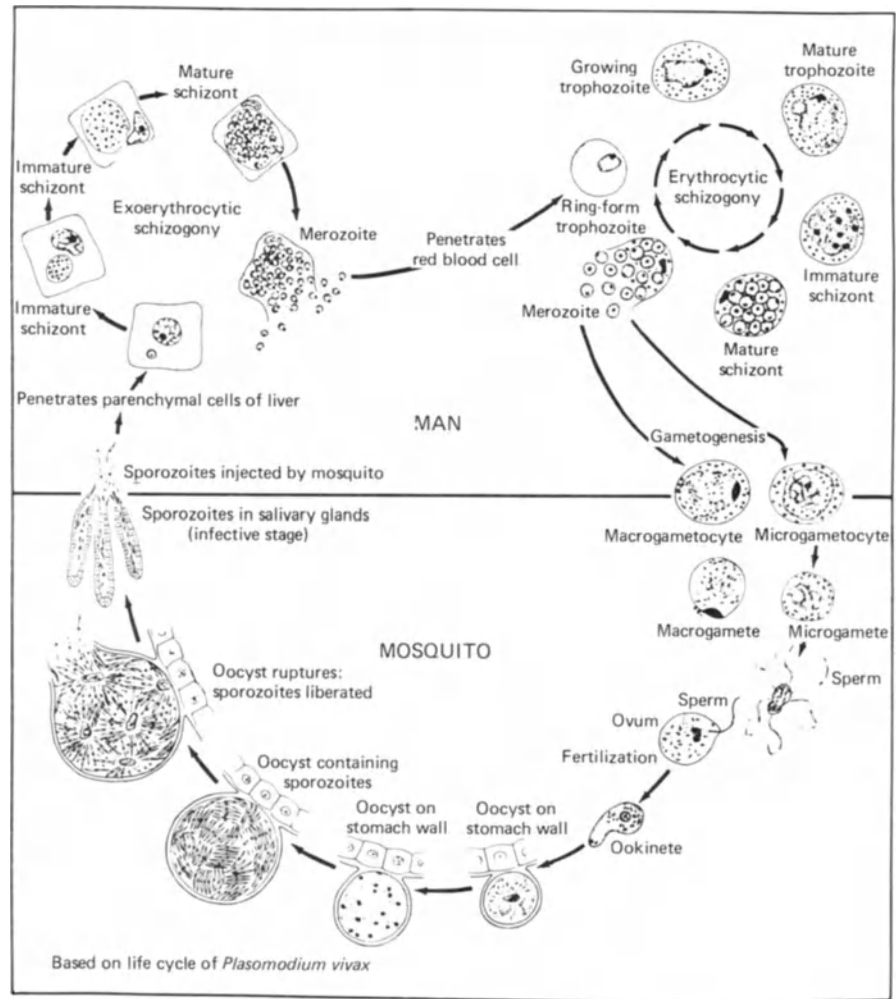


Figure 11–10. Life cycle of a malarial parasite. (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.)

Table 11–10). *Note.* The thick films should routinely be flooded with immersion oil and scanned under low power (×100) for detecting microfilariae prior to using the oil immersion objective (×1000) for detecting malaria.

Table 11–10. Comparison of *Plasmodium* Species Affecting Man[a]

	Appearance of erythrocyte		Appearance of parasite			
Species	Size	Schüffner's stippling	Cytoplasm	Pigment	Number of merozoites	Stages found in circulating blood
Plasmodium vivax	Enlarged. Maximum size (attained with mature trophozoites and schizonts) may be 1½–2 times normal erythrocyte diameter.	+ With all stages except early ring forms.	Irregular, ameboid in trophozoites. Has "spread-out" appearance.	Golden-brown, inconspicuous.	12–24 Average is 16.	All stages. Wide range of stages may be seen on given film.
Plasmodium malariae	Normal.	— (Ziemann's dots rarely seen.)	Rounded, compact trophozoites with dense cytoplasm. Band-form trophozoites occasionally seen.	Dark-brown, coarse, conspicuous.	6–12 Average is 8. "Rosette" schizonts occasionally seen.	All stages. Wide variety of stages usually not seen. Relatively few rings or gametocytes generally present.
Plamodium ovale	Enlarged. Maximum size may be 1¼–1½ times normal red blood cell diameter. Approximately 20% or more of infected red blood cells are oval and/or fimbriated (border has irregular projections).	+ With all stages except early ring forms.	Rounded, compact trophozoites. Occasionally slightly ameboid. Growing trophozoites have large chromatin mass.	Dark-brown conspicuous.	6–14 Average is 8.	All stages.
Plasmodium falciparum	Normal. Multiply infected red blood cells are common.	— (Maurer's dots occasionally seen.)	Young rings are small, delicate, often with double chromatin dots. Gametocytes are crescent or elongate.	Black. Coarse and conspicuous in gametocytes.	6–32 Average is 20–24.	Rings and/or gametocytes. Other stages develop in blood vessels of internal organs but are not seen in peripheral blood except in severe infections.

[a] From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.

B. Filaria and Trypanosomes (Figures 11–11 to 11–13)

Procedure:

1. Examination of whole blood
   Place a drop of the blood on a glass microscope slide, cover it with a 22-mm coverglass and scan the entire slide under low power (×100) magnification for motile microfilariae or trypanosomes.
2. Concentration[13]
   a. Add approximately 10–12 ml of 4% acetic acid to a 15 ml conical centrifuge tube.
   b. Add 1 ml of the well-mixed, citrated blood sample to the acetic acid.
   c. Shake the mixture vigorously.
   d. Centrifuge at 2000 rpm for 10 min.

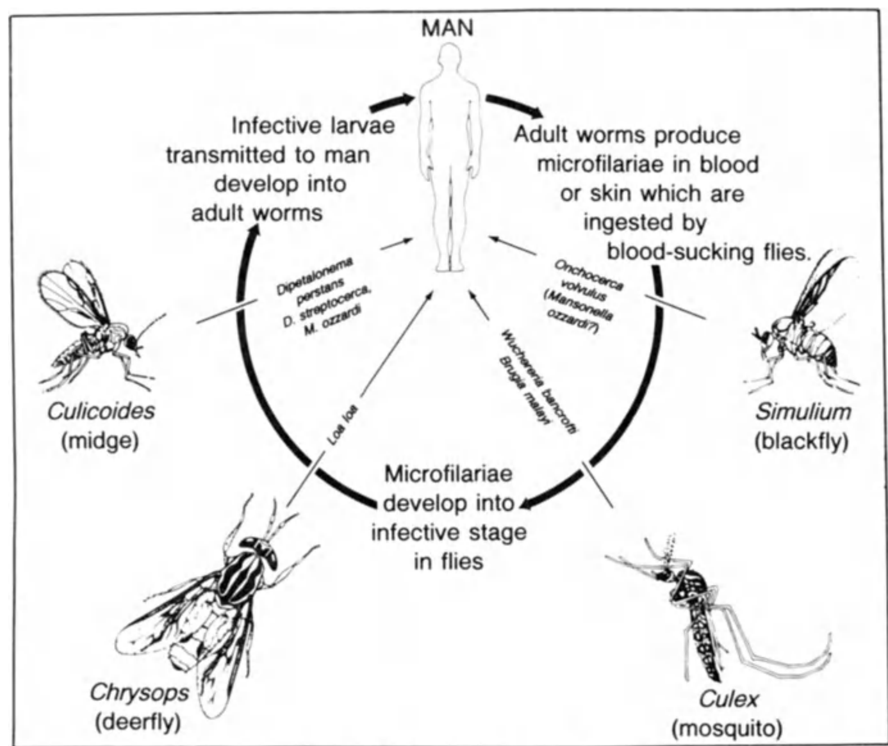


Figure 11–11. Life cycle of filariae. (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.)

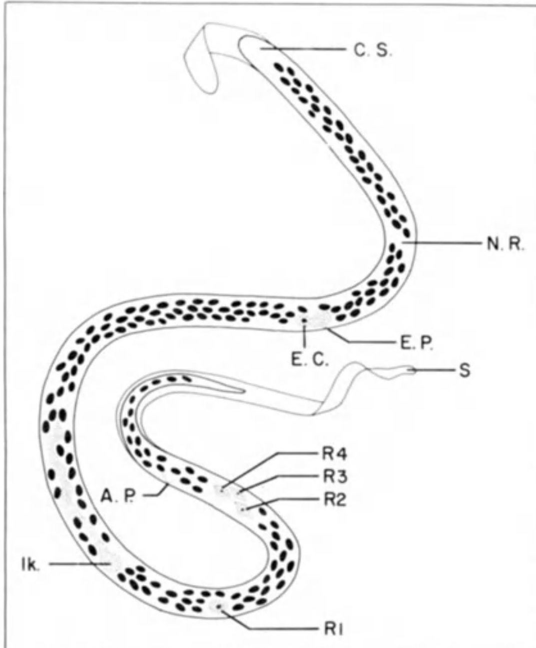


Figure 11–12. Morphology of a microfilaria. This diagrammatic representation of a microfilaria shows characteristic anatomical features, including the sheath (S), cephalic space (CS), nerve ring (NR), excretory pore (EP), excretory cell (EC), Innenkörper (Ik), rectal cells (R1–R4), and anal pore (AP). (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.)

e. Decant the supernatant fluid from the sediment.
f. Examine drops of the sediment microscopically as wet preparations.
g. If the concentration is positive for microfilariae, smear some of the sediment on a slide, allow to air dry, fix in methyl alcohol, and stain with Giemsa to aid in species identification.

3. Alternative procedures
   a. Buffy coat[29,30]
   Wet preparations of the buffy coat may be examined for trypanosomes following centrifugation of citrated blood for 30 min at 3000 rpm.
   b. (CSA) Citrate-saponin-acid technique[18]
   This procedure is based on the fact that most of the erythrocytes will be destroyed as the citrated blood comes into contact with saponin, but the microfilariae are unharmed and continue to demonstrate motility.
      (1) Mix 10 ml blood with 2 ml of freshly prepared sodium citrate (two evacuated blood collection tubes with EDTA may be used). The specimen should be mixed immediately and inverted at intervals to avoid clotting.
      (2) Centrifuge for 10 min at 1000 rpm and carefully remove and discard plasma.

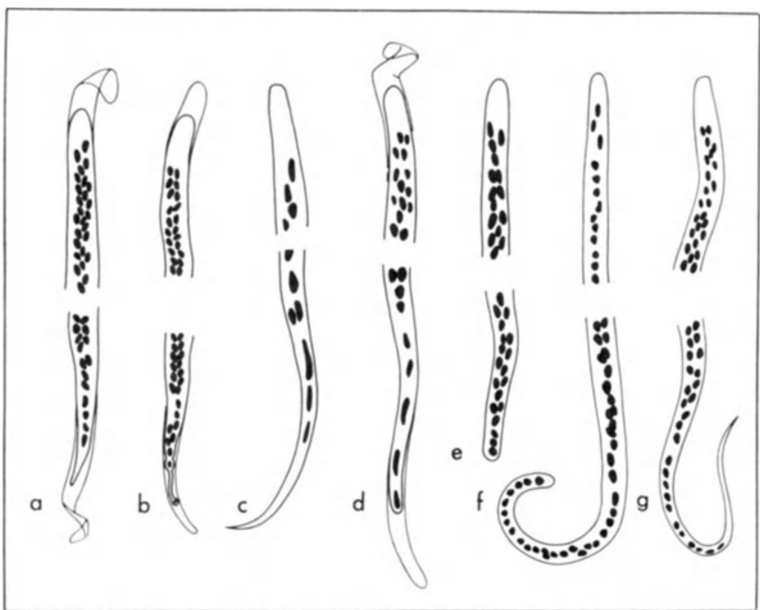


Figure 11–13. Anterior and posterior ends of microfilariae found in man. (a) *Wuchereria bancrofti,* (b) *Brugia malayi,* (c) *Onchocerca volvulus,* (d) *Loa loa,* (e) *Dipetalonema perstans,* (f) *Dipetalonema streptocerca,* and (g) *Mansonella ozzardi.* (From Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H. *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.)

(3) Transfer all packed cells (approximately 8 ml) to 50 ml tubes containing freshly made saponin (0.5% w/v in saline).
(4) Mix gently at intervals and let stand for 15 min.
(5) Centrifuge at 3500 rpm for 10 min, decant supernatant and discard.
(6) Examine the sediment under low power (×100) magnification by spreading a few drops on a slide without coverslip. This examination should be done quickly to observe the live, motile microfilariae before allowing the slides to air dry for staining. Stained microfilariae will appear coiled or curved.
(7) Mix the remainder of the sediment with two drops of 1.0% acetic acid solution either on slides or by adding the drops of acid to the sediment which is then removed to make slides. These slides are then thoroughly air dried before staining (the microfilariae on these slides will be killed by the acid and will be straightened).
(8) Dip the slides into methylene blue phosphate, rinse in two changes of distilled water, and then place in aqueous Giemsa stain for 8 to 10 min.

(9) Rinse the slides in distilled water and allow to air dry.
(10) Flood the slides with oil to scan under low power (×100) magnification and then under oil immersion (×1000) when microfilariae are detected.

V. Examination of Specimens Other Than Feces and Blood (Table 11–11)[5,23,28–30]

A. Urine

1. *Schistosoma haematobium*

a. Collect urine for 24 hr without preservative.
b. Allow the specimen to sediment completely overnight. (Procedure for single specimen is the same.)
c. Carefully aspirate supernatant until the sediment is clearly visible in remaining liquid.

Table 11–11. Nonfecal Specimens for Diagnosis[a]

Specimen	Infectious Agent	Stage
Sputum	*Paragonimus westermani*	Eggs
	Occasionally, larvae of *Ascaris lumbricoides,* hookworm, or *Strongyloides stercoralis*	Larvae
Anal swab or cellulose tape preparation	*Enterobius vermicularis*	Eggs
	Taenia spp.	Eggs
Duodenal aspirate	*Strongloides stercoralis*	Larvae
Muscle biopsy	*Trichinella spiralis*	Larvae
Cyst fluid	*Echinococcus granulosus*	Hydatid sand
Rectal biopsy	*Schistosoma japonicum* or *Schistosoma mansoni*	Eggs
Urine or urinary bladder biopsy	*Schistosoma hematobium*	Eggs
Liver biopsy	*Schistosoma* spp.	Eggs
	Nonhuman ascarids and hookworms (visceral larva migrans)	Larvae
	Echinococcus granulosus	Hydatid cyst
	Capillari hepatica	Eggs
Other tissue biopsy	*Taenia solium*	Cysticercus
	Multiceps multiceps	Coenurus
	Echinococcus granulosus	Hydatid cyst

[a] Smith, J. W., Ash, L. R., Thompson, J. H., McQuay, R. M., Melvin, D. M., and Orihel, T. C. *Intestinal Helminths.* Chicago, American Society of Clinical Pathologists, 1976.

d. Pour sediment into 15 ml conical centrifuge tube and spin at 2000 rpm for 10 min.
e. Carefully remove supernatant and place a couple of drops of the sediment on a glass microscope slide and apply a coverslip.
f. Examine the wet preparation for the terminally spined eggs of *Schistosoma haematobium.*

2. *Trichomonas vaginalis* in Males

a. Process a single voided specimen as described above (V,A,1); however, wet preparation may be examined microscopically before centrifugation.
b. The wet preparation and sediment are examined microscopically for the motile trichomonads (Fig. 2–11).

3. Filaria

a. Process a single voided or 24 hr urine collection as described above (V,A,1).
b. Examine microscopically for microfilariae, especially those of *Onchocerca volvulus.*
c. If positive, make a smear of the sediment, stain with Giemsa, and examine microscopically.

B. Anal Swabs

1. Scotch Tape Swab

a. Apply the sticky side of the Scotch tape to a glass microscope slide.
b. Examine under low power (×100) magnification for the asymmetrical pinworm eggs (*Enterobius vermicularis*).

2. Swube

a. Remove the sticky paddle from the plastic tube.
b. Place it lengthwise (sticky side up) on a glass microscope slide and examine as with a Scotch tape specimen (V,B,1).

C. Vaginal Swabs

1. Fresh Wet Preparation

a. Place several drops of normal saline or eosin-saline solution on a glass slide.

b. Swirl the tip of the swab (Culturette, Marion Scientific Products) in the saline. *Note.* Several drops of saline are required to make a wet preparation because the swab tip soaks up some fluid, allowing the preparation to dry up during examination.
c. Apply a coverslip and examine microscopically for the motile *Trichomonas vaginalis* (Fig. 2–11).

D. Biopsies, Tissue Specimens, and Specimens for Identification (Arthropods and Artifacts)

1. Jejunal or Duodenal Biopsies (Aspirates)

Procedure:

a. Place a few drops of the specimen on a glass microscope slide, apply a coverslip, and examine microscopically as a wet preparation.
b. Place the remainder of the specimen in a conical centrifuge tube and spin at 2000 rpm for 10 min.
c. Examine both the wet preparation and the sediment microscopically for the motile forms of *Giardia lamblia* and the larvae of *Strongyloides stercoralis.*

2. Rectal Biopsies

Procedure:

a. Place tissue in a few drops of saline to prevent drying on a glass microscope slide.
b. Carefully tease the tissue apart with a dissecting needle.
c. Apply a coverslip and examine microscopically for schistosome eggs and the trophozoites of *Entamoeba histolytica.*
d. If the wet preparation is positive for amebae and a permanent mount is desired, stain the preparation directly with Trichrome or embed the tissue in paraffin, prepare sections, and stain with iron hematoxylin.

3. Bladder Biopsies

Procedure:
Make a wet preparation as described above (V,D.2) and examine microscopically for *Schistosoma haematobium* eggs.

4. Bone Marrow Biopsy

Procedure:

a. Fix the specimen or slide in methyl alcohol.
b. Stain with Giemsa.
c. Examine under oil immersion (×1000) for *Leishmania donovani.*

5. Muscle Biopsy or Meat Products

a. Compression Technique—"Trichinoscope"[25]

The device consists of two plate glass or heavy plastic plates (approximately 8 cm in diameter) mounted in heavy metal frames equipped with bolts and wing nuts.

(1) Place a small piece of tissue on glass plate of trichinoscope.
(2) Cover with matching glass plate and tighten them together until the specimen is compressed down to a single layer of cells. It is important that the metal frames are tightened evenly so the tissue will stay in one place and the whole specimen can be screened.
(3) Screen for the encysted *Trichina* larvae under the dissecting microscope.

b. Digestion Technique

See p. 676.

6. Skin Biopsies and Specimens from Skin

a. *Leishmania tropica, Leishmania braziliensis*

(1) Divide the specimen aseptically when it is received.
(2) Prepare an impression smear (touch preparation) with the cut surface of one portion of tissue on a clean, glass microscope slide. Allow the smear to air dry, fix in alcohol, stain with Giemsa, and examine microscopically.
(3) Culture the other portion of tissue as described elsewhere (p. 685).

b. Onchocerciasis

(1) Make a wet preparation of skin snips.
(2) If microfilariae are present, fix the slide in alcohol, stain with Giemsa, and examine microscopically.

c. Cutaneous Amebiasis

(1) Make a wet preparation and examine microscopically.
(2) If amebae are present, immediately place tissue in Schaudinn's fixative.
(3) Prepare a smear of a portion of the fixed tissue on a glass microscope slide for either Trichrome or iron hematoxylin staining.
(4) If there is sufficient material, after fixation, one may wish to proceed with paraffin embedding, sectioning, and subsequent Trichrome or iron hematoxylin staining.

d. Arthropods

(1) Make a wet preparation and examine microscopically for *Sarcoptes scabiei* ("scabies"). Examine *Demodex folliculorum* or deeply embedded ticks with a 4× objective on a conventional microscope or with a dissecting microscope.
(2) If there is a large amount of surrounding tissue, it may be necessary to digest the tissue first (p. 676).

7. Sputum

Procedure:

a. Make a wet preparation and examine it microscopically primarily for amebae and other organisms, such as helminth eggs (*Paragonimus westermani*), the protoscolices and hooklets of *Echinococcus,* and possibly, the larvae of hookworm, *Ascaris,* and *Strongyloides.*
b. Add 2% NaOH and let stand until sputum is completely digested.
c. Centrifuge at 2000 rpm for 10 min.
d. Examine sediment microscopically.

8. Drainage Material, Aspirates, and Spinal Fluid

Procedure:

a. Make a wet preparation and examine it microscopically primarily for the presence of amebae of the *Naegleria–Acanthamoeba–Hartmanella* group.
b. Centrifuge the material at 2000 rpm for 10 min and examine the sediment to detect the organisms.
c. If organisms are seen, prepare smears and stain with Trichrome or Giemsa as necessary.

9. Bile

Procedure:

a. Make a wet preparation and examine it microscopically.
b. Centrifuge a portion of the specimen at 2000 rpm for 10 min.
c. Examine the sediment for the hooklets and protoscolices of *Echinococcus,* the large operculated eggs of *Fasciola hepatica,* and the trophozoites of *Giardia lamblia.*

10. Lymph Nodes

Procedure:
Process as described previously for skin biopsies.

11. Specimens for Identification (Arthropods and Artifacts)

Specimens sent to the laboratory for identification are placed in a petri dish containing normal saline solution to rehydrate them, although for live arthropods, this step may not be necessary. A wet preparation is made and examined with a 4× objective on a conventional microscope or with a dissecting microscope. Some specimens may have to be teased apart or dissected to aid in identification. Specimens retained for teaching purposes can be placed in Roudabush fixative or 10% formalin. (See Table 11–1 for brief summary of above.)

VI. Serology for Parasitic Diseases

Most parasitic organisms are identified by detecting a stage in the life history of the organism, either by simple gross inspection of a stool specimen or by examining material microscopically.

In some instances, establishing current or previous infection requires serological, skin testing, and/or chemical tests. Radioisotopic scanning techniques may also be used in certain cases. Such procedures are especially helpful in the early stages of some infections where products of the life cycle (e.g., helminth eggs in a stool specimen or malaria and microfilariae in a blood specimen) are not yet present or in instances in which the organism is either difficult or impossible to detect by any routine microscopic examinations, including ones of tissue [e.g., echinococcosis, trichinosis, toxoplasmosis, visceral larva migrans, cysticercosis, and South American trypanosomiasis (Chagas Disease)].

Furthermore, it is often desirable to follow a course of treatment of a parasitic disease to determine the results of treatment, as, for example, in malaria in which stable titers may indicate incomplete treatment of a mixed infection or failure of the laboratory to detect and report a mixed infection. Treatment of *Plasmodium falciparum* only because of

failure to detect a mixed infection with *Plasmodium vivax* will allow *P. vivax* to continue its exoerythrocytic cycle in the liver, leading to recrudescence of disease.

Likewise, the Casoni skin test remains positive following removal of a hydatid cyst; however, the complement-fixation or indirect hemagglutination tests become negative, provided excision was complete and no other immature growing cysts remained in the patient.

Serology is important epidemiologically by providing estimates of disease and implementing and evaluating control measures and public health programs based on those estimates.[14]

Serological tests must meet the following criteria: (1) simple and rapid performance; (2) objective interpretative criteria; (3) economy; (4) sensitivity and specificity; and (5) reliability when performed in different laboratories.

In many instances the materials and reagents for performing serological tests are available only in reference laboratories; however, a listing of commercially available tests and their manufacturers has been published by Walls and Smith.[31]

The details of methodology and analysis of the various procedures are described elsewhere.[12] The preferred tests and significant titers for certain parasitic diseases are listed in Table 11–12.[31]

Table 11–12. The Test of Choice and Significant Titers for Selected Parasitic Infections as Performed at the Center for Disease Control[a,b]

Infection	IHA	BFT	IIF	CF	DAT	Others
Amebiasis	1:128					
Ascaris	1:128[c]	1:5[c]				1:32-ELISA
Chagas				1:32		
Cysticercosis	1:128[c]	1:5[c]				
Echinococcus	1:128					
Filariasis	1:128[c]	1:5[c]				
Leishmaniasis					1:64	
Malaria			1:64			
Paragonomiasis				1:16		
Pneumocystosis			1:16			
Schistosomiasis			Pos			
Strongyloidiasis	1:64					
Toxocariasis	1:128[c]	1:5[c]				1:32-ELISA
Toxoplasmosis			1:256			
Trichinella		1:5				

[a] From Walls, K. W., and Smith, J. W.: *Serology of Parasitic Infections.* Laboratory Medicine **10**:329–336, 1979. Pursuant to 17USC, Section 105, the foregoing is a U.S. Government work for which copyright protection is not available.

[b] IHA, indirect hemagglutination; BFT, bentonite flocculation; IIF, indirect immunofluorescence; CF, complement fixation; DAT, direct agglutination; ELISA, enzyme-linked immunospecific assay.

[c] Both must be positive.

VII. Miscellaneous Procedures

These will be given in alphabetical order using a common term or author's name.

A. Baerman Digestion Technique[1]

Purpose:
To recover larval worms from feces, soils, or, in the case of suspected *Trichinella spiralis* infection, infected tissue or meat.

Principle:
Motile larvae migrate from digested material and are recovered for microscopic examination.

Reagents:

Digestion Fluid

Pepsin	10 g
Water	2 liters
HCl, conc.	14 ml

Procedure:

1. Connect a funnel (15 to 17 cm in diameter) to a conical centrifuge tube by a short length of rubber tubing with a pinch clamp.
2. Place this assembly in a ring stand.
3. Fill the funnel with water when feces or soil are being examined and digestion fluid when meat or tissue is being examined.
4. Place a sheet of wire gauze or a sieve (20 mesh, 12 cm in diameter by 4 cm in depth) over the funnel in contact with the fluid.
5. Lay two or three layers of gauze across the sieve.
6. Place a large amount of feces, soil, or tissue on the gauze. If hard, feces should first be emulsified in water.
7. Allow feces or soil to remain in contact with the water (preferably warm to enhance migration) for 2 hr. Meat or tissue should digest in the fluid for 16 to 18 hr before proceeding.
8. At intervals thereafter, open the pinch clamp on the tubing to allow 5 to 10 ml of fluid to drain into the centrifuge tube.
9. Centrifuge the fluid and examine the sediment for larvae. Steps 8 and 9 are repeated until all of the fluid in the funnel has been examined.

B. Beaver Egg-Counting Technique[2]

This technique, described in detail elsewhere, is a highly accurate method for estimating worm burden by measuring egg production photoelectrically.

C. Casoni Skin Test[7]

Purpose:
To determine skin test reactivity to *Echinococcus*.

Principle:
When hydatid cyst fluid is injected intradermally, patients with hydatid cyst disease manifest hypersensitivity by developing redness and wheal at the injection site. In strongly reactive cases, pseudopodia appear at the periphery of the wheal. Although usually an immediate reaction, some are delayed so that the injection site should be observed for 30 min and again in 24 hr. A saline control should be injected intradermally below the test site.

Reagent:
The hydatid cyst fluid is obtained at the time of excision of a cyst from an infected human. (Animal sources should be avoided because of nonspecific reactions, as should fluid from cysts which have been injected with formalin because of loss of antigenicity.) The contents of the excised cyst are aspirated aseptically, centrifuged to remove hydatid sand (protoscolices), filtered (0.45 μm), and then tested for sterility. If sterile, merthiolate is added to a final concentration of 1:50,000, and the fluid is placed into sterile vaccine bottles. The shelf life at 25°C is ≥25 years and is longer at 5°C.

Procedure:

1. Obtain blood for serological testing prior to injecting the antigen intradermally.
2. Inject 0.05 ml of antigen intradermally into the volar surface of one forearm.
3. Inject 0.05 ml of saline (control) intradermally into the volar surface of the forearm at a distance of ≥10 cm from the site of injection of the antigen.

Interpretation:

Positive test: Appearance of redness and wheal at the injection site of the antigen.
Negative test: No redness or wheal at the injection site of antigen.

D. Hatching Test for Schistosomiasis[16]

Purpose:
To diagnose active infection or ascertain the effectiveness of treatment.

Principle:
Viable eggs seen in fresh, wet preparations can be studied for the presence of active flame cells (solenocytes), which can be observed with the oil immersion objective (×1000) after the coverslip has thoroughly dried down on the slide to avoid movement of the fluid. These cells will appear to be flickering as the flame of a candle in the anterior and posterior third of viable organisms.

Further evidence of viability can be derived from actual hatching of the egg with liberation of the free-swimming, ciliated, larval stage called the miracidium.

Procedure:

1. Homogenize the entire stool specimen (or a 24-hr collection) in saline and pour through three to four layers of gauze into a tall glass cylinder.
2. Allow the specimen (or specimens of urine or prostatic secretions) to sediment for several hours, decant the supernatant fluid, and resuspend in saline (schistosome eggs will not hatch in saline). This process is repeated at least twice to get a fairly clear supernatant fluid.
3. Decant the supernatant fluid.
4. Place the sediment in a 500 ml flask (either Erlenmeyer or Florence) with a side arm of glass tubing. This flask should be completely painted with black enamel (except for the side arm) or covered with aluminum foil to keep out light.
5. Fill the flask containing the sediment completely to include the sidearm with chlorine-free, well-aerated water. (Tap water can be dechlorinated by boiling for 5 to 10 min and cooling or allowing it to stand overnight in a wide-mouth container. Water can be aerated by vigorous shaking of the container or by pouring back and forth using two containers. Hatching will occur more rapidly in well-aerated, chlorine-free water.)
6. Allow the flask to stand several hours or overnight with a bright light shining on the exposed sidearm.

7. Examine the sidearm with a hand lens for the miracidia (known to be negatively geotropic and positively phototropic). These free-swimming, ciliated, larval worms will have been attracted to the upper layers of water and to light falling on the side arm of the covered flask. (Demonstration and teaching material from stool or segments containing eggs of *Diphyllobothrium latum* can be similarly processed to hatch the free-swimming larval stage called a coracidium.)

E. Hookworm Culture[9]

Purpose:
To recover larval stages from eggs in feces or develop larval stages of *Strongyloides stercoralis* and *Trichostrongylus* sp.

Principle:
Life cycles can be demonstrated in a simple charcoal mixture that simulates conditions found in natural soil.

Procedure:

1. Add water (chlorine-free) to the stool specimen and mix into a thick paste.
2. Transfer this mixture to the bottom of a petri dish about half-filled with granulated charcoal. Mix the charcoal and feces thoroughly to produce a smooth even paste. Add water (chlorine-free) to increase the moisture content, but not in an amount that leaves water standing at the edges of the mixture. The mixture should be moist enough to glisten brightly.
3. Mold the edges of the mixture in the center of the dish to form a cone that is slightly higher than the edges of the dish. When the lid of the petri dish is placed in position, it should barely make contact with this cone.
4. Place the dish in the darkness of an incubator, cabinet, or desk drawer.
5. Check the dish daily to ensure that the surface of the mixture (especially the cone) remains sufficiently moist. Water may be dropped or sprinkled on the surface daily to keep surface moist. Care should be exercised in handling the material because skin-penetrating, infective larval stages may be present at the cone and in the water of condensation that accumulates on the lid in contact with the cone. This is especially true at about 5 to 6 days of incubation when hookworm and *Strongyloides* will have reached their filariform infective stages.
6. Expose the covered dish to light. After a few minutes to an hour of light exposure, carefully remove the lid and pipette off the water of condensation which will contain the larval stages for microscopic study.

Also, water may again be dropped on the cone and recovered for larval stages still present in the cone.

F. Roudabush Fixative[20]

See p. 683.

G. Staining of Worms

See p. 684.

H. Stoll Egg-Counting Technique[24]

Purpose:
To estimate worm burden and ascertain the effectiveness of treatment for worm infections.

Procedure:

1. Weigh a 24 hr collection of feces.
2. Remove a sample of exactly 4 g.
3. Place the 4 g sample in a calibrated bottle or large tube and add 0.1 *N* NaOH to bring the volume to 60 ml.
4. Add a few glass beads and shake vigorously to produce a uniform suspension. If the specimen is formed and hard, it is best to allow the suspension to soften overnight in a refrigerator.
5. Remove 0.15 ml rapidly from the suspension with a pipette and drain the contents onto a glass microscope slide. *Do not apply a coverslip.*
6. Examine microscopically and count all eggs.
7. Multiply the actual egg count by 100 to give the number per gram of feces and by the weight of the total specimen to give the number of eggs in the total 24 hr specimen.
8. Depending on the consistency of the specimen, the estimate (eggs/g) varies. The following correction factors should be used to convert the estimate to a formed stool basis:

Mushy formed stools	× 1.5
Mushy	× 2.0
Mushy diarrhea	× 3.0
Flowing diarrhea	× 4.0
Watery	× 5.0

Interpretation:
Some workers prefer to use these estimates of worm burden as the basis for treating a patient (e.g., about 30,000 eggs of *Trichuris trichiura* indicate the presence of several hundred worms which may cause definite clinical symptoms or about 2500 to 5000 hookworm eggs/g may indicate a clinically significant infection). We prefer the concept (especially since excellent and quite tolerable drugs are now available) that until someone proves that *any* worm burden is beneficial to the patient, the patient should be treated and made free of his worms! This is especially true if one considers that a single adult *Ascaris lumbricoides* can migrate and, if agitated by drugs or fever, perforate the intestine.

I. Tapeworm Recovery[26]

Purpose:
To ascertain success of treatment, since if the head (scolex) is not recovered and identified, the success of treatment remains in doubt for at least 12 weeks before new segments (proglottids) develop or eggs pass in the stools.

Procedure:

1. Place the patient on a liquid diet for 24 hr (preferably in the hospital for strict supervision).
2. Administer a cleansing enema in the morning of the day of treatment.
3. Give 400 mg of atabrine p.o.
4. Repeat the 400 mg dose of atabrine in 30 min (to prevent vomiting, it may help to give sodium bicarbonate, 600 mg).
5. Give 15 g of magnesium citrate 2 hr later.
6. Instruct the patient not to use the toilet (in hospital the room should be locked) and to use either a large chamber pot (referred to in times past as a "thunder mug" or "white owl") or a bedpan.
7. Instruct the patient not to place any toilet paper or other tissue in the collection vessel.
8. Collect all stools following ingestion of the magnesium citrate and deliver them immediately to the laboratory. This should continue until the scolex is found or for several hours in the hope that the scolex will be found. If the procedure has been carried out as described, the receptacle used for collection will contain several quarts of fluid, shreds of mucus, and the tapeworm.
9. Decant the fluid carefully through a 20 mesh sieve (at least 12 cm in diameter and 4 cm in depth) and run warm tap water through the sieve to remove debris, leaving only the tapeworm.
10. Invert the sieve carefully over a black enameled pan measuring 25

by 30 cm and backwash the sieve with warm tap water into the pan so that nothing remains in the meshes of the sieve.

11. Search for the scolex by examining under a strong light the entire specimen and any portions broken from it.
12. Usually, one will recover a complete and intact worm from scolex to gravid proglottids (Figure 11–14); however, mechanical injury in

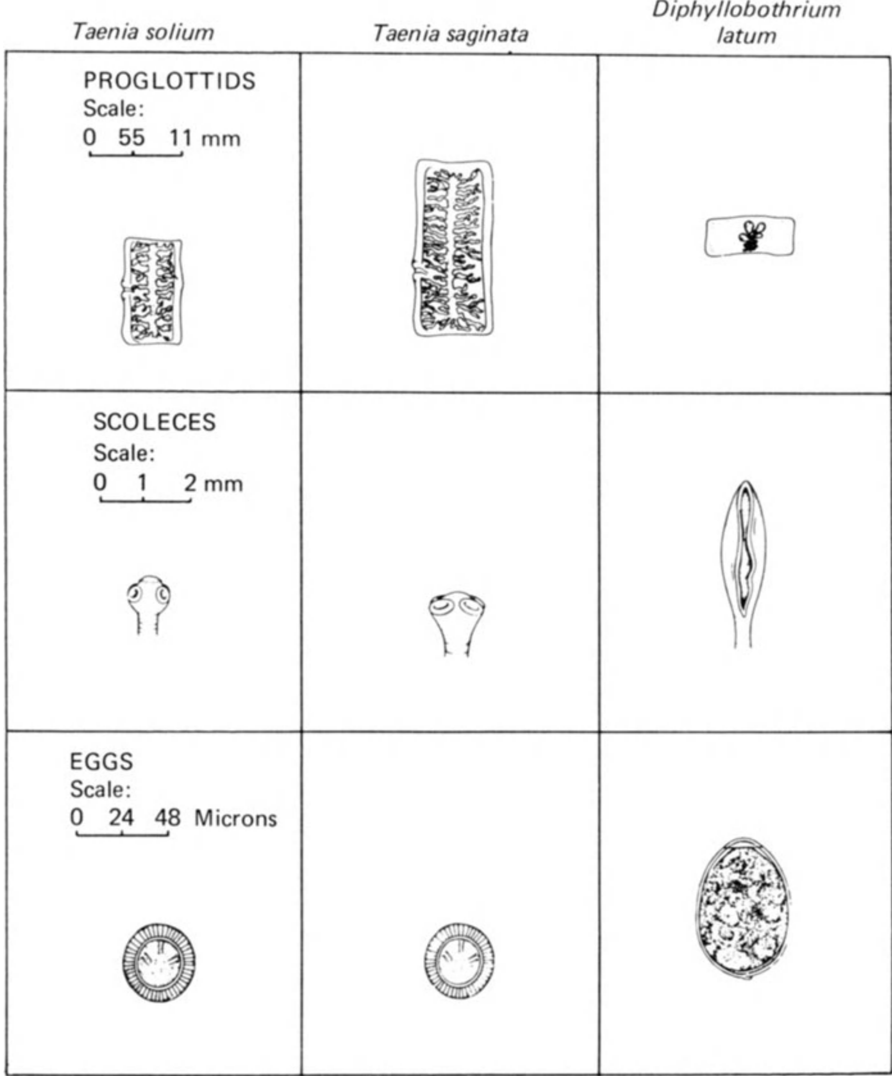


Figure 11–14. Diagnostic features of large tapeworms. (From Smith, J. W., Ash, L. R., Thompson, J. H., McQuay, R. M., Melvin, D. M., and Orihel, T. C. *Intestinal Helminths.* Chicago, American Society of Clinical Pathologists, 1976.)

passage and the active peristalsis resulting from the treatment may cause specimens to be broken in several places. The head may be present in the washings but may be broken off so near the first segment that extreme care in the search is required. For these reasons no food particles or toilet paper should be present in the recovered material. We have been confronted on occasions with the presence of food particles and by diligent searching and good fortune have found the head inside the undigested shell of a kernel of corn or a pea; once it was found adhering to a small clump of disintegrated toilet paper that was in several pieces in the container.

13. if the patient has vomited the drugs or if there is any indication that the protocol was not followed, the patient should be allowed a respite of 24 hr and then the complete protocol repeated.

J. Worm Fixation

Reagent:

Roudabush Fixative[20]

Ethyl alcohol	24 parts
Formalin (commercial)	15 parts
Glacial acetic acid	5 parts
Glycerine	10 parts
Tap water	46 parts

Grenacher's Alcoholic Borax–Carmine Stain[5]

Carmine	3 g
Borax	4 g
Distilled water	100 ml

Boil or allow to stand at room temperature until the carmine has dissolved. Add 100 ml of alcohol (70%) and allow to stand for several days, filter.

Ehrlich's Hematoxylin Stain[5]

Hematoxylin	2.5 g
Alcohol, absolute	50 ml
Potash alum	50 g
Distilled water	1000 ml

Dissolve the hematoxylin by gently heating in the absolute alcohol in a test tube. Dissolve the alum by gently heating in water in an evaporating dish. Mix the two solutions in a bottle, place in strong sunlight or expose to ultraviolet light until the sides of the bottle are deeply stained. Add

a thymol crystal to prevent mold growth. Prepare the final staining solution by mixing one part of the ripened stain with three parts of 35% alcohol to produce a deep wine coloration.

Fixation Procedure:

1. After removing the worm from the specimen, wash in tap water.
2. Carefully transfer the worm, about a foot at a time, from the water to a position above the dish to be used for fixation.
3. Pour warmed (50° to 60°C) fixative over the worm into the dish.
4. Repeat this process with segments of about a foot until the entire specimen is covered with the fixative. Extreme care must be exercised in handling the more terminal segments as their greater weight may cause them to break away from the remainder of the specimen.
5. After fixing, either store the worm in a jar containing the fixative or, 70% alcohol or, if staining is desired, wash it free of the fixative with 70% alcohol and stain with Grenacher's alcoholic borax-carmine or Ehrlich's hematoxylin.

Staining Procedure:

a. Grenacher's Stain

1. Transfer the fixed material to the staining solution and allow to stand overnight.
2. Add HCl (concentrated) dropwise while agitating the container until all of the carmine has precipitated as a heavy, brick-red substance. The supernatant should become a transparent red. Allow to stand overnight.
3. Add an equal volume of 3% HCl in 70% alcohol and mix thoroughly.
4. Allow the material to settle and remove the carmine which was resuspended during mixing.
5. Examine the carmine for organisms.
6. If no organisms are present, refill the container with acid-alcohol, allow the material to settle, and remove the supernatant. Repeat until all carmine has been removed.
7. Place the residual material in a watch glass and cover with acid-alcohol. Examine microscopically. Replace the acid-alcohol as often as necessary since the acid extracts the stain and the acid-alcohol becomes deeply colored. Destaining extracts most of the stain from the cytoplasm, leaving eggs, gland cells, the digestive tract, trachea, etc., deeply colored. When destaining has progressed far enough, transfer several organisms to absolute alcohol for several minutes, clear in creosole, and examine microscopically.
8. When structures appear well differentiated, replace the acid-alcohol in the watch glass with 80% neutral alcohol. Change several times

to remove all traces of acid. Allow material to stand in last change of neutral alcohol for at least 1 hr.

9. Dehydrate, clear, infiltrate with balsam, and mount.

b. Ehrlich's Stain

1. Transfer the fixed material to staining solution. Allow to stand overnight.
2. Proceed as in a.7 above.
3. When structures appear well differentiated, blue the material by briefly dipping into a Coplin jar containing water with a few drops of ammonia.
4. Dehydrate in a series of neutral alcohol changes, clear, infiltrate with balsam, and mount.

K. Culture Media

The majority of clinical microbiology laboratories cannot afford the time, expense, and personnel to examine cultures routinely for parasitic organisms.

In certain instances (for teaching or when an unusual organism comes into the laboratory) culture media can be of great value in keeping the organism for demonstration.

The following references will provide the laboratory with media to account for those organisms which would be of help in teaching.

1. McQuay's charcoal medium (diphasic).[17] This will support growth of stock cultures of *Entamoeba histolytica* and all intestinal amebae and flagellates (except *Giardia lamblia*). *G. lamblia* is extremely difficult to maintain in culture; therefore, for demonstration purposes, it is best to use an animal source (mice, rats, rabbits).
2. Warren's modification of NNN medium.[32] This is an excellent medium for blood and tissue parasites (*Leishmania*, trypanosomes). It is a good maintenance medium for stock organisms, and can be used to culture for clinical material (blood, biopsies, aspirates, spinal fluid) that may be harboring such organisms.
3. Fouts and Kraus modification of Diamond's medium.[10] This will support the growth of *Trichomonas vaginalis* for teaching and demonstration. It may also be used for culture of the organism from clinical material.

References

1. Baerman, G. Eine einfache Methode zur Auffindung von Ankylostomum (Nematoden) Larven in Erdproben. *Meded. Geneesk. Lab. Weltverden.* Festbundel, Batavia, 1917, p. 41.

2. Beaver, P. C. The standardization of fecal smears for estimating egg production and worm burden. *J. Parasitol.* **36:**451, 1950.
3. Brooke, M. M., and Goldman, M. Polyvinyl alcohol fixative as preservative and adhesive for protozoa in dysenteric stools and other liquid materials. *J. Lab. Clin. Med.* **34:**1554, 1949.
4. Camp, R. R., Mattern, C. F. T., and Honigberg, B. M. Study of *Dientamoeba fragilis* Jepps and Dobell. I. Electronmicroscopic observations of the binucleate stages. II. Taxonomic position and revision of the genus. *J. Protozool.* **21:**69, 1974.
5. Cowdry, E. V. *Laboratory Technique in Biology and Medicine.* Baltimore, Williams and Wilkins, 1948, 269 pp.
6. Current, W. L. Human cryptosporidiosis (letter). *N. Engl. J. Med.* **309:**1326–1327, 1983.
7. Dew, H. R., Kellaway, C. H., and Williams, F. E. The intradermal reaction in hydatid disease and its clinical value. *Med. J. Australia* **I:**471, 1925.
8. Faust, E. C., Sawitz, W., Tobie, J., Odom, V., Peres, C., and Lincicome, D. R. Comparative efficiency of various technics for the diagnosis of protozoa and helminths in feces. *J. Parasitol.* **25:**241, 1939.
9. Faust, E. C. *Human Helminthology.* Philadelphia, Lea and Febiger, 1949, 744 pp.
10. Fouts, A. C., and Kraus, S. J. *Trichomonas vaginalis.* Reevaluation of its clinical presentation and laboratory diagnosis. *J. Infect. Dis.* **141:**137, 1980.
11. Harris, J. C., DuPont, H. L., and Hornick, R. B. Fecal leukocytes in diarrheal illness. *Ann. Intern. Med.* **76:**697, 1972.
12. Kagan, I. G., and Norman, L. Serodiagnosis of parasitic diseases. *In* Rose, N. R., and Friedman, H. (eds.), *Manual of Clinical Immunology.* Washington, D.C., American Society for Microbiology, 1976, p. 382.
13. Knott, J. A method for making microfilarial surveys on day blood. *Trans. R. Soc. Trop. Med. Hyg.* **33:**191, 1939.
14. Lobel, H. O., and Kagan, I. G. Seroepidemiology of parasitic diseases. *Ann. Rev. Microbiol.* 32:329, 1978.
15. Mathieson, D. R., and Stoll, A. M. Comparison of methods for detecting eggs of *Schistosoma japonicum* in feces. Dept. No. 1, *Naval Med. Res. Inst.* Bethesda, MD, 1945, 6 pp.
16. McMullen, D. B., and Beaver, P. C. Studies on schistosome dermatitis. IX. The life cycles of three dermatitis-producing schistosomes from birds and a discussion of the subfamily Bilharziellinae (Trematoda: Schistosomatidae). *Am. J. Hyg.* **42:**128, 1945.
17. McQuay, R. M. Charcoal medium for growth and maintenance of large and small races of *Entamoeba histolytica. Am. J. Clin. Pathol.* **26:**1137, 1956.
18. McQuay, R. M. Citrate-saponin-acid method for the recovery of microfilariae in blood. *Am. J. Clin. Pathol.* **54:**743, 1969.
19. Ritchie, L. S. An ether sedimentation technique for routine stool examinations. *Bull. U.S. Army Med. Dept.* **8:**326, 1948.
20. Roudabush, R. L. The fixation of tapeworms. *J. Parasitol.* **33** (Sec. 2):17, 1947.
21. Smith, J. W., Melvin, D. M., Orihel, T. C., Ash, L. R., McQuay, R. M., and Thompson, J. H., Jr. *Atlas of Diagnostic Medical Parasitology,* Vol. I, *Blood and Tissue Parasites.* Chicago, American Society of Clinical Pathologists, 1976.
22. Smith, J. W., McQuay, R. M., Ash, L. R., Melvin, D. M., Orihel, T. C., and

Thompson, J. H., Jr. *Atlas of Diagnostic Medical Parasitology,* Vol. II, *Intestinal Protozoa.* Chicago, American Society of Clinical Pathologists, 1976.

23. Smith, J. W., Ash, L. R., Thompson, J. H., Jr., McQuay, R. M., Melvin, D. M., and Orihel, T. C. *Atlas of Diagnostic Medical Parasitology,* Volume III, *Intestinal Helminths.* Chicago, American Society of Clinical Pathologists, 1976.
24. Stoll, N. R. An effective method of counting hookworm eggs in feces. *Am. J. Hyg.* **3**:59, 1923.
25. Thompson, J. H., Jr. Is immunity to trichinosis being naturally acquired? *Minn. Med.* **39**:174, 1956.
26. Thompson, J. H., Jr. Successful expulsion of tapeworms. *Minn. Med.* **40**:859, 1957.
27. Thompson, J. H., Jr. Examination of blood smears for malarial and other organisms. *Bull. Pathol.* **10**:9, 37, 61, 85, 1969.
28. Thompson, J. H., Jr. How to detect intestinal parasites. *Lab. Med.* **1**:31, 1970.
29. Thompson, J. H., Jr. How to detect blood and tissue parasites. *Lab. Med.* **2**:42, 1971.
30. Thompson, J. H., Jr. The Laboratory Evaluation of Parasitic Diseases Prevalent in the U.S.A. *In* Homburger, H. A., and Batsakis, J. G. (eds.), *Clinical Laboratory Annual,* Vol. 2. Norwalk, Connecticut, Appleton-Century-Crofts, 1983.
31. Walls, K. W., and Smith, J. W. Serology of parasitic infections. *Lab. Med.* **10**:329, 1979.
32. Warren, L. G. Metabolism of *Schizotrypanum cruzi* chagas. I. Effect of culture age and substrate concentration on respiratory rate. *J. Parasitol.* **46**:529, 1960.
33. Wheatley, W. B. A rapid staining procedure for intestinal amoebae and flagellates. *Am. J. Clin. Path.* **21**:990, 1951.
34. Young, K. H., Bullock, S. L., Melvin, D. M., and Spruill, C. L. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. *J. Clin. Microbiol.* **10**:852, 1979.

12

Special Tests of Antimicrobial Activity

Section 12.1

Antimicrobial Assays

John P. Anhalt, Ph.D., M.D.

I. Overview

A. Indications for Assay

The purpose of measuring antibiotic concentrations in serum or other fluids is to ensure that dosage is sufficient for adequate therapy, while avoiding excessive concentrations that may be associated with increased adverse reactions. The relationship of efficacy to serum concentrations of antibiotics is complex and difficult to study. The clinical response of a patient to a specific antibiotic depends on several factors besides the serum concentration, including the susceptibility of the infecting organism, site of infection, age and immunological status of the patient, presence of other diseases, and whether concomitant therapy is being given. One particularly troublesome aspect of measuring efficacy is the fact that many patients with an infectious disease will recover with inappropriate or no antimicrobial therapy, while other patients, who receive presumably appropriate therapy, will succumb.[19] Thus, it is impossible to define accurately a therapeutic concentration for an antibiotic.

The margin between effective concentrations and toxic concentrations of many antibiotics is large. Recommended dosages for these drugs virtually ensure effective levels, and toxicity is rarely a concern except when elimination mechanisms are severely impaired or when the possibility of a mistake in dosage exists. Under unusual circumstances, attainment of the desired and supposedly effective level may be in doubt. For example, assays may be useful when treatment is directed toward an organism that is only moderately susceptible, when infection involves a site to

which drug penetration is variable or uncertain, and when a patient fails to respond clinically to an antibiotic that is active *in vitro* against the presumptive pathogen. In the latter case, however, the clinical failure can often be traced to factors other than the serum concentration of drug. Lastly, assays can be useful to detect noncompliance by patients taking oral antibiotics; however, the serum level attained in a patient who takes a single dose immediately prior to when a blood specimen is obtained often cannot be distinguished from the level attained in a patient who takes the antibiotic consistently as prescribed.

A relatively small number of antibiotics have a narrow margin between effective and toxic concentrations. This list of drugs includes the aminoglycosides (gentamicin, tobramycin, sisomicin, netilmicin, amikacin, streptomycin, and kanamycin), vancomycin, and flucytosine, which are excreted almost entirely by the kidney. For these drugs, assays often are indicated to ensure efficacy,[28] and potentially toxic levels can accumulate even when renal function is only mildly impaired. In fact, the volumes of distribution and elimination half-lives for aminoglycosides vary so greatly between individuals[2,3,5,23,46] that some authors have recommended individualized dosages based upon measured serum concentrations for most patients, including those with normal renal function.[4] Neomycin is also an aminoglycoside, which though not administered parenterally, can accumulate to toxic levels after oral administration or wound irrigation, especially if the patient has severe renal impairment. Finally, chloramphenicol should be considered as a drug with a narrow therapeutic margin. Because it is metabolized in the liver before excretion by the kidney, adult patients with impaired renal function do not require adjustment of dosage.[4] In neonates, who normally metabolize the drug poorly and have decreased glomerular function,[25] and in adults with coexisting liver disease and renal function impairment, however, serum levels are necessary to avoid potential toxicity.

B. Specimen Timing*

Most antimicrobials are administered at intervals that are greater than the half-lives for excretion. A steady state is reached after only two or three doses (i.e., after five half-lives) and is characterized by a large difference between the maximal concentration ("peak") and minimal concentration ("trough") attained in serum between doses. These large changes mandate that times for administration of doses and for obtaining blood for assays are carefully planned and recorded accurately. Samples drawn at inappropriate times or when the times are uncertain will result in values that are misleading or uninterpretable. For only a few antibiotics,

* Adapted from Hermans et al.[15]

namely gentamicin, amikacin, and other aminoglycosides, is the usefulness of both a peak and trough measurement established. For most other antibiotics, a determination of either the peak or trough concentration will suffice for dosage adjustments and will be more cost-effective. Of the two choices, trough levels provide a more sensitive indication of decreased clearance and drug accumulation. Peak levels in a stable patient will tend to vary more because of uncontrolled variables, such as rate of drug infusion, rate of absorption, and differences in sampling time. For chloramphenicol, vancomycin, and flucytosine, we recommend that dosage adjustments be based on trough levels, as long as the dose interval is not more than twice the usual interval (Table 12.1–1). In patients with *Pneumocystis carinii* pneumonia, clinical response has been correlated with peak serum levels of trimethoprim-sulfamethoxazole, and measurement of either agent in the combination is sufficeint for dosage adjustment.

C. Specimen Collection and Handling

The exact time that a peak level occurs varies depending on the drug, route of administration, and clinical status of the patient. It is impossible to predict accurately the time at which the peak level occurs. As a general practice, blood for peak levels should be obtained 30 min after completion of an intravenous infusion, 1 hr after an intramuscular dose, and 1 to 2 hr after an oral dose. The delay after an intravenous dose allows for a short distribution phase, and levels in serum collected then more nearly represent tissue levels than the actual peak concentration, which occurs immediately at the end of a constant infusion. Absorption of an oral medicine is particularly variable. Therefore, if the peak level for an oral dose is unexpectedly low, and there is no other apparent reason for the result, the possibility of slow absorption should be considered. This can be tested by obtaining a sample of blood 3 hr after a dose. The tetracyclines and erythromycin typically are absorbed slowly and produce peak concentrations 2 to 4 hr and 2 to 6 hr after oral doses, respectively. Some dosage forms of erythromycin, however, may produce peak levels as soon as 1 hr after a dose. Blood for trough levels should be obtained immediately before a dose.

The blood should be collected in tubes that do not contain heparin and allowed to clot. The serum should be separated as soon as possible and assayed immediately or stored at −20°C or lower for no longer than 24 hr. Excessive storage time can cause a reduction in the concentration of certain drugs. In particular, some cephalosporins degrade rapidly in serum,[7,30] and aminoglycosides are unstable when a β-lactam drug is also present.[19]

Table 12.1–1. Factors Affecting Monitoring of Selected Antimicrobials[a]

Antimicrobial	Usual dose	Dose interval (hr)	Maximum dose (per 24 hr)	Normal serum half-life (hr)	Major route of elimination	Removed by dialysis: Hemo-dialysis	Removed by dialysis: Peritoneal dialysis	Therapeutic range (μg/ml): Peak	Therapeutic range (μg/ml): Trough	Recommended basis for dosage adjustment[b]
Amikacin	5–7.5 mg/kg	8–12	15 mg/kg	2–3	Renal	Yes	Yes	20–25	5–10	P,T
Gentamicin	1.7 mg/kg	8	5 mg/kg	2–3	Renal	Yes	Yes	4–8	1–2	P,T
Kanamycin	5–7.5 mg/kg	8–12	15 mg/kg	2–3	Renal	Yes	Yes	20–25	5–10	P,T
Netilmicin	2–2.5 mg/kg	8	7.5 mg/kg	2–3	Renal	Yes	Yes	6–10	0.5–2	P,T
Streptomycin	0.5–1 g	8–12	2 g	2–3	Renal	Yes	—	5–20	<5	P,T
Tobramycin	1.7 mg/kg	8	5 mg/kg	2–3	Renal	Yes	Yes	4–8	1–2	P,T
Chloramphenicol	0.5–1 g	6	4 g	4[c]	Hepatic/Renal	Yes	No	15–25	8–10	T
Flucytosine	37.5 mg/kg	6	150 mg/kg	4	Renal	Yes	Yes	100	50	T
TMP/SMX[d]										
TMP	5 mg/kg	6	20 mg/kg	11	Renal	Yes	—	≥5	—	P
SMX	25 mg/kg	6	100 mg/kg	13	Renal	Yes	—	≥100	—	P
Vancomycin	0.5–1 g	8–12	2 g	6	Renal	No	No	20–40	5–10	T

[a] From Hermans et al.[15]

[b] P = peak level, T = trough level. These recommendations are valid when dosage interval is no greater than twice the usual dosage interval. Blood for peak levels should be drawn 30 min after completion of an IV infusion, 1 hr after an IM dose, and 1 to 2 hr after an oral dose.

[c] Half-life in children less than 4 weeks old can be prolonged greatly. Half-life is affected only slightly in renal failure, but can be greatly prolonged with liver disease.

[d] TMP/SMX = trimethoprim/sulfamethoxazole. Serum half-life is shortened in adolescents and children. Measurement of either TMP or SMX alone is sufficient for dosage adjustment.

D. Methodology

Before 1970, one could accurately say that microbiological assays were the most widely used methods and offered the best choice for routine measurement of antibiotic concentrations in biological fluids. Following the introduction and widespread use of gentamicin, a variety of assay methods were developed that did not depend on measurement of a biological response. These newer methods were generally more precise and accurate than microbiological methods, and most important, the newer methods were more rapid and far more specific. Among the first of these newer methods were radioimmunoassays[24] and radioenzymatic assays.[14,37,38] Subsequently, both immunoassays and enzymatic assays have been developed that do not use radioisotopes.[8,17,18,20,35,36,43,44,45] Immunological and radioenzymatic assays for gentamicin, tobramycin, netilmicin, kanamycin, amikacin, streptomycin, sisomicin, and vancomycin are available in kit form from various manufacturers.

Immunological methods are ideally suited for batch testing of large numbers of samples. As the number of samples tested each day diminishes, immunological procedures become increasingly costly per sample because several standards must be run concurrently with each batch of tests. Some automated instruments, for example the TDx (Abbott Diagnostics), have addressed this problem by greatly reducing the frequency that standards must be measured. With these instruments, a standard curve is determined and used for subsequent batches of analyses as long as control results are within acceptable limits. A remaining limitation of immunologic assays is that they have been developed for only relatively few antibiotics. In contrast, chromatographic assays have been developed for almost all antibiotics and are suited particularly to testing small numbers of samples.[11] As for immunoassays, the instrumentation for chromatographic assays can be expensive; however, once the instrumentation is acquired, the method can be used for many antibiotics, and reagent costs are low. In general, liquid chromatography is preferable to other chromatographic techniques because volatility is not required and, therefore, derivatization is unnecessary.

II. Bioassays

A. General Considerations

Bioassays are based on comparison of the response of a susceptible organism to an unknown concentration of antibiotic with the response of the same organism, under identical test conditions, to a known concentration of antibiotic. In essence, a bioassay is the converse of a susceptibility

test of a standard organism, and the response is usually measured by either broth dilution[31] or agar diffusion techniques.[21,26] In order to avoid the inherent inaccuracies of a broth dilution method using serial twofold dilutions, various continuous measures of growth in broth have been used. These methods include turbidimetric assays, potentiometric[29] and titrimetric assays,[6] radiometric assays,[13] and assays based on measurement of intracellular bacterial adenosine triphosphate concentration.[27] Comprehensive reviews of the theory and practice of bioassays have been prepared by Grove and Randall,[12] Kavanagh,[21,22] and Reeves and associates.[34]

Because bioassays depend only on the inhibitory effects of an antimicrobial substance on the indicator organism, they lack specificity and cannot differentiate between antimicrobials present in mixtures. It is essential, therefore, that accurate and complete information be obtained about concurrent antimicrobial therapy. Unfortunately, accurate information may not always be provided. Reeves and Holt,[33] for example, found undisclosed antimicrobials in 19% of the serum specimens submitted to their laboratory for assay. In addition, metabolites of antimicrobials may be present in sera.[9,34] Some of these metabolites are inactive, or have only a small portion of the antimicrobial activity of the parent drug, and would not be expected to cause interference; however, not all metabolites can be disregarded. Carbenicillin, for example, degrades to benzylpenicillin, which is more active than the parent compound against some indicator organisms. Thus, whenever practical, an indicator organism that is multiply resistant should be used to improve specificity; however, the possibility of synergistic or antagonistic activity must be ruled out before this organism is used to assay antibiotics in mixtures. Specificity can also be improved by antagonism or inactivation of other antimicrobials present or by separation of the antimicrobials in a mixture prior to assay.[34] The most common of these methods are the addition of a β-lactamase to inactivate penicillins and cephalosporins, addition of 50 mg/liter of *p*-aminobenzoic acid and 5 mg/liter of thymidine to antagonize the activity of sulfonamides and trimethoprim, and addition of calcium salts or cation-exchange resins[39] to antagonize or remove aminoglycoside activity.

The lack of specificity of bioassays can also be viewed as an advantage inasmuch as a single organism and basic procedure can be used to measure a wide variety of antimicrobials. Two common bioassay techniques, disk-plate assay and broth-dilution assay, are described below. A third common technique, cylinder-plate assay, will not be described in detail. This method differs from the disk-plate assay principally in that the solution to be assayed is contained in a metal cylinder placed on the agar surface rather than adsorbed onto a paper disk. The metal cylinder provides a relatively large reservoir of antibiotic solution in which the concentration does not fall appreciably during the critical phase of diffusion and growth inhibition.[21] This condition provides optimal accuracy and precision and

allows measurement of antibiotics present in low concentration. For clinical monitoring, cyclinder-plate assays have generally been replaced by disk-plate assay, because of greater simplicity of the latter method. The indicator organisms and test conditions for drugs not described in the examples are summarized.

B. Disk-Plate Bioassays

1. General Considerations

Several variations of the disk-plate bioassay exist, but all are characterized by the use of absorbent paper disks to contain the specimens and standards (Figure 12.1–1). The variations differ principally in the choice of medium, test organism, and calibration method. The method for penicillin G is described in detail and is representative of the other methods.

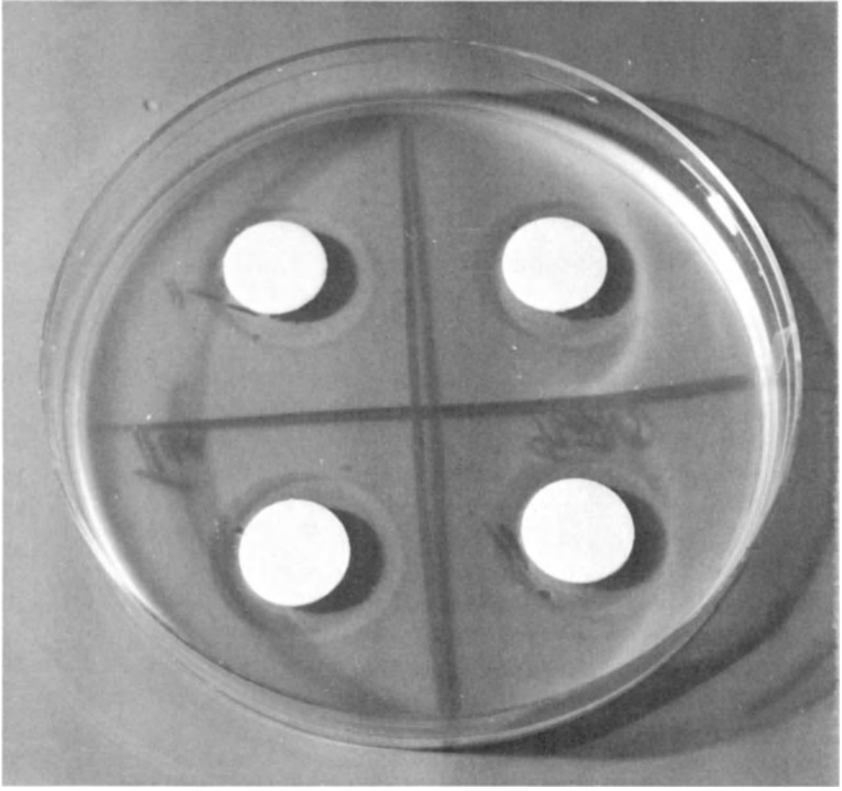


Figure 12.1–1. Disk-plate bioassay.

2. Representative Procedure

Penicillin G Assay

Purpose:
To measure the concentration of penicillin G in serum, urine, or cerebrospinal fluid.

Principle:
Sarcina lutea is inhibited by many antibiotics, including penicillins, cephalosporins, clindamycin, and chloramphenicol. A dispersion of *Sarcina lutea* is prepared in agar. The fluid to be tested for antibiotic and solutions containing known concentrations of penicillin G are deposited onto paper disks that are placed then on the agar surface. Antibiotic diffuses from the disks and inhibits growth of *S. lutea* in the agar (Figure 12.1–1). After a suitable incubation period, zones of inhibition around each disk are measured. The inhibition zones produced by the known concentrations are plotted against concentration to form a standard curve. The drug concentration in the sample corresponding to the zone of inhibition produced is determined from the standard curve. Because an aminoglycoside is often administered along with penicillin and would interfere with the assay, calcium chloride is added to the assay medium to prevent the effects of the aminoglycoside.[10]

Specimen:
Serum, urine, or cerebrospinal fluid may be tested.

Materials and Reagents:

1. Sterile petri dishes, 100 by 15 mm, with flat bottoms (Falcon Optilux, cat. no. 1001).
2. Paper disks, 6.35 mm in diameter (Schleicher and Schuell, cat. no. 740E). Sterilize in a glass petri dish in a hot air oven.
3. Sterile, disposable, 20-μl capillary pipettes with bulbs (Becton, Dickinson and Company, cat. no. 5878) or sterile Eppendorf-type pipettes of the same capacity.
4. Antimicrobial stock solutions are prepared as described in Chapter 4 (Table 4.4–3). Stock solutions are diluted in a suitable buffer (Table 12.1–2) to a concentration at least tenfold greater than needed. Further dilutions are made in serum for assays of serum or in buffer for assays of cerebrospinal fluid and urine, according to the scheme in Table 12.1–3. Appropriate concentrations to use for the standard curve are shown in Table 12.1–4.
5. Sterile, pooled, normal human serum (Gibco Laboratories). The serum

Table 12.1–2. Diluents for Stock Antibiotic Solutions, Urine, and Cerebrospinal Fluid for Disk-Plate Assays

Antibiotic	Diluent pH[a]
Aminoglycosides	8
Cephalosporins	6
Penicillins	
Amoxicillin	7
Ampicillin	7
Carbenicillin	7
Cloxacillin	6
Dicloxacillin	6
Methicillin	6
Mezlocillin	6
Nafcillin	6
Oxacillin	6
Penicillin	7
Piperacillin	6
Ticarcillin	7
Miscellaneous	
Chloramphenicol	6
Clindamycin	8
Erythromycin	8
Metronidazole	7
Streptomycin	8
Trimethoprim	7

[a] All diluents are phosphate buffers at a concentration of 0.1 mol/liter. See Table 4.4–4 for methods of preparation.

must be negative for HB_sAg by a third-generation test and must not possess antibacterial activity or inhibit the activity of penicillins or cephalosporins (p. 853). The pH is adjusted to 7.4 before use.

6. *Sarcina lutea* ATCC 9341 is maintained on a Trypticase soy agar slant or a Penassay Seed Agar slant (Difco Laboratories) in a refrigerator and is subcultured every 2 weeks to a fresh agar slant. The subculture is incubated overnight at 30°C before being replaced in the refrigerator for storage.
7. Phosphate buffer, 0.1*M* at pH 7, is prepared as described in Table 4.4–4.

Media:

1. Antibiotic medium no. 5 (Streptomycin Assay Agar, Difco, cat. no. 0277) to which 10 g/liter of $CaCl_2$ is added. Adjust to pH 7.0 after autoclaving and dispense into bottles containing 100 ml each.

Table 12.1–3. Dilution Scheme for Preparation of Standards Used in Disk-Plate Assays

Antibiotic solution[a]			
Amount (ml)	Concn (μg/ml)	Sterile serum (ml)[b]	Final antibiotic concn (μg/ml)
For standards containing 2 μg/ml to 50 μg/ml:			
0.5	1000	4.5	100
1.0	100	1.0	50
0.8	100	1.2	40
0.6	100	1.4	30
0.5	100	1.5	25
0.5	100	2.0	20
0.3	100	1.7	15
0.2	100	1.8	10
0.5	10	0.5	5
0.2	10	0.8	2
For standards containing 1μg/ml to 10 μg/ml:			
0.2	1000	1.8[c]	100
0.5	100	4.5	10
0.9	10	0.1	9
0.8	10	0.2	8
0.75	10	0.25	7.5
0.7	10	0.3	7
0.6	10	0.4	6
0.5	10	0.5	5
0.4	10	0.6	4
0.3	10	0.7	3
0.25	10	0.75	2.5
0.2	10	0.8	2
0.1	10	0.9	1
For standards containing 0.1 μg/ml to 1.0 μg/ml:			
0.2	1000	1.8[c]	100
0.2	100	1.8[c]	10
0.5	10	4.5	1
0.9	1	0.1	0.9
0.8	1	0.2	0.8
0.7	1	0.3	0.7
0.6	1	0.4	0.6
0.5	1	0.5	0.5
0.4	1	0.6	0.4
0.3	1	0.7	0.3
0.2	1	0.8	0.2
0.1	1	0.9	0.1

[a] Stock solutions containing 1000 μg/ml of antibiotic are prepared as described in Table 4.4–3.

[b] A suitable buffer (Table 12.1–2) should be substituted for serum to prepare standards for assays of urine or cerebrospinal fluid.

[c] Use a suitable buffer (Table 12.1–2) to prepare these dilutions. Prepare further dilutions for serum assays in sterile serum.

Table 12.1–4. Suggested Concentrations for Disk-Plate Assays

Antibiotic	Standards (μg/ml)	Reference (μg/ml)	Approximate reference zone (mm)
Cephalosporins			
Cefaclor	1, 2, 3, 4, 5	3	25.8
Cefazolin	5, 10, 15, 20, 25	15	14.3
Cefamandole	1, 2, 3, 4, 5	3	22.5
Cefoperazone	1, 2.5, 5, 7.5, 10	5	17.4
Cefoxitin	5, 10, 15, 20, 25	15	15.8
Ceftazidime	2, 5, 5, 10, 15, 20	10	16
Ceftizoxime	1, 2.5, 5, 7.5, 10	5	19.1
Cefuroxime	1, 2, 3, 4, 5	3	—
Cephalexin	1, 2, 3, 4, 5	3	20.5
Cephalothin	1, 2, 3, 4, 5	3	17.7
Cephapirin	1, 2, 3, 4, 5	3	23
Cephradine	1, 2, 3, 4, 5	3	22.3
Moxalactam	1, 2.5, 5, 7.5, 10	5	14
Penicillins			
Amoxicillin	0.3, 0.4, 0.5, 0.6, 0.7	0.5	—
Ampicillin	0.1, 0.2, 0.3, 0.4, 0.5	0.3	23.3
Carbenicillin	10, 20, 30, 40, 50	20	16.1
Cloxacillin	1, 2, 3, 4, 5	3	13.5
Dicloxacillin	2, 4, 6, 8, 10	6	—
Methicillin	0.5, 0.6, 0.7, 0.8, 0.9, 1.0	0.7	15.6
Nafcillin	0.5, 0.6, 0.7, 0.8, 0.9, 1.0	0.7	17.8
Oxacillin	0.6, 0.7, 0.8, 0.9, 1.0	0.8	19.7
Penicillin	0.1, 0.2, 0.3, 0.4, 0.5	0.3	23.0
Piperacillin	5, 10, 15, 20, 25	15	19.6
Ticarcillin	5, 10, 15, 20, 25	15	27.5
Miscellaneous			
Chloramphenicol	5, 10, 15, 20, 25	15	24.4
Clindamycin	1, 2, 3, 4, 5	3	17.3
Erythromycin	1, 2, 3, 4, 5	3	20.4
Metronidazole	5, 10, 16, 20	15	17.7
Streptomycin	1, 2, 3, 4, 5	3	15.1
Trimethoprim	1, 2.5, 5, 7.5, 10	5	20.3

2. Trypticase soy broth (BBL Microbiology Systems).

Preparation of Assay Plates:

1. Heat bottles containing antibiotic medium no. 5 until the agar is melted and allow to equilibrate at 50°C in a water bath until used. One bottle of 100 ml of agar is needed for the base layer of 10 plates. One bottle of 100 ml is required for the seeded agar of 23 to 25 plates.

2. To form the base layer, pipette 9 ml of uninoculated agar into each 100 by 15 mm petri dish. Tilt the dish back and forth to distribute the agar evenly and then place it on a level surface while the agar solidifies. Leave the covers slightly ajar to permit escape of excess moisture.
3. Add 2.0 ml of an overnight culture of *Sarcina lutea* incubated at 30°C in Trypticase soy broth to a bottle containing 100 ml of melted agar maintained at 50°C in a water bath. (The temperature of the agar should be measured with an alcohol-flamed thermometer to ensure that it does not exceed 50°C.) Mix the contents thoroughly by inverting the bottle several times. This seeded agar should contain 1×10^7 CFU/ml.
4. Pipette 4 ml of seeded agar into each of the dishes to overlay the base layer. Tilt each plate back and forth rapidly during pipetting to distribute the seed layer evenly over the surface of the base layer. Place the dishes on a level surface while the seed layer hardens.
5. The covered plates may be stored at 4°C and should be used within 7 days after preparation.

Procedure:

1. Estimate the concentration of antibiotic in the specimen to be tested on the basis of the dosage and time interval between administration and specimen collection. Dilute the specimen so that its concentration will be within the range of the standard curve, preferably near the reference concentration (Table 12.1–4). Serum specimens are diluted in sterile human serum. Urine and cerebrospinal fluid are diluted in 0.1 mol/liter phosphate buffer, pH 7.0.
2. Using alcohol-flamed forceps, place the blank sterile paper disks in a single layer in a sterile petri dish. Pipette 20 μl of the appropriate standard solution or test fluid dilution onto each disk. It is important to fill the stem of the capillary pipette completely to ensure that 20 μl of fluid is delivered. Do not pipette fluid onto more than six disks before transferring them to the seeded agar plate.
3. Transfer the disks onto the surface of the seeded agar plates and gently tap with the forceps to ensure complete contact.
4. On each of three plates, place disks with each of the standards. Onto each of three additional plates, place the disks with the undiluted and diluted specimen, as appropriate, and a disk with the reference concentration (Table 12.1–4). The disks should be evenly spaced and about 2 cm from the center of the plate.
5. Incubate the plates, without stacking, at 30°C for 18 hr.

Interpretation:

1. Measure diameters of the zones of inhibition for each calibration concentration, reference concentration, and specimen dilution. Use ver-

nier calipers and oblique or darkfield lighting to make the measurements. Zone diameters may also be read with a Fisher-Lilly antibiotic zone reader (Fisher Scientific Co., cat. no. 7–906).

2. Calculate means of the zone diameters for each calibration concentration on the three plates with just standards. Construct a standard curve by plotting the mean zone diameter on the abscissa against logarithms of concentration on the ordinate.
3. Calculate means of the zone diameters of the reference concentration and of each specimen dilution. Do not include zone diameters from the plates containing standards in the mean for the reference concentration.
4. Correct the mean zone diameter of each specimen dilution by adding algebraically the difference between the mean zone of the reference concentration on the plates containing specimen and the zone corresponding to the reference concentration as determined from the standard curve. Because the best line for the standard curve usually does not go through each point, the zone corresponding to the reference concentration will be slightly different from the mean zone diameter of the reference concentration.
5. Locate the corrected zone diameter for each specimen dilution on the standard curve and read the corresponding concentration. Multiply this concentration by the dilution factor to calculate the antibiotic concentration in the original specimen. Report the result from the specimen dilution that gives a zone diameter nearest the reference zone diameter. An example of these calculations is shown in Table 12.1–5.

Comments:
In practice, one specimen dilution should give a zone diameter similar to the zone diameter of the reference concentration. Calculations may be done with this single dilution and reported. Results from other dilutions will often be outside the range of the standard curve. These results should be checked for consistency to protect against gross errors, but exact calculations do not need to be made with them. The standard curve should never be extrapolated to accommodate zones larger or smaller than the largest or smallest zones observed. When this situation occurs, the test must be repeated using different specimen dilutions.

Specifications for disk-plate assays of other antimicrobials are given in Table 12.1–6. For most disk-plate assays, specimen dilutions of 1:4, 1:10, 1:20, and 1:40 will suffice. For trough levels, an undiluted serum specimen is usually tested, and for peak levels of penicillins, a 1:100 dilution may be necessary. These dilutions may be prepared conveniently as follows:

1:4 = 0.6 ml specimen + 1.8 ml diluent
1:10 = 0.4 ml specimen + 3.6 ml diluent

1:20 = 1.0 ml of 1:10 dilution + 1.0 ml diluent
1:40 = 0.5 ml of 1:10 dilution + 1.5 ml diluent
1:100 = 0.2 ml of 1:10 dilution + 1.8 ml diluent

3. Inactivation of β-Lactam Antibiotics

a. General Considerations

The presence of β-lactam antibiotics in a specimen can interfere with the bioassay of other antimicrobials, for example, clindamycin. This interference can be avoided by enzymatic inactivation of the penicillin or cephalosporin with a β-lactamase prior to assay. The β-lactamase can

Table 12.1–5. Example of Calculations for Disk-Plate Assay of Penicillin Assay of Penicillin

A. Calculation of Mean Zone Diameters

	Standard curve concn (μg/ml)					Specimen plates	
	0.1	0.2	0.3	0.4	0.5	Serum dilution (1:100)	Reference concn (0.3 μg/ml)
Zone diameter (mm)	14.8	18.9	21.9	23.9	25.0	20.7	22.3
	14.9	19.2	22.4	24.0	24.8	20.3	23.2
	16.1	19.3	21.9	23.2	25.3	21.6	22.3
Mean zone diameter (mm)	15.3	19.1	22.1	23.7	25.0	20.9	22.6

B. Calculation of Corrected Zones

Reference zone diameter from standard curve	21.9 mm
Mean reference zone with specimen	22.6 mm
Correction factor	−0.7 mm
Corrected zone for specimen at 1:100 dilution	20.9 mm + (−0.7 mm) = 20.2 mm

C. Determination of Specimen Concentration

Concn corresponding to zone of diluted specimen (20.2 mm)	0.23 μg/ml
Multiply by dilution factor (1:100)	23 μg/ml of penicillin in specimen

Table 12.1–6. Specifications for Disk-Plate Assays

Antibiotic	Medium			Test organism			Sample volume (μl)	Incubation[a]	
	Description	Base layer (ml)	Seed layer (ml)	Description	Growth conditions	Vol (ml) of 16–18 hr broth culture per 100 ml seed layer		Duration (hr)	Temperature (°C)
Cephalosporins[b] Penicillins Erythromycin Clindamycin	Antibiotic assay medium no. 5 with $CaCl_2$ (10 g/liter) added, pH 7.0	9	4	*S. lutea* ATCC 9341	TSB at 30°C	2	20	18	30
Carbenicillin Piperacillin Ticarcillin	Nutrient agar with $CaCl_2$ (10 g/liter) added, pH 7.0	None	10	*P. aeruginosa* ATCC 25619	MHB at 35°C	1 of a 1:10 dilution	20	22	30
Moxalactam	Antibiotic assay medium no. 1, pH 6.6	None	10	*E. coli* ATCC 10536	TSB at 30°C	0.25	20	18	30
Streptomycin	Antibiotic assay medium no. 11, pH 7.9	None	10	*B. subtilis* ATCC 6633	TSB at 30°C	1 of a 1:10 dilution	20	18	30
Trimethoprim	Mueller-Hinton agar with lysed horse blood (5% v/v) and *p*-aminobenzoic acid (100 μg/ml), pH 7.4	None	10	*B. subtilis* ATCC 6633	TSB at 30°C	1 of a 1:10 dilution	20	18	30
Metronidazole	Heart infusion agar with lysed horse blood (5% v/v), pH 7.4	None	5	*Clostridium bifermentans* (Mayo)	Thio at 35°C	1	20	18	35 (Anaerobic)

[a] Equilibrate assay plates to room temperature before applying samples.

[b] When measuring cephalothin, results with *S. lutea* will be falsely elevated because of interference from a metabolite, desacetylcephalothin. The interference can be avoided by substituting *B. subtilis* ATCC 6633 and keeping other conditions the same. This substitution should not be done when an aminoglycoside is also present.

be obtained from a commercial supplier or prepared in the laboratory. A simple preparation of β-lactamase from *Enterobacter cloacae* that will inactivate most cephalosporins has been described.[40]

b. Representative Procedure

Inactivation of β-Lactam Antibiotics

Purpose:
To allow the biossay of antibiotics present in mixture with penicillins or cephalosporins.

Principle:
A β-lactamase is added to the specimen before assay. The mixture is incubated briefly to destroy the antimicrobial activity of the β-lactam drug.

Materials and Reagents:

1. Penicillinase concentrate containing 10×10^6 kinetic (Kersey) units per ml (BBL Microbiology Systems, cat. no. 11898).
2. Type IV Penicillinase (Cephalosporinase) from *Enterobacter cloacae* (Sigma Chemical Co., cat. no. P4524). Dissolve this material in water according to the potency provided with each lot to give a solution containing 10 units of cephalosporinase activity per ml. Store at −15°C or lower in 0.5-ml amounts. Expiration date: 1 month.
3. Sterile, pooled, normal human serum.

Procedure:

1. Add 0.1 ml of penicillinase or 0.2 ml of cephalosporinase, 1 ml of specimen, and sufficient normal human serum to give a final volume of 2 ml to a small test tube and mix.
2. Incubate for 30 min at 35°C. The specimen can then be used in the various bioassays without concern from interference from the β-lactam drug. The 1:2 dilution of the specimen must be considered in calculating results.

Comments:
When only penicillins are present, use the penicillinase concentrate. When a cephalosporin or a cephalosporin and a penicillin are present, use the broad spectrum β-lactamase. Cefamandole, moxalactam, and cefoxitin are not fully inactivated under the conditions described. Cephradine, cefazolin, cefoperazone, cephalothin, and cephalexin are fully inacti-

vated. Other cephalosporins, particularly second- and third-generation agents, should be tested for complete inactivation before relying on this procedure.

D. Broth-Dilution Bioassays

1. General Considerations

The broth-dilution bioassay is used infrequently to measure antimicrobial concentrations because it is inherently less accurate than diffusion-based bioassays. Usually, serial twofold dilutions of antimicrobial are tested in dilution procedures. Any given end point, therefore, represents a true value somewhere between the preceding lower dilution and the next succeeding higher dilution, which gives an inherent error of 50 to 100%. An attempt can be made to decrease this error by testing intermediate dilutions, but these dilution schemes add to the complexity of the test. Broth-dilution bioassays are useful for drugs that diffuse poorly in agar and for which alternative methods are not available or are impractical because of the infrequency with which the drug is measured. For example, colistin (polymyxin E) can be measured by agar-diffusion assay using *Bordetella bronchiseptica* ATCC 4617, but because of the infrequency that this drug is assayed, a broth-dilution assay using the more easily maintained *Escherichia coli* ATCC 25922 is more practical. Finally, a variation of the broth-dilution assay, the serum bactericidal or Schlichter test, is used to monitor therapy in some conditions, particularly in endocarditis (Section 12.2).

2. Representative Procedure

Broth-Dilution Assay of Polymyxin

Purpose:
To measure the concentration of polymyxin in serum or other fluid.

Principle:
Dilutions of the fluid to be tested are prepared in broth, and the highest dilution that causes complete inhibition of growth of the test organism is determined. In a parallel test, the lowest concentration of drug that causes complete inhibition of growth is also determined. That is, an MIC is determined concurrently using the same inoculum and incubation conditions as are used for the test specimen. The product of the MIC and

the reciprocal of the highest test dilution that gave inhibition gives the drug concentration.

Specimen:
Serum, urine, or cerebrospinal fluid may be tested.

Materials and Reagents:

1. Sterile, disposable plastic test tubes (12 by 75 mm; Falcon, cat. no. 2054), cotton-plugged or capped.
2. *Escherichia coli* ATCC 25922 is obtained from the daily susceptibility control or is maintained on a Trypticase soy agar slant in a refrigerator and is subcultured every 2 weeks to a fresh agar slant. The subculture is incubated overnight at 35°C before being replaced in the refrigerator for storage.
3. Sterile, pooled, normal human serum (Gibco Laboratories). Precautions for use are described above for the disk-plate assay for penicillin.
4. Antimicrobial stock solutions are prepared as described in Chapter 4 (Table 4.4–3). For assay of serum, dilute the stock solution with normal human serum to give a solution containing 20 μg/ml of polymyxin. For assay of urine or cerebrospinal fluid, dilute the stock solution with sterile water to give a solution containing 20 μg/ml of polymyxin.

Medium:
Trypticase soy broth (BBL Microbiology Systems).

Procedure:

1. Place 12 test tubes in a rack for each specimen to be tested.
2. Add 2 ml of Trypticase soy broth to each tube.
3. Add 2 ml of the test material to the first tube.
4. With a fresh 2 ml pipette, mix the contents of the first tube and transfer 2 ml of the diluted material to the second tube. Discard the pipette.
5. Using a fresh pipette for mixing and transferring each time, continue as in the above step to make a series of twofold dilutions in the remaining tubes. Discard 2 ml of the mixture from the last tube.
6. Similarly, twofold dilutions of polymyxin are prepared in 9 tubes, each containing 2 ml of Trypticase soy broth. Add 2 ml of the appropriate solution containing 20 μg/ml of polymyxin to the first tube to give a series with concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 μg/ml. A tenth tube with 2 ml of Trypticase soy broth is prepared as a control.
7. Prepare the inoculum by diluting with broth a 6 hr culture of *Escherichia coli* ATCC 25922 to a turbidity equivalent to a McFarland No. 0.5 standard. Then dilute this 1:100 in broth.
8. Using a single, 0.2 ml pipette for each series of tubes, add 0.04 ml

of the diluted inoculum to each tube. Begin the inoculation with the control or the highest sample dilution and proceed, in order, with each successive tube. The final inoculum density is 2×10^4 CFU/ml.
9. Incubate all tubes at 35°C for 16 hr.

Interpretation:
The reciprocal of the greatest dilution of the specimen completely inhibiting the test organism is multiplied by the lowest concentration in the standards set that completely inhibited the test organism (MIC). The result is the concentration of polymyxin in the specimen.

III. Liquid Chromatographic Assays

A. General Considerations

Liquid chromatography is basically a method for separation. Quantitation is achieved by subsequent analysis of the separated moieties. In modern instrumentation, the functions of separation and quantitation are combined to give a continuous quantitative measure of each analyte in a mixture as it is separated. Liquid chromatographic procedures have been developed to measure almost all of the antibiotics in clinical specimens.[11] Most of the procedures are similar and involve either extraction of the antimicrobial from the specimen or precipitation of protein followed by chromatography of the extract or protein-free fluid on a reversed-phase column. The chromatography is performed with a predominately aqueous mobile phase to which either methanol or acetonitrile has been added to adjust the retention time. Quantitation is usually based upon absorption of ultraviolet light, although in the following procedure for aminoglycosides, quantitation is based on fluorescence of products formed in a reaction occurring after the separation is completed.

One advantage of liquid chromatography over other methods of assay is that a standard can be mixed with the specimen prior to assay. This standard is called an *internal standard.* Because both the drug of interest and the internal standard are subjected then to exactly the same procedure, subsequent small errors in pipetting or changes in test conditions do not affect results greatly. Quantitation is based solely on the ratio of the drug to the internal standard. The selection of a liquid chromatographic procedure should place particular emphasis on whether an internal standard is used in the assay. The review by Gerson and Anhalt[11] provides detailed instructions for assay of most antimicrobials, including the aminoglycosides, vancomycin, and chloramphenicol.

B. Specific Procedure[3]

Aminoglycoside Assay*

Purpose:
To measure the concentration of gentamicin, tobramycin, netilmicin, kanamycin, neomycin, or amikacin in serum.

Principle:
Aminoglycoside antibiotics at pH 6–7 carry a positive charge because several amino groups are on these molecules. These positively charged molecules bind tightly to a weak cation-exchange resin, CM-Sephadex, and can be extracted from serum in this manner. An internal standard is added to serum, and the mixture is applied to a small column containing CM-Sephadex. The resin is washed with excess buffer to remove serum components and interfering substances. An alkaline buffer then is used to remove the bound aminoglycoside from the resin. This step also functions to regenerate the resin for subsequent use. The alkaline extract containing the aminoglycoside is analyzed. The chromatographic eluate is continuously mixed with *o*-phthalaldehyde reagent, which reacts with primary amino groups to form fluorescent products. In this way, the aminoglycoside is converted to fluorescent products that can be quantitated. The optimal wavelength for fluorescence excitation is 340 nm and for emission is 455 nm.

Specimen:
Serum (0.4 ml) is the preferred specimen. If plasma is used, it should not be heparinized, because heparin can bind aminoglycosides. The specimen should be stored refrigerated to prevent microbial growth, but storage at 25°C for 2 days does not affect gentamicin levels. Various drugs, such as carbenicillin and ticarcillin, react with aminoglycosides during storage to form inactive addition products. Serums containing a β-lactam antibiotic in combination with an aminoglycoside should be stored frozen, preferably at −20°C or below, and tested within 2 days.

Materials and Reagents:
Water should be deionized and distilled. Suitable sources for other reagents are as follows: methanol (Burdick and Jackson Laboratories); acetic

* Portions from Gerson, B., and Anhalt, J. P. *High-Pressure Liquid Chromatography and Therapeutic Drug Monitoring.* Chicago, IL, American Society of Clinical Pathologists, 1980. And, Anhalt, J. P. Aminoglycoside antibiotics by HPLC. *In* Sunshine, I., and Jatlow, P. (eds.), *Methodology for Analytical Toxicology,* Vol. II. Boca Raton, FL, CRC Press, 1982, p. 31. Used by permission.

acid ("Photrex" reagent) and H_3BO_3 (granular) (J. T. Baker Chemical Co.); $NaH_2PO_4 \cdot H_2O$ (granular), Na_2HPO_4 (granular), Na_2SO_4 (granular), KOH and NaOH (Mallinckrodt); sodium 1-pentanesulfonate monohydrate (Eastman Kodak Co.); *o*-phthalaldehyde, 2-mercaptoethanol, and polyoxyethylene-23-lauryl ether (Brij 35) (Sigma Chemical Co.); and pooled human serum (Gibco Laboratories). Sodium pentanesulfonate is of variable purity; powders that give yellow solutions should be recrystallized from a water–ethanol mixture before use. Synthesis of 1-*N*-acetylgentamicin C_1, the internal standard for gentamicin, is described elsewhere.[1]

1. *Gentamicin and Netilmicin Mobile Phase*

Na_2SO_4 (MW 142.04)	56.816 g
Sodium pentanesulfonate·H_2O (FW 192.21)	7.688 g
Acetic acid	2 ml
Water	1940 ml
Methanol	60 ml

Dissolve salts in water. Add acetic acid. After solution is complete, add methanol. Filter through a Millipore HAWP (0.45 μm) membrane filter (Millipore Corp.). Expiration date: 3 months. Depending on the age of the chromatographic column and the brand used, the amount of methanol required may vary.

2. *Amikacin, Kanamycin, Neomycin, and Tobramycin Mobile Phase*

Na_2SO_4 (MW 142.04)	28.048 g
Sodium pentanesulfonate·H_2O (FW 192.21)	7.688 g
Acetic acid	2 ml
Water	2 liters

Dissolve salts in water. Add acetic acid. Filter through a Millipore HAWP (0.45 μm) membrane filter. Expiration date: 3 months.

3. *Potassium Borate Buffer (0.40 mol/liter)*

Boric acid (MW 61.83)	49.46 g
KOH (MW 56.11)	42.64 g
Water	1950 ml

Add water to boric acid. Then add KOH pellets and stir until solution is complete. Adjust pH to 10.40 ± 0.02 with KOH. Filter through a Millipore HAWP (0.45 μm) filter. Expiration date: 6 months.

4. o-Phthalaldehyde Reagent

o-Phthalaldehyde (Fluoropa)	80 mg
2-Mercaptoethanol-Brij 35 mixture	0.5 ml
Methanol	1 ml
Potassium borate buffer	100 ml

Prepare a mixture containing three parts of 30% (w/v) Brij 35 in water and two parts of 2-mercaptoethanol. This mixture may be stored at room temperature if the container is tightly closed. Dissolve the *o*-phthalaldehyde in methanol. Be sure all crystals are completely dissovled. Add the 2-mercaptoethanol-Brij 35 mixture and gently mix until completely decolorized. Add potassium borate buffer and mix well. Filter through a Millipore HAWP (0.45 μm) filter or a Nalgene filter unit. Immediately place the solution in the reagent reservoir and flush with nitrogen. Take care to avoid excessive exposure of *o*-phthalaldehyde crystals to humid atmosphere or light. Expiration date: 1 week.

5. Sodium Sulfate Solution (0.2 mol/liter)

Na_2SO_4 (MW 142.04)	56.816 g
Water	2 liters

Dissolve Na_2SO_4 in water. Filter through a Millipore HAWP (0.45 μm) filter. Expiration date: 6 months.

6. Diluent for Internal Standards (0.2 mol/liter Phosphate Buffer, pH 6.9)

$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	4.83 g
Na_2HPO_4 (MW 141.96)	9.23 g
Water	500 ml

Mix ingredients and check for pH 6.8–7. Adjust pH with 1 mol/liter NaOH if necessary. Filter through a Millipore HAWP (0.45 μm) filter.

7. Alkaline Elution Buffer (0.01 mol/liter NaOH, 0.2 mol/liter Na_2SO_4)

NaOH (MW 40.00)	0.2 g
Na_2SO_4 (0.2 mol/liter)	500 ml

Dissolve NaOH in 500 ml of 0.2 mol/liter sodium sulfate solution. Filter. Expiration date: 2 months.

8. Ion-pair Concentrate

Sodium pentanesulfonate·H_2O (FW 192.21)	5.00 g
Acetic acid	2 ml
Water	100 ml

Mix ingredients and filter (HAWP, 0.45 μm). Expiration date: 6 months.

9. Antimicrobial Stock Solutions
Stock solutions are prepared as described in Chapter 4 (Table 4.4–3). These solutions may be divided into aliquots and stored frozen for up to 1 year. After thawing, solutions should be used within 1 week and should not be refrozen.

10. Internal Standard Solutions
Prepare working dilutions of the internal standards by diluting with the phosphate buffer, pH 6.9, the stock solutions containing 1000 μg/ml of appropriate internal standards as shown in Table 12.1–7.

Table 12.1–7. Internal Standard Solutions

Drug	Internal standard	Volume of stock (ml)	Final volume (ml)	Final concn (μg/ml)
Gentamicin	1-*N*-acetyl-gentamicin C_1[a]	0.8	100	8
Netilmicin	1-*N*-acetyl-gentamicin C_1[a]	0.8	100	8
Kanamycin	Tobramycin	0.8	100	8
Amikacin	Tobramycin	0.8	100	8
Tobramycin	Amikacin	0.4	100	4
Neomycin	Amikacin	0.4	100	4

[a] If tobramycin is used as internal standard, prepare a 2 μg/ml solution by diluting 0.2 ml of stock to 100 ml.

11. Calibration Standard Solutions
The chromatographic system is calibrated using aqueous standards prepared in the mobile phase for amikacin. Since the instrument response is linear over the range of concentrations usually encountered clinically, it is more important to ensure accuracy of the ratio of drug to internal standard than the absolute concentration of each in the calibration standard. Because a small dilution occurs during extraction, the absolute concentrations of drugs in the calibration standards have been adjusted to give peak heights that are on scale and are similar to those produced

Table 12.1–8. Calibration Standard Solutions

Assay	Internal standard	Actual concn assay drug/ Internal standard (μg/ml)	Fictitious concn assay drug/Internal standard[a]
Gentamicin	1-*N*-acetylgentamicin C_1	4/8	4/8
Netilmicin	1-*N*-acetylgentamicin C_1	2/4	4/8
Kanamycin	Tobramycin	6/6	8/8
Amikacin	Tobramycin	6/6	8/8
Tobramycin	Amikacin	3/3	4/4
Neomycin	Amikacin	4/4	4/4

[a] See text for description and use of fictitious concentrations.

by clinical specimens. Preparation of these calibration standards is described in Table 12.1–8. In each case, a stock solution containing the proper *ratio* of aminoglycoside and internal standard is prepared in water. These are then diluted to the proper *concentration* with amikacin mobile phase (Table 12.1–8) and stored in 2-ml aliquots at −20°C for up to 1 year. For example, prepare the amikacin calibration mixture by mixing 3 ml of amikacin stock solution (1000 μg/ml), 3 ml of tobramycin stock solution (1000 μg/ml), and 4 ml of water. The resulting solution will contain 300 μg/ml each of amikacin and tobramycin. Dilute 2 ml of this solution to 100 ml with amikacin mobile phase to give a working calibration standard for amikacin containing 6 μg/ml each of amikacin and tobramycin. Depending on the data system used with the chromatograph, methods may be entered using the actual concentrations in the calibration standards and an appropriate dilution factor or fictitious concentrations that already take into account the dilution factor can be entered. The Hewlett-Packard 3390A data system allows either method to be used. We have used the latter method for several years, and the appropriate fictitious concentrations that should be used are shown in Table 12.1–8.

12. Serum Controls

Dilute 0.4 ml of each antibiotic stock solution (1000 μg/ml) with 4.6 ml of phosphate buffer (pH 6.9) to give intermediate dilutions containing 80 μg/ml of drug. For gentamicin, netilmicin, neomycin, and tobramycin, mix 0.4 ml of the intermediate dilution with 7.6 ml of pooled human serum to give a serum control containing 4 μg/ml of drug. For amikacin and kanamycin, mix 0.8 ml of the intermediate dilution with 7.2 ml of pooled human serum to give a serum control containing 8 μg/ml of drug. Serum controls should be stored in a refrigerator and used within 1 month.

An increase in cloudiness of a control may be evidence of deterioration before the expiration date.

13. Serum Extraction Columns

Suspend 20 g of CM-Sephadex (C-25) (Pharmacia Fine Chemicals) in 200 ml of 0.2 mol/liter Na_2SO_4 solution and let swell overnight in a refrigerator. Resuspend the Sephadex, allow the Sephadex to settle, and decant off fines. Add additional Na_2SO_4 solution to replace the volume lost when decanting the fines and repeat the procedure twice or until fines are no longer present. Adjust the volume of suspension to 200 ml with Na_2SO_4 solution and place the mixture in a 250 ml culture flask with a single side arm and a suspended magnetic stir bar (Celstir model 356539; Wheaton Scientific). Place a paper disk cut from Whatman No. 54 filter paper in the bottom of a polypropylene column (model QS-U; Isolab) as a bed support. Add the Sephadex suspension until the bed volume after settling is level with or slightly below the third ring on the inside of the column. Be sure the bed is homogeneous after adjusting the bed height, if necessary. When using the Celstir and a magnetic stirrer, the volume of suspension necessary can be determined emipirically (usually about 2.5 ml is adequate), and adjustment of the bed height will be unnecessary. After the Sephadex settles, add a second disk of filter paper (Whatman no. 54) to the top. Then using an inverted disposable pipette or glass rod, press the paper disk to compress slightly (about 1 mm) the column packing. The final bed volume is about 1.0 ml with a height of 1.6 cm. Allow additional Na_2SO_4 solution (2 ml) to run through the column to remove any fine particles. The columns can be tightly capped and stored in a refrigerator for up to 6 months. Before use, warm the column to room temperature and allow excess Na_2SO_4 solution to drain until flow stops.

14. Miscellaneous

Because aminoglycosides in dilute aqueous solutions bind to glass surfaces causing significant losses, various sizes of plastic pipettes, test tubes, and flasks are required whenever protein-free solutions are handled.

Liquid Chromatograph:

A diagram of the chromatographic equipment used for continuous-flow, post-column derivatization and analysis is shown in Figure 12.1–2. The following components were used: Constametric IIG pump with pulse dampener (Laboratory Data Control); Rheodyne model 7120 injection valve with a 20-μl sample loop (Rheodyne Inc.); μBondapak C_{18} column (30 cm $\times$ 3.9 mm i.d., Waters Associates); Schoeffel model 970 fluorometer (Kratos/Schoeffel Instruments); flowmeter (model F1100, Roger Gilmont Instruments); mixing tee (Cheminert model CJ3031, Laboratory Data Control); reagent valve (modified type 50–11, Rheodyne Inc.); assorted sizes of Teflon tubing and fittings (Laboratory Data Control; Ace Glass);

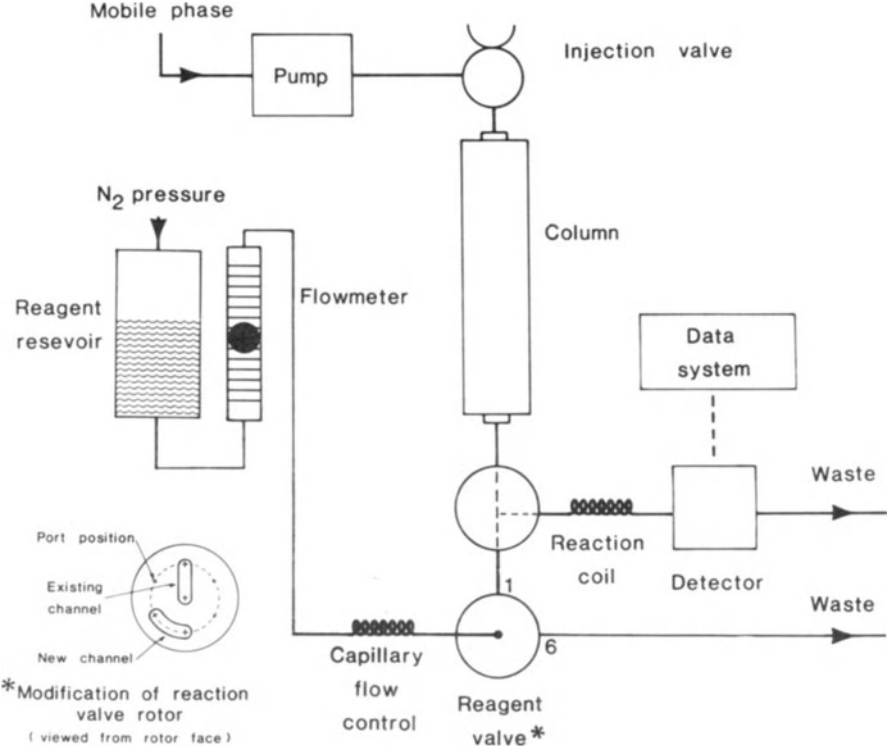


Figure 12.1–2. Diagram of chromatographic system. (From Anhalt and Brown.[1] Used by permission.)

and model 3390A chromatographic data system (Hewlett-Packard Co.). The reagent reservoir (20 cm × 19 mm i.d.) was constructed from glass tubing with a wall thickness of 3 mm. Reagent flow rate was controlled with a capillary restrictor made from Teflon tubing (9.0 m × 0.3 mm i.d.) and was varied by adjusting the nitrogen pressure applied to the reservoir. The reaction coil consists of a Teflon tube (2.0 m × 0.6 mm i.d.) wound on a rod 6.5 mm in diameter. A more detailed description of construction of a suitable system has been published.[1] Modifications of the system that have been shown to work include the use of comparable equipment from other manufacturers and substitution of the pressurized vessel and capillary flow control with a second liquid chromatographic pump. The chromatographic pump is operated at a flow rate of 2 ml/min, and *o*-phthalaldehyde reagent is delivered at 0.5 to 0.6 ml/min. The detector is operated with an excitation wavelength of 340 nm and a KV418 filter is used as the emission filter. Different detector sensitivity settings are used depending on the aminoglycoside being measured and the settings used for the data system. These settings are chosen to allow

a maximal detector response without detector overload for drug concentrations at the upper limit of the therapeutic range.

Procedure:

1. Pipette 400 μl of serum and 400 μl of the appropriate internal standard into a small, plastic test tube. Mix solutions and transfer the mixture into a well drained CM-Sephadex column. Let the mixture drain completely.
2. Add 2 ml of Na_2SO_4 buffer and let drain completely. Then add an additional 2 ml of Na_2SO_4 buffer and let drain completely.
3. Add 600 μl of alkaline elution buffer and let drain completely (discard drainage).
4. Add 400 μl of alkaline elution buffer and collect entire fraction in a plastic container.
5. Add 50 μl of ion-pair concentrate to collected fraction. After mixing, a 20 μl aliquot is used for analysis (Figure 12.1–3 and 12.1–4).
6. Regenerate the CM-Sephadex column by flushing with 1 ml of alkaline elution buffer once followed by 2 ml of Na_2SO_4 solution twice. Degradation of the column is evidenced by a decrease in recovery of the internal standard or by a marked decrease in flow rate. A single column usually can be used 5 times. The column should be discarded after each use with lipemic or hemolyzed specimens.

Interpretation:
Approximate retention times for each drug or standard are shown in Table 12.1–9. These times will vary with the age and brand name of column used. New columns give the longest retention times and may require an increase in the methanol concentration in the mobile phase used for gentamicin. A standard curve comparing relative peak areas or peak heights of drug to internal standard with concentration can be prepared in the usual manner using several standards prepared in either serum or mobile phase. Experience has shown, however, that a single-point calibration using a standard prepared in mobile phase is adequate. Either peak area or peak height may be used for calculations. The calculations for gentamicin present a unique problem because the drug is composed of three major and several minor components. Each component gives a similar, but not identical, response in the detector. Calculations should be based on the individual components with the concentration of each component calculated separately and then summed to give the total gentamicin concentration. Samples of purified components are available for research, but it is neither practical nor advisable to use purified components for calibration because of cumulative errors in the potency values for each component. The best option is to obtain from the manufacturer the component ratio in the specific lot of gentamicin that is used

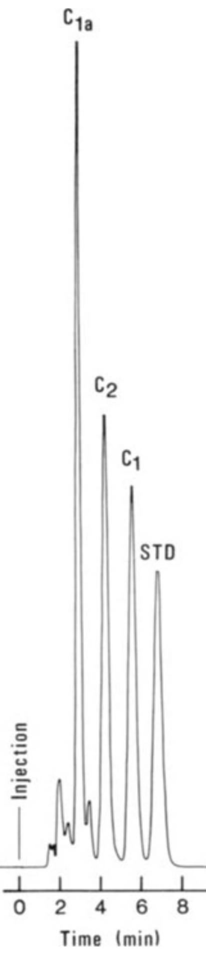


Figure 12.1–3. Representative chromatogram of gentamicin (10 mg/liter) and 1-*N*-acetylgentamicin C_1 (STD) isolated from serum. (From Anhalt and Brown.[1] Used by permission.)

for calibration. Response factors are then calculated for each component and are used for analyses of clinical specimens that may contain gentamicin with different component ratios. These calculations are simplified considerably when a data system is used. Alternatively and with a small loss of accuracy, the total area or height of the peaks in the gentamicin complex can be used to standardize the instrument. The calibration standard is tested after every fifth specimen and the results are averaged with the current calibration to give a revised calibration.

Quality Control:
As one aspect of quality control, serum controls are tested daily. The between-day coefficient of variation should be 5% or less, and 10% deviation for two, consecutive determinations necessitates remedial action.

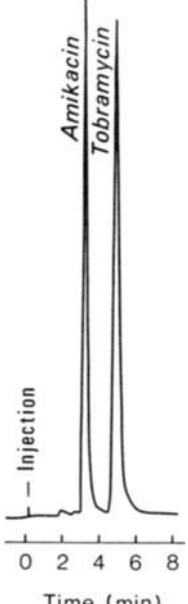


Figure 12.1–4. Representative chromatogram of amikacin (5 mg/liter) and tobramycin (5 mg/liter) isolated from serum. (From Anhalt and Brown.[1] Used by permission.)

Table 12.1–9. Approximate Retention Times of Aminoglycosides

Drug	Retention time (min)
Gentamicin C_{1a}[a]	2.55
Gentamicin C_2	3.70
Gentamicin C_1	4.75
1-*N*-Acetylgentamicin C_1	6.51
Amikacin	2.56
Tobramycin	3.69
Kanamycin A[b]	2.47
Kanamycin B	3.85
Neomycin B[c]	4.05
Neomycin C	3.31
Netilmicin	3.57

[a] Gentamicin complex consists of three major components and several minor components. The minor components, gentamicin C_{2a} and gentamicin C_{2b}, may appear as shoulders or as separate peaks between gentamicin C_{1a} and gentamicin C_2. The first of these, which is probably gentamicin C_{2b}, is added to the area of gentamicin C_{1a}. The second of these, which is usually not resolved, is added to the area of gentamicin C_2.

[b] Quantitation is based on measurement of kanamycin A only. Kanamycin B constitutes less than 5% of the clinical drug and often is not resolved as a distinct peak.

[c] The major component is neomycin B. Quantitation is based on this peak only.

Comments:

1. Drug concentrations and peak area ratios are linearly related over a wide range of concentrations beginning at 0.5 mg/liter. For gentamicin and tobramycin, concentrations in excess of 10 to 13 mg/liter require injection of a smaller sample (e.g., 10 μl) or dilution of the extract. For amikacin, concentrations in excess of 15 mg/liter require a smaller sample or dilution, which extends the upper range of concentration to 40 mg/liter.
2. Tobramycin can be used as the internal standard in the gentamicin assay. Tobramycin elutes before gentamicin C_{1a}. Thus, the methanol concentration in the mobile phase should be reduced or eliminated to provide adequate resolution of tobramycin from other substances that also elute prior to gentamicin C_{1a}. Alternatively, the mobile phase for amikacin and tobramycin can be used.
3. The CM-Sephadex can be replaced by more rigid cation exchange resins such as CG-50 (Rohm and Haas Co., Philadelphia) or Bio-Rex 70 (Bio-Rad Laboratories). This substitution allows the use of pressure, centrifugation, or vacuum to increase the flow rate through the column used for serum extraction. The higher exchange capacity of these resins, however, necessitates increasing the NaOH concentration in the elution buffer. With small columns prepared with CG-50 ion-exchange resin, a NaOH concentration of 0.20 mol/liter gave adequate elution and did not cause rapid degradation of the aminoglycosides.

Vancomycin Assay

Purpose:
To measure the concentration of vancomycin in serum.

Principle:
Serum is acidified and diluted with a solution containing the internal standard, ristocetin. The diluted solution is passed through a weak cation-exchange resin, CM-Sephadex. Vancomycin and ristocetin bind to this resin, and interfering substances are removed by washing with a sodium sulfate solution. An alkaline borate buffer is then used to elute the bound antibiotics. The alkaline extract is analyzed directly, and quantitation is based on the ratio of peak areas obtained for ristocetin and vancomycin.

Specimen:
Serum (0.4 ml) is the preferred specimen.

Materials and Reagents:
Materials are available from the same sources as described for aminoglyco-

side assays (p. 710). Ristocetin is available from Sigma Chemical Co. (cat. no. R7752).

1. Sodium Sulfate Solution (0.2 mol/liter):

Na_2SO_4 (MW 142.04)	56.816 g
Water	2 liters

Dissolve Na_2SO_4 in water. Filter through a Millipore HAWP (0.45 μm) filter. Expiration date: 6 months.

2. Borate Elution Buffer (0.05 mol/liter Borax, 0.2 mol/liter Na_2SO_4, pH 9.45):

Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$, FW 381.42)	7.628 g
Na_2SO_4 (MW 142.04)	11.36 g
Water	400 ml

Dissolve sodium borate in 300 ml of water and adjust with 1.0 mol/liter NaOH to pH 0.6. Dilute to 400 ml with water, dissolve Na_2SO_4, and filter. The final pH should be 9.45. Expiration date: 6 months.

3. Serum Diluent with Internal Standard (0.02 mol/liter Acetic Acid, 0.16 mol/liter Na_2SO_4, 25 mg/liter Ristocetin):

Ristocetin (1000 mg/liter)	2.5 ml
Acetic acid (0.1 mol/liter)	25.0 ml
Na_2SO_4 solution (0.2 mol/liter)	100 ml

Prepare 0.1 mol/liter acetic acid by diluting 5.75 ml of glacial acetic acid (17.4 mol/liter) to 1.0 liter. Mix 25 ml of 0.1 mol/liter acetic acid with 100 ml of 0.2 mol/liter Na_2SO_4. Prepare according to potency a stock solution containing 1000 mg/liter ristocetin in water. Dilute 2.5 ml of this stock solution to 100 ml with the solution of acetic acid in 0.16 mol/liter Na_2SO_4 prepared above. The diluent is stable for at least two weeks at 2° to 8°C.

4. Mobile Phase for Vancomycin (0.05 mol/liter Phosphate Buffer, 0.1 mol/liter Na_2SO_4-acetonitrile Mixture, 91 : 9):

Na_2SO_4 (MW 142.04)	42.61 g
KH_2PO_4 (MW 136.09)	17.90 g
$K_2HPO_4 \cdot 3H_2O$ (FW 228.23)	4.21 g
Water	3.0 liters
Acetonitrile	297 ml

Dissolve phosphate salts and Na_2SO_4 in 3.0 liters of water to give a buffer, pH 5.75. The pH does not appear to be critical. Add 297 ml of acetontrile to 3.0 liters of buffer, mix, and filter through a Millipore FHUP (0.5 μm)

Teflon membrane filter. It is necessary to first wet the filter with a few drops of methanol before filtration of the mobile phase mixture. Expiration date: 6 months.

5. *Calibration Standard (25 mg/liter Vancomycin, 25 mg/liter Ristocetin)*:

Ristocetin (1000 mg/liter)	2.50 ml
Vancomycin (1000 mg/liter)	2.50 ml
Water	95.00 ml

Prepare calibration standard in a 100-ml plastic volumetric flask. Measure vancomycin and ristocetin with plastic pipettes. Mix well and aliquot 5 ml into plastic screw-cap tubes. Store at −20°C. One tube can be kept thawed at 2–8°C for two weeks. Dilute calibration standard 1:2 with borate elution buffer before injecting. The diluted calibration standard must be used within 30 min.

6. *Serum Control (20 mg/liter Vancomycin in Pooled Human Serum)*:

Vancomycin (1000 mg/liter)	1.0 ml
Pooled human serum	49.0 ml

Prepare serum control in a 50-ml plastic volumetric flask. Use a plastic pipette to measure vancomycin. Mix for 30 min at room temperature and aliquot 400 μl into 1.5-ml polypropylene centrifuge tubes. Store at −20°C for no longer than 1 year.

7. *Serum Extraction Columns:*

Prepare CM-Sephadex columns for extraction as described for aminoglycoside assays (p. 715).

Liquid Chromatograph:

A conventional liquid chromatographic system is used consisting of a Constametric IIG pump with pulse dampener (Laboratory Data Control), Rheodyne model 7120 injection value with a 20-μl sample loop (Rheodyne, Inc.), μBondapak C_{18} column (30 cm × 3.9 mm i.d., Water Associates), Schoeffel model SF770 ultraviolet detector (Kratos/Schoeffel Instruments), and a model 3390A chromatographic data system (Hewlett-Packard Co.). The chromatographic pump is operated at 2 ml/min, and analysis is at ambient temperature. Detection is by absorbance at 210 nm with a full-scale sensitivity of 0.02 absorbance unit.

Procedure:

1. Mix 400 μl of serum with 400 μl of serum diluent containing internal standard. Add this mixture to the top of a well-drained CM-Sephadex column and let drain completely.

2. Add 2.0 ml of Na_2SO_4 solution and let drain completely, twice.
3. Add 400 μl of borate elution buffer and let drain completely. Discard this fraction.
4. Add 800 μl of borate elution buffer. Collect this fraction for analysis.
5. Fill a 100-μl gas-tight syringe with sample to be injected and discard. Refill the syringe and inject all 100 μl into the injector. This is to ensure complete flushing of the 20 μl injection loop with sample.
6. Regenerate CM-Sephadex columns after each use by washing with 1.0 ml of borate elution buffer once and 2 ml of Na_2SO_4 solution twice. Degradation of the columns is evidenced by a decrease in recovery of internal standard. A single column can usually be used five times. The column should be discarded after each use with lipemic or hemolyzed specimens.

Interpretation:
Ristocetin elutes before vancomycin. In typical chromatograms, ristocetin appears after 4 to 5 min, and vancomycin after 8 to 9 min. Elution time can be adjusted by changing the amount of acetonitrile in the mobile phase. Minor peaks may appear in the chromatogram between ristocetin and vancomycin. These are presumably impurities or degradation products of vancomycin. Calibration is done as described for the aminoglycosides using a standard with each set of specimens.

Quality Control:
The serum control is tested with each batch of specimens, and the result for vancomycin must be between 18 and 22 μg/ml.

Comments:

1. The syringe used for sample injection must not have a metal plunger in contact with the solution. A 100-μl gas-tight syringe is specified, because this version uses a Teflon-tipped plunger, which prevents the metal contact.
2. Some lots of optically clear, defibrinated human serum seem to irreversibly bind up to 20% of the vancomycin. This binding has not been observed with human serum that is not specifically optically clear or with recalcified serum prepared in-house.

Chloramphenicol Assay

Purpose:
To measure concentration of chloramphenicol in serum.

Principle:
Serum is alkalinized to pH 10 with a solution containing the internal standard. The mixture is extracted with ethyl acetate and the upper or-

ganic phase is transferred to a clean tube. Ethyl acetate is evaporated and the residue containing chloramphenicol and internal standard is dissolved in mobile phase and analyzed by liquid chromatography.

Specimen:
Serum (0.1 ml) is the preferred specimen. The method can be scaled-down to use 20 μl of serum.

Materials and Reagents:
Materials are available from the sources described for aminoglycoside assays.

1. Mobile Phase for Chloramphenicol (50 mmol/liter Phosphate Buffer-Acetonitrile Mixture, 78:22):

KH_2PO_4 (MW 136.09)	17.90 g
$K_2HPO_4 \cdot 3H_2O$ (FW 228.23)	4.21 g
Water	3.0 liters
Acetonitrile	846.0 ml

Dissolve phosphate salts in water. The pH should be 6.0 ± 0.1. Add acetonitrile, mix, and filter through a Millipore (FHUP) Teflon membrane. It is necessary to first wet the filter with a few drops of methanol before filtration of the mobile phase mixture. Alternatively, an Ultipor $N_{66}X$ (Pale Trinity Micro Corp.) 0.45 μm membrane filter can be used. This latter filter is made of nylon and does not require wetting with methanol before use. Expiration date: 6 months.

2. Internal Standard:
The internal standard is an analogue of chloramphenicol in which the dichloroacetyl group has been replaced by a pivaloyl group. This compound ("pivamphenicol") is prepared by heating a mixture of chloramphenicol base [2-amino-1-(*p*-nitrophenyl)-1,3-propanediol, Aldrich Chemical Co.] with trimethylacetic anhydride (pivalic anhydride) according to the method of Rebstock.[32] The product is recrystallized from either ethylene dichloride-toluene or chloroform-toluene mixtures.

3. Serum Diluent with Internal Standard (0.8 mol/liter Tris (hydroxymethyl)aminomethane, 20 mg/liter Pivamphenicol):

Tris(hydroxymethyl)aminomethane (MW 121.136)	9.69 g
Pivamphenicol (1000 mg/liter)	2.0 ml
Water	98 ml

Prepare serum diluent-internal standard solution in a 100-ml plastic volumetric flask. Dissolve Tris in about 90 ml distilled water. The pH should be 11.0 ± 0.2. Add pivamphenicol and q.s. to 100 ml with water. Mix

well, and aliquot into 5-ml plastic screw-cap tubes. Store at −20°C. One tube can be kept at 2–8°C for 1 month.

4. Calibration Standard (20 mg/liter Chloramphenicol, 20 mg/liter Pivamphenicol):

Chloramphenicol stock (1000 mg/liter)	2.0 ml
Pivamphenicol stock (1000 mg/liter)	2.0 ml
Water	96.0 ml

Mix solutions and aliquot into 5-ml plastic screw-cap tubes. Store at −20°C. One tube can be stored at 2–8°C for 1 month.

5. Serum Control (12 mg/liter Chloramphenicol in Pooled Human Serum):

Chloramphenicol (1000 mg/liter)	100 μl
Pooled human serum	9.23 ml

Mix chloramphenicol and pooled human serum for 30 min at room temperature. Aliquot 100 μl into 1.5 ml polypropylene centrifuge tubes, and store at −20°C.

Liquid Chromatograph:
The liquid chromatograph and analytical conditions are as described for vancomycin (p. 722), except detection is by absorbance at 278 nm with a full-scale sensitivity of 0.02 absorbance unit.

Procedure:

1. Mix 100 μl of serum and 100 μl of serum diluent containing internal standard in a 1.5-ml polypropylene centrifuge tube.
2. Add about 1 ml of ethyl acetate with a Pasteur pipette.
3. Vortex for 30 sec.
4. Centrifuge for 1 min at 8000 × *g*.
5. Remove about 800 μl of the upper layer (ethyl acetate) with a Pasteur pipette, and place in another 1.5-ml polypropylene tube.
6. Evaporate ethyl acetate to dryness under a stream of nitrogen at 40°C.
7. Dissolve the residue in 100 μl of chloramphenicol mobile phase.
8. Inject 50 μl of the samples or calibration standard into the injector to ensure complete flushing and filling of the loop.

Interpretation:
Pivamphenicol elutes after chloramphenicol (Figure 12.1–5). The succinate esters of chloramphenicol are not extracted by this method. With the mobile phase used, however, they elute before chloramphenicol and do not interfere with the analysis. Calibration is based on ratios of peak area as described for aminoglycoside assays (p. 717).

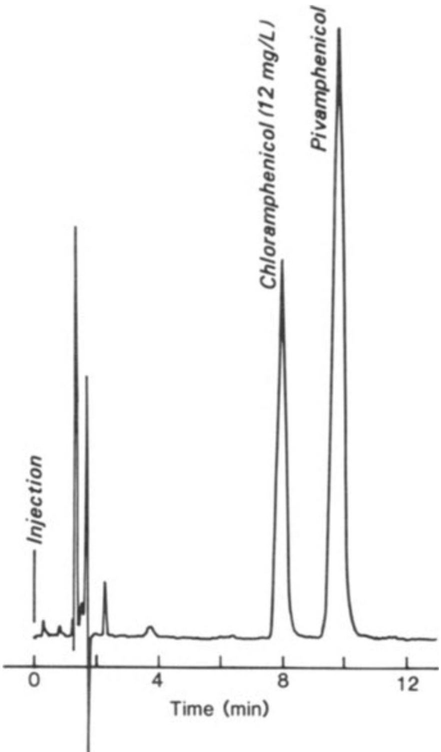


Figure 12.1-5. Representative chromatogram of chloramphenicol (12 μg/ml) and pivamphenicol isolated from serum.

Quality Control:
A serum control is tested with each batch of specimens. The result must be between 10.8 and 13.2 μg/ml of chloramphenicol.

Comments:

1. If extraction is done under acidic conditions (e.g., at pH 4.6 with sodium acetate buffer), the succinate esters are extracted and can be quantitated.
2. To scale-down the procedure to use 20 μl of serum, use 20 μl of internal standard solution and 0.5 ml of ethyl acetate. Redissolve the residue after evaporation in 40 μl of mobile phase and analyze at a full scale sensitivity of 0.01 absorbance unit.

References

1. Anhalt, J. P., and Brown, S. D. High-performance liquid-chromatographic assay of aminoglycoside antibiotics in serum. *Clin. Chem.* **24**:1940, 1978.

2. Barza, M., Brown, R. B., Shen, D., Gibaldi, M., and Weinstein, L. Predictability of blood levels of gentamicin in man. *J. Infect. Dis.* **132**:165, 1975.
3. Barza, M., and Lauermann, M. Why monitor serum levels of gentamicin? *Clin. Pharmacokinet.* **3**:202, 1978.
4. Bennett, W. M., Singer, I., Golper, T., Feig, P., and Coggins, C. J. Guidelines for drug therapy in renal failure. *Ann. Intern. Med.* **86**:754, 1977.
5. Bootman, J. L., Zaske, D. E., Wertheimer, A. I., and Rowland, C. Cost of individualizing aminoglycoside dosage regimens. *Am. J. Hosp. Pharm.* **36**:368, 1979.
6. Bourne, P. R., Phillips, I., and Smith, S. E. Modification of the urease method for gentamicin assays. *J. Clin. Pathol.* **27**:168, 1974.
7. Broughall, J. M., Bywater, M. J., Holt, H. A., and Reeves, D. S. Stabilization of cephalosporins in serum and plasma. *J. Antimicrob. Chemother.* **5**:471, 1979.
8. Burd, J. F., Wong, R. C., Feeney, J. E., and Carrico, R. J., and Boguslaski, R. C. Homogeneous reactant-labeled fluorescent immunoassay for therapeutic drugs exemplified by gentamicin determination in human serum. *Clin. Chem.* **23**:1402, 1977.
9. Drayer, D. E. Active drug metabolites and renal failure. *Am. J. Med.* **62**:486, 1977.
10. Ervin, F. R., and Bullock, W. E., Jr. Simple assay for clindamycin in the presence of aminoglycosides. *Antimicrob. Agents Chemother.* **6**:831, 1974.
11. Gerson, B., and Anhalt, J. P. *High-Pressure Liquid Chromatography and Therapeutic Drug Monitoring.* Chicago, American Society of Clinical Pathologists, 1980, Chapter 4.
12. Grove, D. C., and Randall, W. A. *Assay Methods of Antibiotics: A Laboratory Manual* (Antibiotics Monograph 2). New York, Medical Encyclopedia, 1955.
13. Gunn, B. A., Brown, S. L., Otey, C. S., Gaydos, C. A., Keiser, J. F., Meeks, F. A., and Trahan, R. G. Serum gentamicin assay by a radiometric procedure. *Am. J. Clin. Pathol.* **73**:259, 1980.
14. Haas, M. J., and Davies, J. Enzymatic acetylation as a means of determining serum aminoglycoside concentrations. *Antimicrob. Agents Chemother.* **4**:497, 1973.
15. Hermans, P. E., Anhalt, J. P., and Washington, J. A., II. *Pocket Guide to Antimicrobial Agents 1984.* Philadelphia, Centrum Philadelphia, 1984.
16. Hewitt, W. L., and McHenry, M. C. Blood level determinations of antimicrobial drugs. *Med. Clin. North Am.* **62**:1119, 1978.
17. Jolly, M. E., Stroupe, S. D. Schwenzer, K. S., Wang, C. J., Lu-Steffes, M., Hill, H. D., Popelka, S. R., Holer, J. T., and Kelso, D. M. Fluorescence polarization immunoassay. III. An automated system for the therapeutic drug determination. *Clin. Chem.* **27**:1575, 1981.
18. Jolley, M. E., Stroupe, S. D., Wang, C.-H. J., Panas, H. N., Keegan, C. L., Schmidt, R. L., and Schwenzer, K. S. Fluorescence polarization immunoassay. I. Monitoring aminoglycoside antibiotics in serum and plasma. *Clin. Chem.* **27**:1190, 1981.
19. Jones, S. M., Blazevic, D. J., and Balfour, H. H., Jr. Stability of gentamicin in serum. *Antimicrob. Agents Chemother.* **10**:866, 1976.
20. Kabakoff, D. S., Leung, D., and Singh, P. An EMIT assay for gentamicin. *Clin. Chem.* (Abstr.) **24**:1055, 1978.

21. Kavanagh, F. *Analytical Microbiology.* New York, Academic Press, 1963.
22. Kavanagh, F. *Analytical Microbiology,* Vol. II. New York, Academic Press, 1972.
23. Kaye, D., Levison, M. E., and Labovitz, E. D. The unpredictability of serum concentrations of gentamicin: Pharmacokinetics of gentamicin in patients with normal and abnormal renal function. *J. Infect. Dis.* **130**:150, 1974.
24. Lewis, J. E., Nelson, J. C., and Elder, H. A. Radioimmunossay of an antibiotic: Gentamicin. *Nature(London)* **239**:214, 1972.
25. McCracken, G. H., Jr. Pharmacological basis for antimicrobial therapy in newborn infants. *Am. J. Dis. Child.* **128**:407, 1974.
26. McGuire, J. M., Davis, W. W., Parke, T. V., and Daily, W. A. A new linear diffusion method for the microbiological assay of streptomycin and dihydrostreptomycin. *J. Clin. Invest.* **28**:840, 1949.
27. Nilsson, L., Höjer, H., Ansého, S., and Thore, A. A rapid semiautomated bioassay of gentamicin based on luciferase assay of bacterial adenosine triphosphate. *Scand. J. Infect. Dis.* **9**:232, 1977.
28. Noone, P., Parsons, T. M. C., Pattison, J. R., Slack, R. C. B., Garfield-Davies, D., and Hughes, K. Experience in monitoring gentamicin therapy during treatment of serious gram-negative sepsis. *Br. Med. J.* **1**:477, 1974.
29. Noone, P., Pattison, J. R., and Samson, D. Simple, rapid method for assay of aminoglycoside antibiotics. *Lancet* **2**:16, 1971.
30. Pitkin, D., Actor, P., Filan, J. J., White, R., and Weisbach, J. A.: Comparative stability of cephalothin and cefazolin in buffer or human serum. *Antimicrob. Agents Chemother.* **12**:284, 1977.
31. Rammelkamp, C. H. A method for determining the concentration of penicillin in body fluids and exudates. *Proc. Soc. Exp. Biol. Med.* **51**:95, 1942.
32. Rebstock, M. C. Chloramphenicol (chloromycetin). IX. Some analogs having variation of the acyl group. *J. Amer. Chem. Soc.* **72**:4800, 1950.
33. Reeves, D. S., and Holt, H. A. Resolution of antibiotic mixtures in serum samples by high-voltage electrophoresis. *J. Clin. Pathol.* **28**:435, 1979.
34. Reeves, D. S., Phillips, I., Williams, J. D., and Wise, R. *Laboratory Methods in Antimicrobial Chemotherapy.* Edinburgh, Churchill Livingstone, 1978.
35. Rubenstein, K. E., Schneider, R. S., and Ullman, E. F. Homogeneous enzyme immunoassay—A new immunochemical technique. *Biochem. Biophys. Res. Commun.* **47**:846, 1972.
36. Shaw, E. J., Watson, R. A. A., Landon, J., and Smith, D. S. Estimation of serum gentamicin by quenching fluoroimmunoassay. *J. Clin. Pathol.* **30**:526, 1977.
37. Shaw, W. V., Carter, J., and Sachs, J. Enzymatic assay of gentamicin and kanamycin in body fluids. *J. Clin. Res.* **20**:83, 1972.
38. Smith, D. H., Van Otto, B., and Smith, A. L. A rapid chemical assay for gentamicin. *N. Engl. J. Med.* **286**:583, 1972.
39. Stevens, P., and Young, L. S. Simple method for elimination of aminoglycosides from serum to permit bioassay of other antimicrobial agents. *Antimicrob. Agents Chemother.* **12**:286, 1977.
40. Stroy, S. A., and Preston, D. A. Specific assay of aminoglycosidic- or polymyxin-type antibiotics present in human sera in combination with cephalosporins. *Appl. Microbiol.* **21**:1002, 1971.

41. Uhl, J. R., and Anhalt, J. P. High-performance liquid chromatographic assay of vancomycin in serum. *Therapeut. Drug Monitor.* **1**:75, 1979.
42. Washington, J. A., II. *Laboratory Procedures in Clinical Microbiology.* Boston, Little, Brown, 1974.
43. Watson, R. A. A., Landon, J., Shaw, E. J., and Smith, D. S. Polarisation fluoroimmunoassay of gentamicin. *Clin. Chim. Acta* **73**:51, 1976.
44. Williams, J. W., Langer, J. S., and Northrop, D. B. A spectrophotometric assay for gentamicin. *J. Antibiot.* **28**:982, 1975.
45. Wills, P. J., and Wise, R. Rapid, simple enzyme immunoassay for gentamicin. *Antimicrob. Agents Chemother.* **16**:40, 1979.
46. Zaske, D. E., Cipolle, R. J., and Strate, R. J. Gentamicin dosage requirements: wide interpatient variations in 242 surgery patients with normal renal function. *Surgery* **87**:164, 1980.

Section 12.2

Bactericidal Tests

John P. Anhalt, Ph.D., M.D.
John A. Washington II, M.D.

Bactericidal tests may be categorized into those in which a determination is made of (1) the minimal concentration of an antimicrobial that is required to kill an organism (minimal bactericidal or lethal concentration or the MBC or MLC), (2) the greatest dilution of a patient's serum that will inhibit or kill an infecting organism (serum inhibitory or bactericidal titer, or SIT or SBT), and (3) the minimal concentrations of two or more antimicrobials in combination that are required to kill an organism.

It should be emphasized that, with the notable exception of the inhibitory phase (minimal inhibitory concentration or MIC) of the test to determine the MBC, no reference or standard method has been described for performing any of these tests. Considerable variation in methodology, therefore, exists in the literature and must be taken into account when reviewed.

Bactericidal or killing activity is not only a function of the specific antimicrobial and microorganism under study but also of the conditions used for performing the test and the end-point criteria selected for interpretation of the test. Bactericidal effects are generally quantified by determining surviving colony-forming units (CFU) per ml after a specified period of exposure to antimicrobial and comparing this number with those in the original inoculum and, in some instances, concurrently with those grown under identical conditions in the absence of antimicrobial. With the exception of timed killing curves, killing activity is determined after 18 to 24 hr of incubation, and the MBC (or MLC) and SBT are usually arbitrarily defined by the lowest concentration of antimicrobial or greatest dilution of serum killing 99.9% of the original inoculum. In order to determine this level of killing accurately, the initial inoculum

and the subculture volume must be sufficiently large such that there will be several colonies on subculture when only 0.1% of the inoculum survives.[18,19]

I. Minimal Bactericidal Concentration (MBC)

Bactericidal therapy is generally advised for infections of the endocardium and bone and in immunosuppressed hosts; however, actual bactericidal tests of clinical isolates are seldom necessary because of the recognized bactericidal activity of the classes of antibiotics (e.g., β-lactams, aminoglycosides) usually administered in such instances.

Inhibition of growth without lysis, otherwise known as tolerance, may occur in certain species of gram-positive cocci exposed to penicillins and cephalosporins.[23,24,30] Tolerance is manifested when the MBC is at least 32 times the MIC. There is evidence that all clinical isolates of *Staphylococcus aureus* contain some tolerant organisms (0.5% to 50%) and that a bacteriophage may mediate tolerance.[4] Other authors have proposed that the percentage of tolerant organisms in a strain, rather than the mere existence of tolerance, is the controlling variable in determining clinical significance of the phenomenon.[10] What relationship exists between tolerance and persistence[17] remains obscure. Although of uncertain clinical significance, the finding of staphylococcal tolerance to penicillins and cephalosporins[23] has resulted in increased bactericidal testing of staphylococci.

A. Bacteriostatic Phase

1. Preparation of Antimicrobial Dilutions in Broth

a. Thaw the frozen stock solution of antimicrobial agent (1000 μg/ml) and dilute it in Mueller-Hinton broth (MHB) to provide a final concentration of 256 μg/ml. For streptococci, use Todd-Hewitt broth with 0.1% L-cysteine, without dextrose, pH 7.0. For some streptococci, addition of vitamin B_6 (pyridoxal or pyridoxamine phosphate at a final concentration of 10 μg/ml) may be required. For fastidious gram-negative organisms, use Schaedler's or Mueller-Hinton broth with 5% Fildes extract. When testing trimethoprim alone or with mixture of other agents, e.g., trimethoprim/sulfamethoxazole, use MHB with 5% laked horse blood for the antimicrobial-containing medium.
b. Label 12 sterile glass test tubes 1, 2, 3, . . . , 12.
c. Pipette 0.5 ml of MHB into tubes 2 through 12.
d. Into tubes 1 and 2, pipette 0.5 ml of the 256 μg/ml solution of antimicrobial. Mix well and then transfer 0.5 ml of the contents of tube 2

to tube 3 and mix; transfer 0.5 ml from tube 3 to the next tube. Repeat the process through tube 11 but *not* tube 12 (control). This will produce the sequence as follows:

Tube	Antimicrobial (μg/ml)
1	128
2	64
3	32
4	16
5	8
6	4
7	2
8	1
9	0.5
10	0.25
11	0.125
12	0

2. Preparation of Inoculum

a. Portions of four or five isolated colonies of the organism to be tested are inoculated into 5 ml of MHB and incubated at 35°C for 6 hr or until the broth's turbidity matches that of a McFarland no. 1 barium sulfate standard.
b. Dilute the broth culture 1:1000 in MHB.
c. For staphylococci, perform a colony count of the diluted broth culture. Prepare 10-fold dilutions in MHB through 10^{-3}. Transfer 0.1 ml of the 10^{-3} dilution in duplicate to plates containing brain heart infusion agar. Spread the inoculum over the surface of the agar using a bent, sterile glass rod.
d. Inoculate each of the 12 tubes with 0.5 ml of diluted (1:1000) inoculum.

3. Incubation

Incubate all tubes at 35°C for 18 hr, with the exception of tests with staphylococci for which the tubes should be incubated for 24 hr[24] in order to detect tolerance. Incubate all plates at 35°C for 72 hr.

4. Results

a. After 18 hr (24-hr for staphylococci) of incubation, examine the tubes macroscopically. The lowest concentration of antibiotic completely

inhibiting visible growth is the minimal inhibitory concentration (MIC) in $\mu g/ml$.

b. Calculate the colony count of the diluted inoculum. For staphylococci, the count is based on the number of colonies on the subcultures. For other organisms, it may be assumed that the diluted inoculum contained 3×10^5 CFU/ml.

B. Bactericidal Phase

1. Streptococci

a. From each tube without visible turbidity and from the first tube exhibiting turbidity, subculture 0.05 ml of the broth culture (use 1.0-ml calibrated pipettes) into correspondingly labeled tubes containing 10 ml of thioglycollate-135C (BBL Microbiology Systems) broth. For some streptococci, the addition of vitamin B_6 as described above may be required.
b. Incubate the subcultures at 35°C for 72 hr and then examine macroscopically for evidence of growth.
c. The lowest concentration of antimicrobial whose subculture exhibits fewer than 15 discrete colonies or no growth in any of the subcultures is reported as the MBC. Colonies will form discretely because of the small amount of agar in thioglycollate medium.

2. Organisms Growing Readily in Subcultures on Conventional Agar Media

a. From each tube without visible turbidity and from the first tube exhibiting turbidity, subculture 0.1 ml of the broth culture (use 1.0-ml calibrated pipettes) onto the surface of a brain heart infusion (BHI) agar plate.
b. Spread the material evenly over the surface of the BHI agar with a bent glass rod which has been dipped into alcohol and flamed.
c. Incubate the plates for 72 hr (48 hr for *Staphylococcus aureus*) at 35°C.
d. Count the colonies at the end of incubation.
e. The lowest concentration of antimicrobial subculture of which results in 99.9% bactericidal activity is reported as the MBC.
f. For staphylococci calculate the MBC/MIC ratio. Organisms with ratios exceeding 32 are considered tolerant.[23,24]

C. Interpretation of Results

In most instances, the number of colonies decreases to zero or to a level representing ≤0.1% of the original inoculum at one concentration and at all higher concentrations. The breakpoint calculated from the initial inoculum as representing ≤0.1% survival must be interpreted flexibly. For example, if 0.1% survival represents 150 CFU/ml (i.e., an inoculum of 1.5×10^5 CFU/ml), multiple 0.1-ml samples would contain on average 15 CFU. It is unlikely, however, that a single subculture would grow exactly 15 colonies. When the subculture contains more colonies, a decision must be made as to whether that number was likely to occur by chance or truly represent more than 0.1% survival. Similarly, when the subculture has fewer than 15 colonies, the possibility that the sample was from a population with more than 0.1% survivors must be considered. These problems can be addressed by application of the statistics of sampling.[1,18,19] When considered exactly, a range of colony counts is defined as one for which a decision cannot be made with certainty as to whether survival is ≤0.1%. This might complicate reporting.

Because tolerance of staphylococci and most other species except streptococci is of uncertain clinical significance, and because reports of tolerance can lead to a great amount of additional testing and use of secondary or more expensive antimicrobials, overreporting tolerance should be carefully avoided, while underreporting of tolerance is more acceptable. Thus, colony counts less than the breakpoint should be interpreted as less than 0.1% survival. Colony counts greater than the breakpoint (n), but less than $n + 2\sqrt{n}$ (or $n + 3\sqrt{n}$) can also be considered as showing less than 0.1% survival.[1] More exact rejection criteria have been developed by Pearson et al.[19] However, even these criteria should be applied cautiously, because other sources of error, such as inaccuracy in determination of the initial inoculum, drug carryover, and adherence of bacteria to surfaces, are not taken into account.[2,16,29] Even when these criteria are applied, test interpretation can be complicated by "skipped tubes." In those instances, the number of surviving organisms decreases to ≤0.1% of the original inoculum at one concentration and then increases above this level at one or more of the higher concentrations before decreasing to the ≤0.1% level in the remaining higher concentrations. This may often be due to technical problems in the test.[16,29] The single skipped tube should be ignored, and the MBC should be reported as the lowest antimicrobial concentration in the series first yielding ≤0.1% survivors. Multiple skipped tubes present more of a problem to interpret and raise the question of a paradoxical ("Eagle") effect. The MBC is usually defined by the lowest antimicrobial concentration at which there first occurs ≤0.1% survivors. Because of uncertainty about the significance of the paradoxical effect, the percentage killing at the higher concentrations may also be reported.

II. Serum Inhibitory Titer (SIT) and Serum Bactericidal Titer (SBT)

This procedure was initially reported by Schlichter and associates[25,26]; however, it is not commonly appreciated that the serum antibacterial activity originally determined in their studies was the inhibitory activity at the anticipated trough level of antibiotic. It is, therefore, a misnomer to apply the term Schlichter test to tests of serum bactericidal activity at the anticipated peak level of antibiotic. There does exist considerable variation in how the test is performed today. The major variables in the test are the timing of collection of the blood, the diluent (broth or serum) for the serum, the inoculum size, the subculture medium, and the end point. Differences in methodology among published studies of serum antibacterial titers are summarized in Table 12.2–1. Proof of the clinical value of the serum bactericidal titer (SBT) is meager. Despite the fact that the test is most frequently used to monitor the antimicrobial therapy of patients with endocarditis, published data are insufficient to show that the SBT has prognostic value.[6] The most impressive evidence of its value has been in studies of the bacteremia in cancer patients.[14,15,27]

The procedure described below is one that has been in use at the Mayo Clinic for many years. Titers usually represent those obtained at anticipated peak antimicrobial levels in serum, and the diluent used for the serum is broth rather than serum. Although advocates of the use of serum as diluent maintain that the resulting titers will more accurately reflect the effects of protein binding of the antimicrobial,[20,22] it is our contention that the use of serum represents yet another uncontrolled variable in a test already beset with many poorly controlled variables. Some organisms may be sensitive to complement in the serum pool[5] so that titers in the patient's serum may reflect bactericidal activity in a donor's serum rather than that in the patient's serum. Moreover, it has been our experience that commercially available serum not infrequently contains detectable antibiotic or antibiotic neutralizing activity that would certainly confound the determination of antibacterial activity in the patient's serum.

A. Bacteriostatic Phase

1. Preparation of Dilutions of Serum

a. Label 11 sterile glass test tubes 1, 2, 3, . . . , 11.
b. Into tubes 2 through 11, pipette 0.5 ml of Mueller-Hinton broth (MHB). For streptococci, use Todd-Hewitt broth with 0.1% L-cysteine, without dextrose, pH 7.0. For some streptococci, addition of vitamin B_6 (pyri-

Table 12.2–1. Variabilities in Published Serum Antibacterial Tests

Variable	Schlichter et al.[26]	Fisher[8]	Geraci and Martin[9]	Jawetz[11]	Tan et al.[28]	Klastersky et al.[14]	Pien et al.[20]	Klastersky et al.[15]	Reller and Stratton[22]
Timing	Trough	NS[a]	Peak	NS	Variable	Peak and trough	NS	Peak and trough	NS
Inoculum (CFU/ml)	3×10^4	$2 \times 10^{2-3}$	4×10^3	$5 \times 10^{3-5}$	10^6	10^4	10^4	10^5	10^5
Diluent	Broth	Broth	Broth	Broth	Broth	Serum	Serum	Broth	Serum-broth
Phase	Static	Cidal	Cidal	Cidal	Static and cidal	Static and cidal	Cidal	Cidal	Cidal
Subculture volume (ml)	—	NS	NS	0.02	0.01	NS	0.02	NS	0.01
End point (%)	No visible growth	NS	NS	≥99.9	≥99.9	100	≥99.9	NS	≥99.9
Titer	≥1:2	≥1:2	≥1:2	≥1:10	NS	≥1:8 (static)	NS	≥1:8 (peak) ≥1:4 (trough)	NS

[a] NS, not specified.

doxal or pyridoxamine phosphate at a final concentration of 10 μg/ml) may be required. For fastidious gram-negative organisms, use Schaedler's or Mueller-Hinton broth with 5% Fildes extract.

c. Into tubes 1 and 2, pipette 0.5 ml of the patient's serum.
d. Serially dilute the serum by removing 0.5 ml from tube 2 and adding it to tube 3; repeat the process through tube 10 but *not* tube 11 (control). This will produce the sequence as follows:

Tube	Serum
1	Undiluted
2	1:2
3	1:4
4	1:8
—	—
—	—
—	—
10	1:1024
11	0

2. Preparation of Inoculum and Incubation

Use the steps described under I,A,2 and 3, except colony counts are not performed on the inoculum for staphylococci.

3. Results

The highest dilution of serum that completely inhibits visible growth represents the serum inhibitory titer (SIT).

B. Bactericidal Phase

1. Streptococci

Use the procedure under I,B,1.

2. Organisms Growing Readily in Subcultures on Standard Agar Media

Use the procedure under I,B,2.

C. Interpretation of Results

The 99.9% killing breakpoint for all organisms is 15 colonies or less on subculture. As demonstrated in Table 12.2–1, the minimum desirable

titer has varied considerably; however, peak bactericidal titers of ≥1:8 are currently regarded as acceptable.[5,15]

III. Combination Studies

The purpose of studies of antimicrobials in combination is to determine whether their interaction is synergistic, antagonistic, or indifferent. As emphasized in an extensive review of the subject by Rahal,[21] however, clinical evidence for synergy or antagonism between antimicrobial agents remains sparse. The principal indications for testing combinations are to develop our understanding of the mechanisms of antimicrobial action and interaction and to explore potentially useful combinations of drugs for the treatment of organisms which are resistant to single antibiotics.

There are two basic methods for testing drug interaction: the two-dimensional broth dilution ("checkerboard" titration) test and the timed killing curve. In the checkerboard titration, serial twofold dilutions of two drugs, alone and in combination, are inoculated with a standardized inoculum of an organism (Table 12.2–2). The lowest concentrations of each drug in combination that inhibit or kill the organism are plotted as an isobologram (Figure 12.2–1). These results may also be expressed as the fractional inhibitory concentration (FIC) of each drug by dividing the minimal inhibitory concentration (MIC) of drug A in the combination by the MIC of drug A alone.[7,13] The fractional bactericidal concentrations (FBCs) are calculated in a similar manner using MBCs. The advantage of plotting FICs, rather than MICs, is to minimize the effects of biological variation. By drawing intersecting lines through the experimental points, there will be a point at which the FICs reach a minimum (i.e., demonstrate maximal effect) and the sum of which provides the FIC index (Figure 12.2–2). The FIC index equals 1.0 when the drug interaction is additive; the smaller the index, the greater the synergistic interaction. The application of these principles to studying the effects of combinations of more than two drugs has been described in detail by Berenbaum.[3] In general, an FIC index ≤0.7 is equivalent to a threefold reduction in the MIC of each drug.[13]

The other approach to testing drug interaction is the timed killing curve[12] in which a plot is made of the bactericidal effect of each drug alone and in one or more combinations (Figure 12.2–3). The definition of synergy in this test is usually based on at least one $\log_{10}$ reduction of organisms relative to the control within a specified time interval.

Each of these procedures is technically demanding. The checkerboard titration provides quantitative results reflecting drug interaction in a conventional mode (i.e., based on MIC or MBC). The killing curve reflects drug interaction at timed intervals that are frequently shorter than the incubation period for the MIC or MBC. Thus, although both methods

Table 12.2-2. Sample Two-Dimensional Broth Dilution "Checkerboard" Protocol[a]

←——Horizontal——→

←——Vertical——→

	Antibiotic A (μg/ml)										
Antibiotic B (μg/ml)	128	64	32	16	8	4	2	1	0.5	0.25	0[b]
32											
16											
8											
4											
2											
1											
0.5											
0.25											
0[c]											[d]

[a] Each square represents a tube containing antibiotic, singly or in combination.
[b] Control column for antibiotic B.
[c] Control row for antibiotic A.
[d] Control tube for viability; contains no antibiotic.

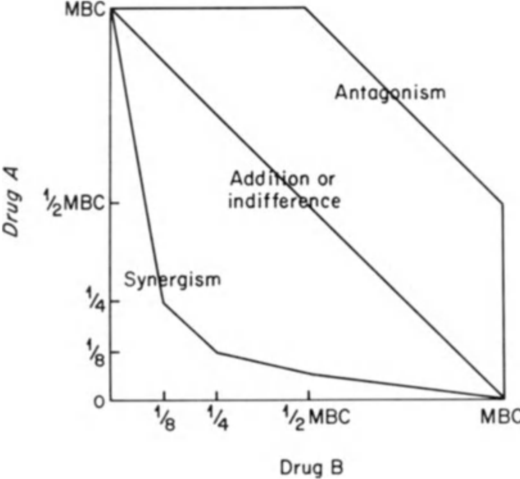


Figure 12.2–1. Isobols representing three types of interactions between drugs A and B. (From Washington, J. A., II. Antimicrobial susceptibility tests. *In* Washington, J. A., II (ed.), *Detection of Septicemia.* West Palm Beach, FL, CRC Press, 1978, p. 101.)

may show the same end result at 18 or 24 hr, the killing curve can show how quickly that end point was reached. For purposes of brevity, only the checkerboard titration will be described.

A. Bacteriostatic Phase

1. Preparation of Antimicrobial Dilutions in Broth

a. Thaw frozen stock solutions of the two antimicrobials to be tested and dilute in broth to yield the desired final concentrations.

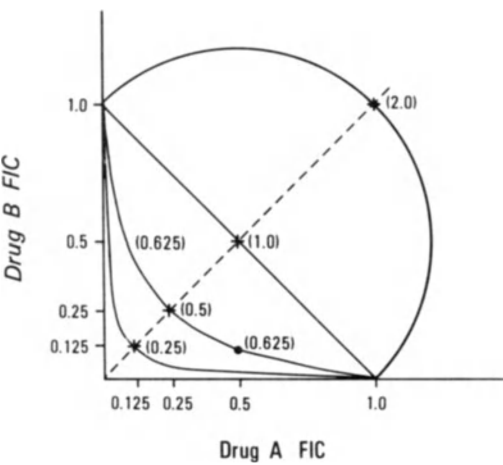


Figure 12.2–2. Inhibitory effect of combination of two agents, expressed as isobols and fractional inhibitory (FIC) indices. FIC index is equal to sum of individual drug FIC values. Points on concave isobols (FIC index <1.0) represent synergism, and points on convex isobols (FIC index >1.0) represent antagonism. The nature of the interaction is adequately revealed by testing combinations lying along dotted line marked +. (From Paisley, J. W., and Washington, J. A., II. *J. Infect. Dis.* **140:**183, 1979.)

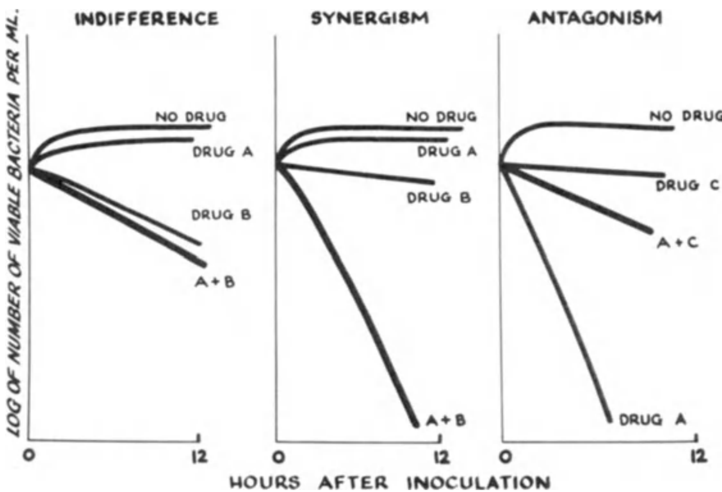


Figure 12.2–3. Schematic representation of bactericidal action in vitro, showing the possible types of results seen when one drug or two drugs act on a homogeneous population of bacteria, under conditions permitting growth. (From Jawetz.[12])

b. Set up a two-dimensional broth dilution checkerboard as illustrated in Table 12.2–2. In order to obtain the desired starting concentrations of each antibiotic (128 μg/ml for A and 32 μg/ml for B), it is necessary to start with concentrations of 512 and 128 μg/ml, respectively, to allow for the subsequent dilutions. Prepare the appropriate serial dilutions of the antibiotics: 1 ml of A at 512 μg/ml is serially diluted to 0.25 μg/ml, and 1 ml of B at 128 μg/ml is serially diluted to 0.25 μg/ml.

c. A rack containing 11 rows of tubes horizontally and 9 columns of tubes vertically is used.

d. To each tube in the first column, add 0.5 ml of A at 512 μg/ml; to each tube in the second column, add 0.5 ml of A at 256 μg/ml; and so on through the tenth column.

e. To each tube in the first horizontal row, add 0.5 ml of B at 128 μg/ml; to each tube in the second horizontal row, add 0.5 ml of B at 64 μg/ml; and so on through the eighth horizontal row.

f. All but one of the tubes in the ninth row will contain only A and all but one of the tubes in the eleventh column will contain B only. To all of the tubes add 0.5 ml of broth. The tube in row 9, column 11 serves as a viability control and it receives 1.5 ml of broth.

2. Preparation of Inoculum

a. Portions of four or five isolated colonies of the organism to be tested are inoculated into broth and incubated overnight.

b. Prepare a 1:1000 dilution of the overnight culture; it should contain 3 to 7 × 10^5 colony-forming unit (CFU)/ml.
c. Add 1.0 ml of inoculum to each tube. The total volume in each tube should be 2.0 ml.

3. Incubation

All tubes are incubated at 35°C for 18 to 24 hr.

B. Bactericidal Phase

1. Subcultures

Follow the procedures described under I,B.

2. Interpretation

a. The MBC is interpreted as described in I,B and I,C.
b. Synergism is established when the FBC index is less than 1.0.[7,13]

C. Modification

In view of the technical complexities involved in performance of synergy studies by the checkerboard technique, it may be desirable, when possible, to abbreviate the procedure by testing a serial dilution of one antibiotic in combination with a fixed concentration of the second antibiotic. Thus, varying concentrations of a penicillin or a cephalosporin, dosages of which may be adjusted considerably, may be tested with a fixed concentration of an aminoglycoside (streptomycin, kanamycin, or gentamicin), dosages of which are limited by toxicity considerations.

References

1. Anhalt, J. P., Sabath, L. D., and Barry, A. L. Special tests: Bactericidal activity, activity of antimicrobics in combination, and detection of β-lactamase production. *In* Lennette, E. H., Balows, A., Hausler, W. J., and Truant, J. P. (eds.), *Manual of Clinical Microbiology,* 3rd ed. Washington, D.C., American Society for Microbiology, 1980, p. 478.
2. Barry, A. L., and Lasner, R. A. In vitro methods for determing minimal lethal concentrations of antimicrobial agents. *Am. J. Clin. Pathol.* **71**:88, 1979.
3. Berenbaum, M. C. A method for testing synergism with any number of agents. *J. Infect. Dis.* **137**:122, 1978.

4. Gradley, H. E., Wetmur, J. G., and Hodes, D. S. Tolerance in *Staphylococcus aureus:* evidence for bacteriophage role. *J. Infect. Dis.* **2**:233, 1980.
5. Bryan, C. S., Marney, S. R., Alford, R. H., and Bryant, R. E. Gram-negative bacillary endocarditis: Interpretation of the serum bactericidal test. *Am. J. Med.* **58**:209, 1975.
6. Coleman, D. L., Horwitz, R. I., and Andriole, V. T. Association between serum inhibitory and bacterial concentrations and therapeutic outcome in bacterial endocarditis. *Am. J. Med.* **73**:260, 1982.
7. Elion, G. B., Singer, S., and Hitchings, G. H. Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. *J. Biol. Chem.* **208**:477, 1954.
8. Fisher, A. M. A method for the determination of antibacterial potency of serum during therapy of acute infections: A preliminary report. *Johns Hopkins Hosp. Bull.* **90**:313, 1952.
9. Geraci, J. E., and Martin, W. J. Antibiotic therapy of bacterial endocarditis. VI. Subacute enterococcal endocarditis: Clinical, pathologic and therapeutic consideration of 33 cases. *Circulation* **10**:173, 1954.
10. Goessens, W. H. F., Fontijne, P., van Raffe, M., and Michel, M. F. Tolerance percentage as a criterion for the detection of tolerant *Staphylococcus aureus* strains. *Antimicrob. Agents Chemother.* **25**:575, 1984.
11. Jawetz, E. The "tough case" of bacterial endocarditis. *Circulation* **20**:430, 1959.
12. Jawetz, E. Combined antibiotic action: some definitions and correlations between laboratory and clinical results. *Antimicrob. Agents Chemother.* **1967**:203, 1968.
13. Kerry, D. W., Hamilton-Miller, J. M. T., and Brumfitt, W. Trimethoprim and rifampicin: In vitro activities separately and in combination. *J. Antimicrob. Chemother.* **1**:417, 1975.
14. Klastersky, J., Daneau, D., Swings, G., and Weerts, D. Antibacterial activity in serum and urine as a therapeutic guide in bacterial infection. *J. Infect. Dis.* **129**:187, 1974.
15. Klastersky, J., Meunier-Carpentier, F., and Prevost, J.-M. Significance of antimicrobial synergism for the outcome of gram-negative sepsis. *Am. J. Med. Sci.* **293**:157, 1977.
16. Lazte, S., Harris, P., and Rolinson, G. N. Factors affecting the apparent regrowth of *Pseudomonas aeruginosa* following exposure to bactericidal concentrations of carbenicillin. *Chemother.* **30**:26, 1984.
17. McDermott, W. Microbial persistence. *Yale J. Biol. Med.* **30**:257, 1958.
18. Miles, A. A., and Misra, S. S. The estimation of the bactericidal power of the blood. *J. Hyg.* **38**:732, 1938.
19. Pearson, R. D., Steigbigel, R. T., Davis, H. T., and Chapman, S. W. Method for reliable determination of minimal lethal antibiotic concentrations. *Antimicrob. Agents Chemother.* **18**:699, 1980.
20. Pien, F. D., Williams, R. D., and Vosti, K. L. Comparison of broth and human serum and the diluent in the serum bactericidal test. *Antimicrob. Agents Chemother.* **7**:113, 1975.
21. Rahal, J. J., Jr. Antibiotic combinations: The clinical relevance of synergy and antagonism. *Medicine* **57**:179, 1978.
22. Reller, L. B., and Stratton, C. W. Serum dilution test for bactericidal activity.

II. Standardization and correlation with antimicrobial assays and susceptibility tests. *J. Infect. Dis.* **136**:196, 1977.

23. Sabath, L. D., Wheeler, N., Laverdiere, M., Blazevic, D., and Wilkinson, B. J. A new type of penicillin resistance of *Staphylococcus aureus. Lancet* **1**:443, 1977.
24. Sabath, L. D. Staphylococcal tolerance to penicillins and cephalosporins. *In* Schlessinger, D. (ed.), *Microbiology—1979.* Washington, D.C., American Society for Microbiology, 1979, p. 299.
25. Schlichter, J. G., and MacLean, H. A method of determining the effective therapeutic level in the treatment of subacute bacterial endocarditis with penicillin: A preliminary report. *Am. Heart J.* **34**:209, 1947.
26. Schlichter, J. G., MacLean, H., and Milzer, A. Effective penicillin therapy in subacute bacterial endocarditis and other chronic infections. *Am J. Med. Sci.* **217**:600, 1949.
27. Sculier, J. P., and Klastersky, J. Significance of serum bactericidal activity in gram-negative bacillary bacteremia in patients with and without granulocytopenia. *Am. J. Med.* **76**:429, 1984.
28. Tan, J. S., Kaplan, S., Terhune, C. A., Jr., and Hamburger, M. Successful two-week treatment schedule for penicillin-susceptible Streptococcus viridans endocarditis. *Lancet* **2**:1340, 1971.
29. Taylor, P. C., Schoenknecht, F. D., Sherris, J. C., and Linner, E. C. Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus:* influence and significance of technical factors. *Antimicrob. Agents Chemother.* **23**:142, 1983.
30. Tomasz, A. From penicillin-binding proteins to the lysis and death of bacteria: A 1979 view. *Rev. Infect. Dis.* **1**:434, 1979.

APPENDIX A

Laboratory Safety

Edward Warren

I. Introduction

Safety is Everybody's Business. It applies to everyone who enters the clinical microbiology laboratory: the director, supervisors, technologists, technicians, secretaries, electricians, custodians, students, and permitted visitors. Each person must be responsible for his own safety and for the safety of others.

Since 1970, employers have had a legal obligation, as well as a personal responsibility, for safety in their places of work. Safety and Health Standards are required by Federal Public Law 91-596 and the Occupational Safety and Health Act (OSHA). Several states also have their own (OSHA) regulations, which by law must be at least as stringent as federal standards. Basically, these regulations require "standard conditions, or the adoption or use of practices, means, methods, operations, or processes, reasonably necessary or appropriate to provide safe or healthful employment and places of employment." These regulations require the employees to familiarize themselves with the provisions of the law and the operating procedures which apply to the work they are engaged in. The law also requires employees to "observe their provisions for [their] own protection and the protection of [their] co-workers."

The laboratory director is directly responsible for safety within the laboratory, including performing risk assessments of the agents and procedures used in the laboratory. The institution must provide the facilities, equipment, and equipment maintenance to ensure a safe working environment. However, the key person in planning, implementing, teaching, and monitoring a good safety program is the laboratory supervisor. The

supervisor is responsible for guaranteeing, on a daily basis, that everyone in the laboratory is properly trained in laboratory safety, observes the rules of the safety program, and uses good common sense, good personal hygiene, and good housekeeping. There should be documentation that the supervisor fulfills these responsibilities. A microbiology laboratory is a place where all people who are there should be properly trained in laboratory safety and where those who are not trained or who are trained but do not observe the rules of safety should not be.

II. Routes of Laboratory-Acquired Infections[15]

Exposure to infectious agents in the laboratory may occur in several ways.

A. Aerosols

Infectious agents may become aerosolized by spillage or breakage of containers and centrifuge tubes or through the use of high-speed blenders. Aerosols may occur on opening evacuated blood collection tubes, specimen containers, and culture tubes or when a needle is withdrawn from a vaccine-stoppered bottle. Aerosols may also occur with pipette spills, by forcing or blowing residual fluid from pipettes, by applying a hot inoculating loop to a culture, and by expelling air bubbles from a syringe containing infectious material.

B. Ingestion

Infectious agents may be accidentally ingested by mouth pipetting, by failing to wash one's hands after handling specimens and cultures, or by smoking, applying cosmetics, or eating in the laboratory.

C. Direct Inoculation

Infectious agents may be inoculated directly by skin puncture with needles or broken glassware, or by scratches or bites of laboratory animals.

D. Direct Contact

Some infectious agents may penetrate unbroken skin or the conjunctiva, or they may enter through small cuts or abrasions in the skin.

E. Vectors

Mosquitoes, ticks, fleas, mites, and other ectoparasites are potential sources of laboratory-acquired infection unless properly contained, whether they are being used in experimental work or happen to be present on animals or in materials brought into the laboratory.

III. Dangerous Chemicals[7]

Dangerous chemicals fall into several categories. Special care must be taken in handling or storing large quantities of these materials, and all containers of chemicals must be properly labeled.

A. Caustic or Corrosive Compounds

Included in this category are acids or bases which may burn or otherwise damage the skin or other human tissue. Such compounds may also cause corrosion of equipment.

B. Poisons

This category includes substances that are sufficiently poisonous that the inhalation or ingestion of relatively small amounts may produce death or other serious effects. Poisons may be in solid, liquid, or gaseous forms.

C. Inflammables

This includes materials that can easily ignite, burn, and serve as fuel for a fire.

D. Explosives

Although many explosive materials are also inflammable, these compounds may explode under special conditions. They must, therefore, be handled in such a way as to eliminate exposure to or attainment of these conditions.

IV. Procedures Following Exposure or Accident

A. General Principles

It is essential that every laboratory develop its own emergency plan which covers circumstances that may arise from the use of hazardous or potentially hazardous materials. When work is hazardous, employees must be well trained in carrying out the laboratory's emergency plan. It is important that everyone in the laboratory be familiar with the potential hazards associated with their work and the emergency plan developed as a safeguard in case of an accident.

B. Specific Incidents

1. Dropped or Spilled Culture

Pour 0.5% sodium hypochlorite (diluted from stock solution daily) over the contaminated area, and cover with paper towels for at least 15 min. Using plastic gloves and paper towels, deposit the contaminated material in covered metal receptacles for autoclaving and place in the autoclave.

2. Dropped or Spilled Fungal Cultures

If a fungal culture suspected of containing a pathogen (e.g., *Coccidioides immitis*) is dropped or spilled, evacuate the room immediately, close the doors to the room involved, and shut off the Mycology Laboratory's ventilating system with the emergency switch. After donning protective clothing and a self-contained breathing apparatus, clean up the spill as described in previous paragraph.

3. Chemical Spills

Chemicals that are potentially toxic and/or inflammable may occasionally be spilled in clinical microbiology laboratories. In order to deal safely with such accidents, laboratory spill kits (J. T. Baker Chemical Co., Fisher Scientific Co.) and self-contained breathing apparatuses (Lab Safety Supply Co., Scott Aviation Co., or local fire department equipment and supply companies) should be used.

4. Puncture Wounds

In the event of accidental puncture with a needle, broken piece of glass, or any object that has been contaminated with a patient's specimen or

culture, immediately scrub the area well with an iodophor handwashing solution and apply tincture of iodine. Then notify the supervisor and document the incident (including name and registration number of patient whose specimen is involved) for the accident report, which must be completed in all such cases. It is recommended that hepatitis B (HB) immune serum globulin be offered to individuals who have had oral or percutaneous exposure to blood or fluids known to be positive for HB_sAg, unless such individuals have had HB infection or vaccine.

5. Centrifuges

In the event of an accident with potentially infectious material in a centrifuge in which safety cups have not been used or in which tubes containing blood are broken, turn off the centrifuge immediately and remove the electrical cord plug from the outlet. Don gloves and mask for cleaning the centrifuge. Remove the carrier, adapter, and broken tube(s) from the centrifuge; pour the blood or other material and pieces of broken glass into a covered pan for autoclaving; place the contaminated parts in a beaker containing sodium hypochlorite and soak for 30 min; remove any glass, and clean the centrifuge parts with a brush and then rinse. Autoclave the parts after they have been exposed to the disinfectant and have been brushed and rinsed. Clean the interior and exterior of the centrifuge with disinfectant, rinse and dry. Wash hands thoroughly and change into a clean laboratory coat.

6. Assistance

When accidents occur that could contaminate an area with dangerous chemicals or infectious agents, it is important to get everyone out of the affected area at once and keep everyone out until there is no doubt about the safety of reentering the area. One must also determine the necessity for treating persons exposed to the dangerous agents. If assistance or additional information is needed in the absence of the laboratory supervisor or director, contact one of the following:

Centers for Disease Control
Attention: Office of Biosafety
Atlanta, GA 30333
Telephone: (404) 329-3883
FTS 236-3883

National Institutes of Health
Attention: Division of Safety
Bethesda, MD 20205
Telephone: (301) 496-1357
FTS 496-1357

National Animal Disease Center
U.S. Department of Agriculture
Ames, IA 50010
Telephone: (515) 862-8258
FTS 862-8258

V. Guidelines for Prevention or Containment of Contamination, Biohazards, and Infectious Agents[5,9-13]

A. Equipment and Facilities

1. Autoclaves

An autoclave must be available and must be operated only by personnel who have been properly trained in its use. Tests for temperature and sterility must be conducted at least once weekly with spore strips or biological indicator ampoules. Each load should be monitored with temperature tape, thermographs, or other means. Heat-resistant gloves must be available for loading and unloading autoclaves.

Malfunction of autoclaves, leading to improperly sterilized media, may preclude isolation of infectious agents from clinical material or provide spurious and misleading results. Inadequate sterilization techniques or time and malfunctioning autoclaves pose hazards to laboratory and other personnel involved in the disposal of laboratory wastes, as well as to the community at large. Minimum sterilization exposure periods are 60 min at 121°C and 15 psi for regular laboratory waste and 90 min at 121°C and 15 psi for material labeled with Creutzfeldt-Jakob precautions.

2. Bench Tops

Bench tops should be impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat. Work areas should be kept clean and free of clutter. This reduces the opportunity for contamination of media and specimens, helps eliminate "false positive" cultures, thereby reducing unnecessary work, and reduces the amount of bacteria-laden airborne dust particles, which may cause laboratory-acquired infection. Bench tops should be decontaminated with 0.5% sodium hypochlorite or a 5% phenolic disinfectant following any spill of potentially infectious material and at the end of each working day.

3. Centrifuges

Tubes should be inspected for cracks and chipping. Aerosol-free (sealed) centrifuge cups are required for processing mycobacteriology, mycology, and virology specimens. Cups should be opened only in a biological safety cabinet. Cups should be kept clean and free of broken glass. A germicidal solution added to the trunnion cup not only serves as a disinfectant in case of breakage but also provides a cushion effect for the tube during centrifugation.

4. Equipment Maintenance

Autoclaves, centrifuges, biological safety cabinets, and fume hoods should undergo regular preventive maintenance by qualified personnel. Air flow rates must be regularly checked in biological safety cabinets and filters changed by qualified personnel when indicated. When filters are changed or when a new biological safety cabinet is installed, the unit must be carefully checked to ensure that there are no air leaks around the edge of the filter before use. Preventive maintenance records must be kept attached to equipment.

5. Floors

Floors should be kept clean and free of clutter. They should be washed with a germicidal solution on a weekly basis and after any spills of infectious material have occurred.

6. Freezers

Freezers used for storage of infectious agents should be monitored for temperature control and connected to a central alarm system in case of power or freezer failure. Freezers should be regularly inspected for the presence of broken vials or tubes containing infectious agents. Broken material should be removed and discarded by a properly attired and gloved individual.

7. Incubators

Incubators may become contaminated in use and thereby pose a hazard to users and to other cultures. The interior of each incubator should, therefore, be cleaned with a germicidal solution on a monthly basis. It is then allowed to air dry for 2 days, washed with soap and water, and again allowed to air dry before use.

Incubators, particularly those used for fungal cultures, may become

infested with mites (*Tyrophagus* spp.), which may migrate from culture to culture, to work areas, to personnel, and to ventilation systems. Often, the first signs of their presence are tracks of bacterial contamination across agar surfaces. Microscopic examination will reveal their presence. Unfortunately, it is necessary to destroy all cultures by autoclaving them and to obtain the services of a professional exterminator as quickly as possible to rid the laboratory and its equipment of mites. Other measures are usually ineffective in eliminating this problem.

8. Pneumatic Tube Systems Used for the Transport of Clinical Specimens[12]

Specimens are placed into leakproof, screw-capped, sterile plastic containers. It is essential that any type of container proposed for specimen transport actually be tested with a water sample in the pneumatic tube for possible leaks, since containers appearing to be leakproof in conventional use may prove not to be under conditions existing within the pneumatic tube. This requirement is often overlooked by cost-conscious purchasing agents when ordering specimen containers but is obviously essential to avoid contamination of either the specimen or the environment.

Specimen containers are double bagged in impervious plastic bags, and the ends of the bags are sealed shut. Red plastic bags are used for containers with specimens from patients suspected or known to have acquired immunodeficiency syndrome (AIDS), Creutzfeldt-Jakob disease (CJD), or hepatitis; these bags are also labeled with "Infection Precautions" and/or "CJD Precautions" stickers.

Bagged specimen containers are placed into a zippered, foam rubber–lined, plastic pouch, which is placed into the pneumatic tube carrier for transport to the laboratory.

The medical technologist, wearing disposable plastic gloves, opens the zippered pouch in a biological safety cabinet. In the event of breakage or leakage, the pouch is autoclaved.

9. Leaking Specimen Containers

Use plastic gloves to handle the material in a biological safety cabinet. Decontaminate the container by placing it in a stainless-steel autoclave pan and pouring 0.5% sodium hypochlorite over it. Transfer the amount of specimen needed for culture to a sterile container and autoclave the remaining material. Contact the ordering physician to report any leaking specimen, which may be contaminated. If another specimen cannot be obtained, proceed with processing. When request forms are contaminated, autoclave them. The report should specify that the specimen was received in a leaking container.

B. Chemicals

1. Inflammables

Small quantities of inflammable materials should be stored in safety cans, while quantities of more than one gallon should be stored in a safety cabinet approved by the National Fire Protection Association. Small quantities of inflammable materials may be stored on open shelves.

2. Storage

Caustic chemicals should be stored at a height not exceeding that of a bench top to reduce the hazard of facial or upper body injury in case of breakage or spillage. In general, only the smallest container compatible with ordinary usage should be stored on open shelves.

3. Transport

Bottle carriers should be used for carrying acids.

4. Ether

Ethyl ether has a flashpoint of −45°C and should not be stored in the refrigerator, including those classified as "explosion-proof." Fumes may accumulate in refrigerators and create a serious risk of explosion if accidentally ignited.

C. Disinfection and Sterilization[2,3]

1. Sterilization

Sterilization signifies destruction of all life and is, for all practical purposes in the laboratory, most effectively accomplished by the use of heat, especially by steam under pressure (autoclaving). Steam sterilization is, however, effective only when properly utilized and is affected by the density, relative size, and position of the load. Despite considerable advances in the design and operation of sterilizers, they should be used only by those trained in their operation and with suitable monitoring devices and controls. The only true indicator of sterilization is the biological type.[2] Indicators with spores of *Bacillus stearothermophilus* are recommended for testing steam sterilizers, while those with spores of *B. subtilis* var. *niger* are used for testing dry heat sterilizers. These indicators are available from several commercial sources and should be used, according to their

manufacturers' directions, on a weekly basis to monitor the performance of sterilizers.

Sterilization without heat is most frequently accomplished in hospitals with ethylene oxide. Radiation is commonly used for sterlization of tissue, foods, fomites, and industrial products. Small volumes of fluids may be sterilized in the laboratory by membrane (usually 0.22 μm) filtration.

2. Disinfectants

There are numerous antiseptics and disinfectants on the market today. The selection of one or more agents for laboratory purposes depends upon the intended application, the types of microorganisms likely to be involved, potentially toxic side effects, and cost. Ordinary soap and water are adequate for routine handwashing purposes; however, in instances in which unusual microbial contamination of the skin may have occurred, washing with an iodophor, which is effective against many groups of infectious agents but is still relatively bland, may be advisable. An iodophor may also be applied to a skin puncture wound or abrasion. Hexachlorophene-containing compounds are effective against staphylococci but not against gram-negative bacilli.

Sodium hypochlorite (freshly prepared 1:10 dilution in water of a 5% solution of household bleach) serves as a useful disinfectant for cleaning inanimate surfaces on a regular basis or in instances of accidental spills of infectious agents. It is presumed to be active against viruses, including those associated with hepatitis and Creutzfeldt-Jakob disease.

Phenolic compounds are also useful for surface disinfection of inanimate objects. Of limited effectiveness are the aqueous quaternary ammonium compounds, and their use should be avoided.

D. Hazardous Infectious Material

1. Aerosols

a. Specimen Containers

Evacuated blood collection tubes and specimen containers should be opened in a biological safety cabinet, as should centrifuge safety cups and high speed blenders. The risk of aerosols is particularly great when breakage of centrifuge tubes has occurred. Lyophilized or frozen cultures should be opened only in a biological safety cabinet. Vials or tubes containing such cultures should be wrapped in cotton soaked with a germicidal solution as they are being broken open, and protective gloves should be worn. When removing infectious material from a vaccine-stoppered bottle, an alcohol-soaked pledget should be placed around the stopper and needle.

b. Syringes and Pipettes

If air needs to be expelled from a syringe containing infectious material, the tip of the needle should be wrapped with an alcohol-soaked pledget, and protective gloves should be worn. Tissue should be ground or homogenized in a biological safety cabinet. Pipettes should be allowed to drain out by gravity flow and should not be used for mixing a culture.

c. Inoculating Loops

Inoculating loops should be allowed to cool before applying them to specimens or cultures. Inoculating loops used for mycobacteria and fungi should be inserted into a small, wide-mouthed jar containing clean sand in phenol before heat sterilizing them. Sterile, disposable inoculating loops and Bacti-Cinerator™ loop and needle sterilizers are available from several commercial sources and are recommended to eliminate dangerous spattering resulting from sterilizing loops and needles in an open flame.

d. Cultures

Cultures yielding mycobacteria, filamentous fungi, or viruses should generally be opened and processed in a biological safety cabinet. The lids of fungal and mycobacterial culture plates should be taped to the bottoms to prevent inadvertent opening. Mycobacterial culture plates are then placed in CO_2-permeable plastic bags and heat-sealed as an added safety measure. Bacterial cultures generally need not be opened and processed in biological safety cabinets, with the exception of cultures for *Bacillus anthracis, Brucella, Francisella tularensis, Legionella, Pseudomonas pseudomallei,* and *Yersinia pestis.* All proficiency testing culture samples should be opened and processed in the biological safety cabinet, since they may contain greater concentrations of organisms than those present in clinical specimens.

e. Tissue Processing

Tissue should be homogenized whenever possible in a Stomacher Lab-Blender (Model no. 80, Tekmar Co., Cincinnati, OH), rather than ground with a mortar and pestle, to eliminate aerosolization.

2. Attire

Laboratory gowns or coats must be worn in the laboratory but should not be worn outside the laboratory and particularly not to eating places. They should be changed daily or when contaminated by spillage of infectious material. Facilities should be available for safely storing personal

effects; purses and outer garments should not be brought into the laboratory. Long hair should be tied back to prevent contact with specimens and cultures. In certain instances masks and gloves should be used for processing particularly hazardous microorganisms[5]; gloves should always be worn in the laboratory when there are fresh cuts or abrasions on hands.

3. Ingestion

Hand-to-mouth transmission of infectious agents should be avoided by frequent handwashing, particularly before eating or drinking. A foot pedal operated dispenser containing an iodophor hand scrubbing solution is highly recommended for this purpose. Hand washing sinks should be equipped with arm-, knee-, or foot-operated controls. Smoking, eating, drinking, and storage of foods or beverages in the laboratory are strictly forbidden, as is mouth pipetting for any purpose.

4. Laboratory Waste

Used pipettes and syringes are placed into a covered metal pan for autoclaving prior to washing for reuse. Waste baskets must be clearly and separately marked for disposal of glass, cloth, and paper to minimize the risk of injury to the custodial staff. Orange or red, waterproof, 6 mil plastic bags are used as waste basket liners for infectious material that is to be disposed of by incineration. Used glass microscope slides are discarded in a jar containing 5% phenol and then autoclaved prior to disposal. Alcohol in jars used for forceps should be replaced daily. Specimens, containers, and used media should be sterilized or disinfected prior to leaving the laboratory to minimize the risk of infection to custodial personnel and sanitary workers. Disposal of laboratory waste must conform to state and federal regulations.

5. Shipment and Distribution of Etiologic Agents[9]

The following code of Federal Regulations (Section 72.25 of Part 72, Title 42) governs the shipment of etiologic agents.

S 72.25 Etiologic Agents

(a) *Definitions* as used in this section:

(1) An "etiologic agent" means a viable microorganism or its toxin which causes, or may cause, human disease.

(2) A "diagnostic specimen" means any human or animal material including, but not limited to, excreta, secreta, blood and its components, tissue, and tissue fluids being shipped for purposes of diagnosis.

(3) A "biological product" means a biological product prepared and manufactured in accordance with the provisions of 9 CFR Part 10, Licensed Veteri-

nary Biological Products; 42 CFR Part 73, Licensed Human Biological Products; 21 CFR 130.3, *New drugs for investigational use in humans;* 9 CFR Part 103, Biological Products for Experimental Treatment of Animals; or 21 CFR 130.3 (a), *New drugs for investigational use in animals,* and which, in accordance with such provisions, may be shipped in interstate traffic.

(b) *Transportation; etiologic agent minimum packaging requirements.* No person may knowingly transport or cause to be transported in interstate traffic, directly or indirectly, any material, including but not limited to diagnostic specimens and biological products containing, or reasonably believed by such person to contain, an etiologic agent unless such material is packaged to withstand leakage of contents, shocks, pressure changes, and other conditions incident to ordinary handling in transportation.

(c) *Transportation; etiologic agents subject to additional requirements.* No person may knowingly transport or cause to be transported in interstate traffic, directly or indirectly, any material, other than diagnostic specimens and biological products, containing, or reasonably believed by such person to contain, one or more of the following etiologic agents unless such material is packaged in accordance with the requirements specified in paragraph (b) of this section, and unless in addition, such material is packaged and shipped in accordance with the requirements specified in other parts of the regulation available on request from the Centers for Disease Control, Atlanta, Ga.

Any etiologic agent that is to be mailed out of the laboratory should be sealed in a screw-capped tube which is placed in a metal can with absorbent material and then packaged in an approved mailing container. Labels designating the package as containing etiologic agents (Figure A–1) should be affixed to the mailing container (Figure A–2). Further information regarding proper packaging, labeling, and mailing of infec-

Figure A.1. Labels to be affixed to mailing container of etiologic agents.

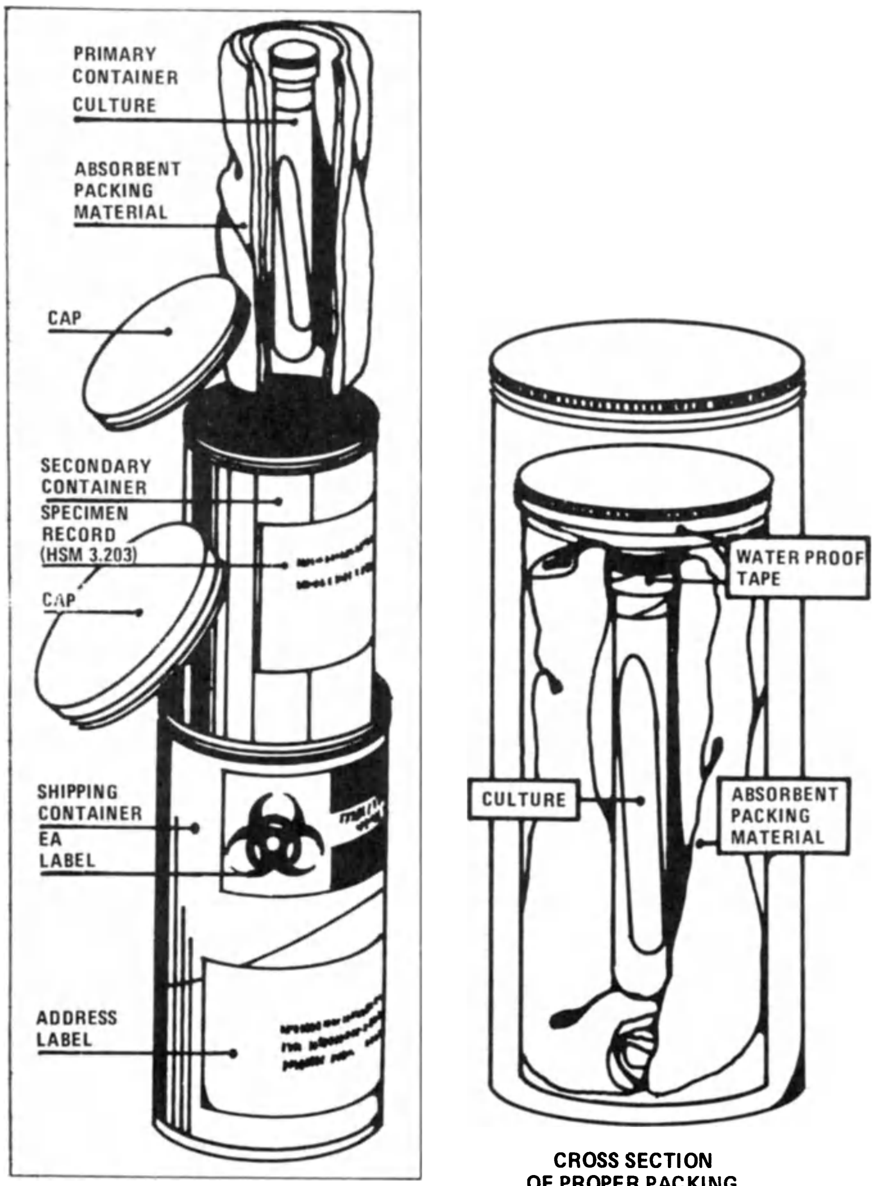


Figure A.2. Diagrams of packaging of etiologic agents in volumes of less than 50 ml.

tious material may be obtained from the Biosafety Office, Centers for Disease Control, Atlanta, GA.

In general, cultures of infectious agents should not be distributed to high school and undergraduate college students. Nonpathogenic microor-

ganisms approved for such use may be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. Advice regarding microbiological studies that are suitable for elementary and secondary schools may be obtained from the American Society for Microbiology.

6. Specimens

Personnel in microbiology laboratories must assume that all specimens received contain potentially infectious material and must, therefore, take all reasonable precautions to minimize the risk of contaminating themselves, others, and the environment with such materials. Instructions regarding specimen transport should be available to physicians, nurses, and other persons submitting material for microbiological examination. Specimen containers should be selected to prevent breakage or leakage, rather than strictly on the basis of price. Certain kinds of specimens warrant special care.

a. Hepatitis

Special care, especially careful handwashing and avoidance of puncture wounds, is important in preventing hepatitis infection. Material contaminated with hepatitis virus is best treated with heat: (1) boiling in water for 10 min, (2) autoclaving at 121°C for 15 min, and (3) dry heat (160°C) for 2 hr.[3] Disinfectants that are presumed to be effective include (1) sodium hypochlorite, 0.5 to 1.0%, for 30 min; (2) 40% aqueous formalin for 12 hr or formalin, 20% in 70% alcohol for 10 hr; (3) alkalinized glutaraldehyde, 2% aqueous, for 10 hr; and (4) ethylene oxide gas sterilization.[3]

b. Transmissible Virus Dementia (Creutzfeldt-Jakob Disease) Precautions[3,8,9]

All specimen containers from patients suspected of having Creutzfeldt-Jakob disease (CJD) should be so labeled and should be considered potentially hazardous. Specimens should be handled with plastic disposable gloves. Simple contamination of the skin by blood or blood products should be dealt with by thoroughly washing the exposed parts with ordinary soap or an iodophor and water. Any instruments of glassware that have come into direct contact with specimens of these patients (e.g., venipuncture needles, test tubes, vials, pipettes, etc.) must be autoclaved for 90 min at 121°C and 15 psi or immersed in 1N sodium hydroxide for 2 hr before being discarded or washed in preparation for use again. Spinal fluid counting chambers should be decontaminated by immersion in 1N sodium hydroxide, phenolic disinfectant, or an iodophor for 2 hr.

Material to be autoclaved, including blood, sera, cerebrospinal fluid,

and brain tissue, should be placed in covered pans used for hazardous infectious material, identified on the outside of the pan by labeling "Creutzfeldt-Jakob Disease Material," and taken directly to the decontamination area.

If a specimen from a patient with CJD or material which has been in contact with such a specimen is dropped or spilled, pour 1N sodium hydroxide over the contaminated area, cover with paper towels, and let stand at least 15 min. Using paper towels and plastic gloves, deposit the contaminated material in the covered pan used for hazardous infectious material to be autoclaved, label "Creutzfeldt-Jakob Disease," and deliver it to the decontamination area.

Great caution must be taken to avoid accidental percutaneous exposure to blood, cerebrospinal fluid, or tissue, particularly brain tissue. If accidental puncture occurs, the wound should be cleaned with 1N sodium hydroxite. The person having the accident, or his/her supervisor, should then contact an environmental medicine or infectious diseases physician.

Alcohol, formaldehyde, β-propiolactone, irradiation, or boiling water are all ineffective against the agent of Creutzfeldt-Jakob disease.

c. Acquired Immune Deficiency Syndrome (AIDS) Precautions[6]

There is presently no evidence of AIDS transmission to hospital or laboratory personnel from contact with affected patients or clinical specimens. Because of concern about a possible transmissible agent, however, interim suggestions are appropriate to guide laboratory personnel.

The following precautions are advised by the Centers for Disease Control for persons performing laboratory tests or studies of clinical specimens or other potentially infectious materials (such as inoculated tissue cultures, embryonated eggs, animal tissue, etc.) from known or suspected AIDS cases:

1. Mechanical pipetting devices should be used for the manipulation of all liquids in the laboratory. Mouth pipetting should not be allowed.
2. Extraordinary care must be taken to avoid accidental wounds from sharp instruments contaminated with potentially infectious material and to avoid contact of open lesions with material from AIDS patients.
3. Needles should not be bent after use, but should be promptly placed in a puncture-resistant container used solely for such disposal. Needles should not be inserted into their original sheaths before being discarded into the container, since this action is a common cause of needle injury.
4. Disposable one-piece syringes and needle units are preferred. If reusable syringes are used, they should be decontaminated before reprocessing.

VI. Environmental Control

A. Work Areas

A minimum of 100 square feet per person should be allowed in microbiology laboratories, including media preparation, sterilization, and office space and taking into consideration all students, residents, secretaries, and professional staff.[1] There should be enough bench top space, in addition to that occupied by instruments, to provide adequate free space for the performance of tests. Bench tops subject to contamination should be covered with nonporous materials and finishes. Each work area should be provided with necessary utilities for efficient operation. Access to work areas should be limited to authorized personnel and should be designed to minimize traffic by others. A warning sign bearing the universal biohazard symbol and "Caution—Biohazard" should be posted on laboratory access doors.

B. Storage Space

There should be sufficient storage space at each work area to stock all necessary glassware, reagents, and supplies to complete the necessary tests. Refrigerated storage space also should be adequate. Records should be stored to facilitate easy retrieval.

C. Ventilation System

The system should provide an adequate amount of fresh air and must be able to remove all toxic and noxious fumes. Temperature and humidity should be adequately controlled.

Ideally, the air pressure in all laboratories should be negative in relation to the pressure in surrounding corridors, thus helping to prevent infectious agents from leaving the work area. Even when cultures are processed under hoods, negative pressure in the general laboratory area relative to that in surrounding corridors is still highly desirable. In addition, doors to all laboratories should be kept closed.

D. Noise

Noise should be kept at an acceptably low level throughout the laboratory to minimize distraction. When feasible, noisy instruments should be

shielded. There should be adequate control of the volume of intercom systems and telephone bells.

E. Fire

Instructions for emergency action in case of fire, including evacuation routes, must be prominently posted and must be required reading for every employee. Fire exists must be clearly marked and frequently inspected to ensure they are not blocked. Sufficient fire extinguishers of a multipurpose dry chemical type must be provided in each work area, and all personnel must be instructed in their use. Accessory firefighting equipment, such as fire hose, fire blankets, and heat-resistant gloves, should be provided as necessary. Automatic sprinkler systems are highly recommended and are required in many areas.

Smoking should be prohibited in laboratories and areas where flammable solvents are being used or stored.

F. Chemical Hazards

Facilities must be provided for rapid flushing of caustic and toxic chemicals from clothing, skin, and eyes. An emergency overhead shower and eyewashing fountain should be immediately available in the laboratory. These flushing facilities should be tested regularly to ensure that they are functioning properly. Safety goggles must be available in the laboratory and workers must use them when indicated. All containers of toxic, caustic, or acidic chemicals must be adequately labeled. It is suggested that before a new employee is introduced to a procedure requiring hazardous chemicals or before a new procedure is introduced to an employee, the following be done:

1. The supervisor should list the compounds to be used (or produced) in the reaction and recommend that each of these be checked in a chemical reference for hazardous properties.
2. The hazardous chemicals, kinds of hazards, and appropriate protective and first aid measures should be discussed in detail with those in the laboratory.

The laboratory should procure and post a wall chart "Emergency Procedures For Dangerous Materials" (Lab Safety Supply Co.) describing health hazards, flammability, reactivity, health hazard ratings, types of hazards, precautions, and fire fighting agents for dangerous liquids, solids, and gases, and the emergency procedures to follow in the event of accident or exposure.

G. Electrical Hazards

All electrical equipment must be grounded, and all personnel should be instructed concerning shock hazards. There should be enough electrical outlets. Gang plugs and extension cords should not be used. Power-failure lights should be installed in areas that would be totally darkened in the event of power failure. Such lights, which are available from commercial sources, are operated by batteries that are kept charged when not in use by being plugged into wall plugs.

VII. Occupational Health

At the Mayo Clinic, the Division of Preventive Medicine provides preemployment physical examinations, immunization programs, and periodic personnel monitoring and health examinations. It also maintains complete medical records of each laboratory employee for occupational or other injury and disease. Immunization is generally recommended for all diseases against which effective, safe, and licensed vaccines have been developed. Women of child-bearing age who are considered for employment in the Virology Laboratory should demonstrate serological evidence of past rubella infection or undergo rubella immunization. The following regular monitoring procedures are used.

1. Fungal Serology

Routinely, all new employees in the mycology and mycobacteriology laboratories have blood drawn for fungal serological tests including tests for *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*. Serological tests are repeated yearly, or more often if there is any accident in the mycology laboratory or if one of the technologists develops an unexplained illness.

2. Tuberculin Skin Testing

Routinely, all new employees in clinical microbiology who are not tuberculin-positive receive the intradermal tuberculin test. This test is repeated yearly.

3. Roentgenograms

Routinely, all new employees have chest roentgenograms. They are repeated yearly on all employees who are tuberculin-positive.

4. Blood Tests for Hepatitis B Virus (HB_sAg)

All new laboratory technologists have blood tests for HB_sAg. Personnel with negative results have the test repeated every 3 months. All new personnel who have frequent contact with blood and other body fluids and who do not have known immunity to hepatitis B (HB) are tested for anti-HB. If negative, such employees are encouraged to receive the HB vaccine.

5. Physical Examinations

Routinely, all new employees have preemployment physical examinations. Appropriate medical follow-up is provided for any person having a positive result in the roentgenogram or skin or blood testing program. Depending on age, employees receive a complete physical examination at 1-, 2-, or 3-year intervals after beginning employment. Female employees have Pap smear and breast examinations annually.

References

1. Bartlett, R. C., Carrington, G. O., and Mielert, C. *Quality Control in Clinical Microbiology,* rev. ed. Chicago, American Society of Clinical Pathologists, Commission on Continuing Education, 1968, 168 pp.
2. Block, S. S. (ed.), *Disinfection, Sterilization, and Preservation,* 3rd ed. Philadelphia, Lea and Febiger, 1983.
3. Bryan, J. A. Recommendations for handling specimens from patients with confirmed or suspected Creutzfeldt-Jakob disease. *Laboratory Medicine* **15**:50, 1984.
4. Center for Disease Control. *Hepatitis Surveillance Report* No. 38. Issued September 1976, p. 21.
5. Center for Disease Control. *Laboratory Safety at the Center for Disease Control* (DHEW Publication No. CDC 76–8118). Atlanta, Center for Disease Control, U.S. Department of Health, Education, and Welfare, 1975.
6. *Morbidity and Mortality Weekly Report.* Centers for Disease Control. Vol. 31:577, 1982.
7. Commission on Inspection and Accreditation. *Microbiology Checklist.* Chicago, College of American Pathologists, 1976.
8. Gajdusek, D. C., Gibbs, C. J., Asher, D. M., Brown, P., Diwan, A., Hoffman, P., Nemo, G., Rohwer, R., and White, L. Precautions in medical care of, and in handling materials from, patients with transmissible virus dementia (Creutzfeldt-Jakob Disease). *N. Engl. J. Med.* **297**:1253, 1977.
9. Richardson, J. H., and Barkley, W. E. (eds.). *Biosafety in Microbiological and Biomedical Laboratories.* Centers for Disease Control and National Institutes of Health, U.S. Department of Health and Human Services, Public Health Service, USHHS Pub. No. (CDC) 84–8395, 1984.

10. Steere, N. V. *CRC Handbook of Laboratory Safety*, 2nd ed. West Palm Beach, FL, Chemical Rubber Company, 1971, 854 pp.
11. Vesley, Donald, *Principles of Biohazard and Injury Control in the Biomedical Laboratory*. Training Course manual. Sponsored by National Cancer Institute and National Institutes of Health. Minneapolis, School of Public Health, University of Minnesota, 1979.
12. Washington, J. A. II, Specimens sent in pneumatic tube systems. *Hospital Infection Control* 9:155, 1982.
13. Wedum, A. G. Laboratory safety in research with infectious aerosols. *Public Health Rep.* 79:619, 1964.

APPENDIX B

Media and Reagents

Pauline K. W. Yu, M.S.

The use of appropriate and dependable media is integral to the isolation and identification of microorganisms. Unfortunately, comparative data documenting the relative efficacy or value of media designed for similar purposes are often lacking. Moreover, one cannot presume identity in composition of a given generic product that is manufactured by several companies because each may supplement the generic products with components, often of a proprietary nature and not specified in the product's labeling. Finally, the actual production of similar products may vary among manufacturers to a sufficient extent to affect their performance. For all of these reasons, therefore, product selection for the laboratory should not be strictly based on cost considerations and should certainly not be based on promotional materials. Evaluations that have been published in the scientific literature should be consulted when available. Alternatively, the prospective buyer should consult a recognized authority in the field.

It is seldom necessary for the laboratory to prepare media using basic components since these are usually available combined in dehydrated form from commercial sources; however, knowledge of a medium's basic components is helpful in understanding how the medium works and what might be wrong when it does not work. Hence, the components have been listed for each medium included in this chapter.

All dehydrated media must be prepared *exactly* according to the manufacturers' directions. Any deviation from these directions may adversely affect or significantly alter a medium's performance. Containers of media should be dated on receipt and when opened, and the media should never be used beyond expiration dates specified by the manufacturers or recommended by quality-control programs.

The media included in this chapter were selected primarily on the basis of efficacy and reliability in use by the authors. This listing is by no means an exhaustive compilation of currently available media. Nearly all the media are available in dehydrated form from commercial sources. Specific brands or commercial sources are cited only when their use is deemed important to the medium's performance.

Also included in the chapter are reagents. Once again, those listed are those in use in this laboratory.

I. Media for Aerobic and Facultatively Anaerobic Bacteria

Acetate Agar

NaCl	5 g
$MgSO_4$	0.2 g
$(NH_4)\ H_2PO_4$	1 g
K_2HPO_4	1 g
Sodium acetate	2 g
Agar	15 g
Bromthymol blue	0.08 g
Distilled water	1000 ml
Final pH 6.7	

This medium is prepared in a similar manner as Simmons' citrate agar. See p. 205 for use of the medium.

Bile-Esculin Agar[1,11]

Bile-esculin agar base (Difco Laboratories)	
Beef extract	3 g
Peptone	5 g
Oxgall	40 g
Ferric citrate	0.5 g
Agar	15 g
Distilled water	1000 ml
Final pH 6.6	
Esculin	1 g

Suspend 63 g of base medium and 1 g of esculin in 1000 ml of distilled water. Heat to boiling to dissolve completely. Dispense into screw-capped tubes and sterilize at 121 °C for 15 min. Let agar solidify in a slant position. See p. 148 for use of the medium.

Blood Agar

Prepare 1000 ml of blood agar base medium—soybean–casein digest [Tryptic Soy Agar (Difco Laboratories), Trypticase Soy Agar (BBL Microbiology Systems), etc.]. Adjust pH to 7.3 Sterilize 121°C for 15 min. Cool to 50°C, and aseptically add 50 ml of sterile defibrinated blood. Mix and pour plates.

This plating medium supports the growth of most medically significant bacteria. It is used for primary plating and for subculturing of colonies and is especially useful for detecting hemolytic activity of bacteria. With certain exceptions, sheep blood is recommended for general use, mainly because colonies of β-hemolytic streptococci show characteristic clear zones on this blood medium. In addition, colonies of streptococci that are neither typically α- nor β-hemolytic on human blood agar appear as typical α-hemolytic colonies after 18 to 24 hr of incubation. One advantage of sheep blood is its inhibitory effect on the growth of bacteria such as *Haemophilus haemolyticus,* the colonial morphology and hemolytic properties of which may resemble those of the β-hemolytic streptococci. In order to isolate *Haemophilus* sp., 5% sterile fresh rabbit or horse blood may be added to the same base in place of the sheep blood. Rabbit and horse blood provide both factors X and V.

Brucella Broth

Polypeptone peptone	20 g
Glucose	1 g
Yeast extract	2 g
NaCl	5 g
$NaHSO_3$	0.1 g
Distilled water	1000 ml
Final pH 7.0.	

Suspend 28 g of dehydrated medium in 1000 ml of distilled water. Heat with agitation until completely dissolved. Sterilize at 121°C for 15 min.

Buffered Charcoal Yeast Extract Agar with α-Ketoglutarate [BCYEα][2,8]-Gibco Laboratories

Basal medium	
Yeast extract	10 g
Ferric pyrophosphate	0.25 g
ACES buffer	10 g

α-Ketoglutarate	1 g
Charcoal, activated	2 g
Agar	15 g
Distilled water	1000 ml

Final pH 6.9 ± 0.1.

Supplement:
10% filter-sterilized solution of L-cysteine·HCl

Suspend 38.3 g of basal medium and approximately 2.8 g of KOH pellets in 1000 ml of water. Heat to boiling with agitation to dissolve. Sterilize at 121°C for 15 min. Allow to cool to 50°C. Add 4 ml of freshly prepared L-cysteine·HCl solution. Adjust pH to 6.9 ± 0.1 with 1 *M* KOH. Pour into petri dishes.

BCYEα Medium with Antimicrobials: BMPAα or BVPAα Agar

Prepare BCYEα medium as described above. Add antimicrobial supplement (cefamandole or vancomycin plus polymyxin B and anisomycin) to agar maintained at 50°C to attain the appropriate final concentration of:

cefamandole	4 μg/ml
or	
vancomycin	0.5 μg/ml
polymyxin B	80 U/ml
anisomycin	80 μg/ml

BCYEα agar is an enriched medium for the isolation of *Legionella* from normally sterile material, e.g., pleural fluid or lung tissue. With 2.5% added glucose, BCYEα agar will support the growth or *Francisella tularensis* better than glucose-cystine blood agar. BMPAα or BVPAα medium facilitates the recovery of *Legionella* from contaminated sources, such as sputum, bronchial washings, or postmortem lung tissues.

Campylobacter (C5) Medium

Brucella agar	43 g
Ferrous sulfate, heptahydrate	0.25 g
Sodium metabisulfite	0.25 g
Sodium pyruvate	0.25 g

Hemin (5 mg/ml)	1 ml
Distilled water	1000 ml
Final pH 7.0	

Antimicrobials to contain final concentration of

Vancomycin	10 μg/ml
Trimethoprim	5 μg/ml
Polymyxin B	8 U/ml
Cefazolin	5 μg/ml

Dissolve the dry ingredients in distilled water with heating and frequent agitation. Boil for approximately 1 min. Sterilize at 121°C for 15 min. Allow to cool to 50°C. Add 50 ml of sterile defibrinated sheep blood, 1 ml of vitamin K_1 solution (10,000 μg/ml) and antimicrobials. Pour into petri dishes. For preparation of hemin and vitamin K_1 solution, see p. 800.

C5 is an enrichment medium containing chemicals (*f*errous sulfate, sodium meta*b*isulfite, and sodium *p*yruvate or FPB supplement) designed to reduce the toxicity of oxygen for *Campylobacter.* It is used for the isolation of *Campylobacter jejuni* from feces, as well as from sites or tissues likely to harbor fecal flora. Since cefazolin does not inhibit *Campylobacter fetus* subsp. *fetus,* C5 medium is also suitable for its isolation.

Carbohydrate Fermentation Broth, Andrade's

a. Broth Base

Peptone	10 g
Meat extract	3 g
NaCl	5 g
Andrade's indicator	10 ml
Distilled water	1000 ml
Final pH 7.6.	

Mix dry ingredients in water and boil to dissolve. Adjust pH to 7.25, then add Andrade's indicator. Dispense in tubes with inverted Durham tubes and sterilize at 121°C for 15 min. Final pH should be 7.6.

Carbohydrates are generally prepared as 10% (w/v) aqueous solutions; less soluble carbohydrate (e.g., dulcitol) is prepared as a 5% solution. Stock solutions are filter sterilized to prevent possible degradation due to autoclaving. They are added to the broth base to achieve a final concentration of 1%. Dulcitol is added to yield a final concentration of 0.5%. See p. 207 for use of the medium.

b. Andrade's Indicator

Acid fuchsin	0.5 g
NaOH, 1*M*	16 ml
Distilled water	100 ml

Dissolve acid fuchsin in distilled water and add NaOH. If the fuchsin is not sufficiently decolorized after several hours, add an additional 1 or 2 ml of the alkali. The amount of NaOH which should be used depends on the dye content of different lots of acid fuchsin. Once decolorization is achieved, let indicator solution stand overnight. Decant off supernatant and discard the sediment. Andrade's indicator improves somewhat on aging and should be prepared 6 months ahead of its anticipated use. The indicator is used in the proportion of 10 ml per liter of medium.

Carbohydrate Fermentation Broth with Brom Cresol Purple

Heart infusion broth	25 g
Brom cresol purple (1.6% in 95% ethyl alcohol)	1 ml
Distilled water	900 ml

Dissolve heart infusion broth in water. Add indicator solution. Dispense 4.5 ml in 16 × 125 mm screw-capped tubes. Autoclave at 121°C for 15 min. When tubes are cooled, aseptically add 0.5 ml of carbohydrate solution to give a final concentration of 1%.

This is a basal medium used in the carbohydrate fermentation test for differentiation of streptococci.

Carbohydrate Utilization Media for Neisseria

a. Serum-Free Medium[3]

(1) Agar Base

GC medium base	36 g
Distilled water	970 ml

Dissolve by boiling, and then add 20 ml of the supplement mixture:

(a) L-Glutamine	1 g
Distilled water	90 ml
(b) $Fe(NO_3)_2$	0.05 g
Distilled water	10 ml
Final pH 7.6.	

(2) Phenol Red Stock Solution

Phenol red	0.2 g
NaOH, O.1 *M*	5.70 ml
Distilled water	94.30 ml

Dissolve dye in NaOH and dilute with water. Add 10 ml of 0.2% phenol red stock solution to the supplemented agar base. Distribute in 90 ml volumes in screw-capped bottles. Sterilize at 121°C for 10 min. Allow to cool to 50°C and add 10 ml of appropriate filter sterilized 10% (w/v) carbohydrate solution to each bottle. Mix well and dispense 3 ml aliquots into sterile small screw-capped vials and slant. See p. 162 for use of the medium.

b. Cystine Trypticase Agar (CTA)

CTA basal medium	28.5 g
Distilled water	1000 ml
Final pH. 7.3.	

Heat to dissolve. Dispense 90 ml amounts in screw-capped bottles. Autoclave at 121°C for 15 min. Cool to 50°C and aseptically add 10 ml of a 10% (w/v) filter-sterilized appropriate carbohydrate solution. Mix well and dispense 5 ml aliquots into small screw-capped vials and allow to harden. See p. 162 for use of the medium.

Cetrimide Medium (Pseudosel Agar)

Gelysate peptone	20 g
$MgCl_2$	1.4 g
K_2SO_4	10 g
Agar	13.6 g
Cetrimide (cetyltrimethylammonium bromide)	0.3 g
Distilled water	1000 ml
Final pH 7.2.	

Dissolve 45.3 g of dehydrated medium in 1000 ml of distilled water by heating. Dispense 6 ml into tubes. Sterilize at 121°C for 15 min. Allow agar to solidify in slant position.

Cetrimide medium is inoculated lightly, with a straight wire, from a young agar slant culture and incubated at 35°C for 7 days. Growth of the organism on this medium is interpreted as a positive result.

Over 95% of strains of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* are cetrimide-tolerant, as are 85 to 90% of *Pseudomonas putida* and 65 to 45% of *Pseudomonas cepacia* and *Pseudomonas pseudoal-*

caligenes. Most strains of other species of *Pseudomonas* are cetrimide-intolerant.

Charcoal Agar[10] (Difco Laboratories)

Beef heart, infusion from	500 g
Peptone	10 g
NaCl	5 g
Soluble starch	10 g
Yeast extract	3.5 g
Activated charcoal (Norit SG)	4 g
Agar	18 g
Distilled water	1000 ml
Final pH 7.3.	

Suspend 62.5 g of dehydrated medium in 1000 ml of distilled water. Heat to boiling with frequent stirring. Dispense 21.5 ml of agar into screw-capped tubes. Sterilize at 121°C for 15 min. Cool and store tubes at 4°C. When needed, melt one tube of charcoal agar and aseptically add 2.5 ml of defibrinated sheep blood and 1 ml of cephalexin (1000 μg/ml). Mix well and pour content into petri dish.

The charcoal neutralizes substances, such as fatty acids and peroxides, that are toxic to *Bordetella pertussis,* and the cephalexin selectively inhibits the growth of bacteria indigenous to the oropharynx.

Chocolate Agar

GC agar base	
Peptone (Polypeptone or Proteose no. 3)	15 g
Corn starch	1 g
K_2HPO_4	4 g
KH_2PO_4	1 g
NaCl	5 g
Agar	10 g
Distilled water	1000 ml
Final pH 7.2.	

Prepare double strength GC agar base by adding 7.2 g to 100 ml of distilled water. Mix well and heat to boiling for 1 min with frequent shaking. Sterilize at 121°C for 15 min. Cool agar to 50°C. Add 2 ml of IsoVitaleX (BBL Microbiology Systems) or 2 ml of supplement B (Difco Laboratories) that has been warmed to room temperature and 100 ml

of 2% hemoglobin solution that is maintained at 50°C. Mix well and pour plates.

Chocolate agar is recommended as a primary plating medium for spinal fluids, eye cultures, gonococcal cultures, and any other specimens that may contain fastidious organisms because it supplies the special growth requirements (X and V factors) of *Haemophilus influenzae* and, when incubated in CO_2, of *Neisseria meningitidis* and *Neisseria gonorrhoeae.*

Citrate Agar (Simmons)

NaCl	5 g
$MgSO_4$	0.2 g
$(NH_4)H_2PO_4$	1 g
K_2HPO_4	1 g
Sodium citrate	2 g
Agar	15 g
Bromthymol blue	0.08 g
Distilled water	1000 ml
Final pH 6.9.	

Dissolve, dispense in tubes, and sterilize at 121°C for 15 min. Allow agar to solidify in a slant position. See p. 193 for use of the medium.

Columbia Colistin–Nalidixic Acid (CNA) Blood Agar

Polypeptone peptone	10 g
Biosate peptone	10 g
Myosate peptone	3 g
Corn starch	1 g
NaCl	5 g
Agar	13.5 g
Colistin	10 mg
Nalidixic acid	15 mg
Distilled water	1000 ml
Final pH 7.3.	

Dissolve 42.5 g of the dehydrated medium in 1000 ml of distilled water with heating and frequent stirring. Boil for 1 min. Sterilize at 121°C for 15 min. Let the medium cool to 50°C, add 50 ml of sterile defibrinated sheep blood. Mix well, and aseptically distribute into petri dishes.

CNA agar is a selective medium for the isolation of staphylococci and streptococci. It inhibits the growth of most gram-negative bacilli; how-

ever, it is useful as a basal medium for the isolation of *Gardnerella vaginalis* from genital material.

Cystine Tellurite Blood Agar

Mueller tellurite base	
Casamino acids, technical	20 g
Casein	5 g
KH_2PO_4	0.3 g
$MgSO_4$	0.1 g
L-Tryptophan	0.05 g
Agar	20 g
Distilled water	1000 ml

Dissolve by boiling 22.5 g of Mueller tellurite base in 500 ml of distilled water. Sterilize at 121°C for 15 min and allow to cool to 50°C. Add 25 ml of sterile, defibrinated rabbit blood, 75 ml of 0.3% sterile potassium tellurite solution, and 22 mg of L-cystine. Swirl the medium while pouring it into petri dishes to keep the cystine suspended. Final pH of the medium is 7.4.

This medium is used for the isolation of *Corynebacterium diphtheriae.* Tellurite inhibits the growth of bacteria indigenous to the oropharynx and imparts the gray or black color characteristic of *C. diphtheriae.*

Decarboxylase Medium (Moeller)

Thiotone peptone	5 g
Beef extract	5 g
Bromcresol purple	15 mg*
Cresol red	5 mg
Glucose	0.5 g
Pyridoxal	5 mg
Agar	3 g†
Distilled water	1000 ml
Final pH 6.0.	

The medium is divided into four 250 ml portions, one of which is tubed for control purposes without the addition of any amino acid. To the re-

* Commercially available dehydrated broth base powder contains 10 mg; an additional 5 mg is added to intensify color change in positive reaction. Dissolve 0.25 g of dye in 46.25 ml of 0.1 *M* NaOH. Add 53.75 ml of distilled water. Use 2 ml for each liter of medium.

† 3 g of agar is added to each liter of broth medium to prepare a semisolid medium.

maining portions of base medium are added L-lysine dihydrochloride, L-arginine monohydrochloride, and L-ornithine dihydrochloride, respectively, to a final concentration of 1%. If DL amino acids are used, they are added to a final concentration of 2%. The pH of the ornithine portion should be readjusted after the addition and prior to sterilization. These media are tubed in 6 ml amounts 16 × 150 mm tubes, properly labeled or color-coded, and sterilized at 121°C for 10 min. On occasion, a small amount of precipitate may be seen in the ornithine medium; however, this does not interfere with the reaction. See p. 196 for use of the medium.

DNase Test Agar

a. For Differentiating Gram-Negative Bacilli

Deoxyribonucleic acid (DNA)	2 g
Phytone peptone	5 g
Trypticase peptone	15 g
NaCl	5 g
Agar	15 g
Toluidine blue O (0.25% aqueous)	40 ml
Distilled water	960 ml
Final pH 7.3.	

Suspend 42 g of the powder in 960 ml of distilled water. Add toluidine blue O and mix thoroughly. Heat with frequent agitation and boil for 1 min. Sterilize at 121°C for 15 min. Cool and pour into petri dishes. See p. 204 for use of the medium.

b. For Differentiating Staphylococci

Prepare agar as in a. above except omit toluidine blue O.

Eosin–Methylene Blue (EMB) Agar, Levine

Peptone (Bacto or Gelysate)	10 g
Lactose	10 g
K_2HPO_4	2 g
Agar	15 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled water	1000 ml
Final pH 7.1.	

Sterilize medium at 121°C for 15 min. Agitate medium frequently while pouring plates.

This differential medium supports the growth of most gram-negative bacilli while inhibiting many of the gram-positive bacteria. It is used both in primary plating and for subculturing (see Tables 3–1, 3–2, 3–4, 3–5, 3–6).

Esculin Agar

Esculin	1 g
Ferric citrate	0.5 g
Heart infusion agar	40 g
Distilled water	1000 ml
Final pH 7.0.	

Sterilize medium at 121°C for 15 min and allow agar to harden in slant position.

Esculin is hydrolized to glucose and esculetin. The latter combines with ferric ion in the medium to form a black complex. Esculin may be hydrolyzed by *Pseudomonas pseudomallei, Pseudomonas cepacia, Pseudomonas putrefaciens,* and *Pseudomonas maltophilia,* but is not hydrolyzed by other species of *Pseudomonas.*

Fletcher's Medium

Peptone	0.3 g
Beef extract	0.2 g
NaCl	0.5 g
Agar	1.5 g
Distilled water	920 ml
Final pH 7.9.	

Dissolve 0.25 g of dehydrated medium in 92 ml of distilled water by heating to boiling. Sterilize at 121°C for 15 min. Cool the medium to 56°C and add 8 ml of reconstituted Leptospira enrichment (Difco Laboratories). Mix well and aseptically dispense 7 ml amounts in screw-capped tubes. Inactivate all the tubes the following day at 56°C for 1 hr.

This medium is used for the isolation, cultivation, and maintenance of *Leptospira.*

Glucose Cysteine Agar

Pancreatic digest of heart muscle	3 g
Papaic digest of soymeal	10 g

NaCl	5 g
L-Cysteine hydrochloride	1 g
Glucose	25 g
Agar	14 g
Thiamine	0.05 mg
Distilled water	1000 ml
Final pH 6.8.	

Suspend 58 g of dehydrated medium in 1000 ml of distilled water. Heat with constant stirring and boil for 1 min. Sterilize at 118 to 121°C for 20 min. Cool the medium to 50°C, and aseptically add 50 ml of defibrinated rabbit blood. Also add 1 ml each of penicillin (100,000 U/ml), polymyxin B sulfate (100,000 U/ml), and cycloheximide (100 μg/ml). Mix well and pour into plates.

This medium is used for the recovery of *Francisella tularensis* from contaminated specimens.

GN (Gram-Negative) Broth

Glucose	1 g
D-Mannitol	2 g
Sodium citrate	5 g
Sodium deoxycholate	0.5 g
K_2HPO_4	4 g
KH_2PO_4	1.5 g
NaCl	5 g
Tryptose	20 g
Distilled water	1000 ml
Final pH 7.0.	

Dissolve ingredients by heat. Dispense in tubes and sterilize at 116°C for 15 min.

This is an enrichment medium for the isolation of *Salmonella* and *Shigella* and is recommended by many for the isolation of *Shigella.* It is inhibitory to gram-positive organisms; coliforms are usually inhibited up to 6 hr.

Heart Infusion Agar

Beef heart, infusion from	500 g
Tryptose	10 g
NaCl	5 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.4.	

Suspend 40 g of dehydrated medium in 1000 ml of distilled water. Heat to boiling to dissolve completely. Sterilize at 121°C for 15 min.

Heart Infusion Broth

This is similar to heart infusion agar with omission of the agar.

Hektoen Enteric (HE) Agar

Lactose	12 g
Sucrose	12 g
Salicin	2 g
Bile salts no. 3	9 g
Proteose peptone	12 g
Beef extract	3 g
NaCl	5 g
Sodium thiosulfate	5 g
Ferric ammonium citrate	1.5 g
Agar	14 g
Thymol blue	0.065 g
Acid fuchsin	0.1 g
Distilled water	1000 ml

Final pH 7.5.

Suspend 76 g of dehydrated medium in 1000 ml of water and boil until it has completely dissolved. Do not autoclave this medium. Cool and pour plates.

This selective medium is used for the isolation and identification of enteric pathogens (Table 3–6).

Human Blood Tween 80 (HBT) Medium

1. Base layer:	
Columbia CNA agar (Difco Laboratories)	44 g
Proteose peptone #3	10 g
Distilled water	1000 ml
2. Overlay:	
Columbia CNA agar	66 g
Proteose peptone #3	15 g
Distilled water	1500 ml
3. Tween 80, 20% aqueous solution	
4. Human whole blood	

Sterilize base layer and top layer at 121°C for 15 min. Let agars cool to 50°C. Aseptically add 0.4 ml of Tween 80 to base layer. Dispense 7 ml per petri dish and allow to solidify. Add 0.6 ml of Tween 80 and 90 ml of human whole blood to overlay. Mix well and dispense 15 ml over the base layer.

This is a satisfactory medium for the isolation of *Gardnerella vaginalis*. The bilayer composition and Tween 80 enhance the α-hemolytic activity of the organism on human blood. Amphotericin B can be added to a final concentration of 2 μg/ml to inhibit the growth of yeasts.

Indole Broth

Peptone (Bacto or Trypticase)	15 g
NaCl	5 g
Distilled water	1000 ml
Final pH 7.1.	

Dispense 2 ml into 13 × 100 mm screw-capped tubes. Sterilize at 121°C for 15 min. See p. 198 for use of the medium.

Loeffler Medium

Veal infusion broth	25 g
Glucose	10 g
Distilled water	1000 ml

Add dry ingredients to water. Heat to dissolve and bring to a boil. Dispense 50 ml amounts in screw-capped bottles. Add five pieces of $CaCO_3$ chips to each bottle and sterilize at 121°C for 15 min. Cool broth to 50°C and add 150 ml of sterile horse serum to each bottle. Mix well and aseptically dispense 5 ml amounts into sterile 16 × 125 mm screw-capped tubes. Slant tubes in a metal rack and inspissate medium at 87°C for 30 min in a sterilizer with an isothermal control.

Lysine-Iron Agar

Peptone (Bacto or Gelysate)	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine	10 g
Ferric ammonium citrate	0.5 g

Sodium thiosulfate	0.04 g
Bromcresol purple	0.02 g
Agar	15 g
Distilled water	1000 ml

Final pH 6.7.

Dispense into screw-capped tubes and sterilize at 121°C for 15 min. Tubes should be slanted to obtain a deep butt and a short slant. See p. 192 for use of the medium.

MacConkey Agar

Peptone (Bacto or Gelysate)	17 g
Peptone (Proteose or Polypeptone)	3 g
Lactose	10 g
Bile salts	1.5 g
NaCl	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1000 ml

Final pH 7.1.

Sterilize at 121°C for 15 min.

Lactose-fermenting colonies are red, and may be surrounded by an opaque zone of precipitated bile. This is due to the action of acids on the bile salts and the subsequent absorption of neutral red. Colonies of nonfermenters are colorless and transparent. This differential medium supports the growth of most gram-negative bacilli and may be used interchangeably with eosin-methylene blue agar.

Malonate Broth, Ewing Modified

Yeast extract	1 g
$(NH_4)_2SO_4$	2 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
NaCl	2 g
Sodium malonate	3 g
Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1000 ml

Final pH 6.7.

Sterilize at 121°C for 15 min. Dispense 1 ml in 13 × 100 mm screw-capped tubes. See p. 206 for use of the medium.

Motility Medium

Casitone	10 g
Yeast extract	3 g
NaCl	5 g
Agar	3 g
Distilled water	1000 ml
Final pH 7.2.	

Mix dry ingredients in water, heat to a boil. Dispense 6 ml per tube. Sterilize at 121°C for 15 min. See p. 174 for use of the medium.

MR-VP Broth

Peptone (Buffered or Polypeptone)	7 g
Glucose	5 g
K_2HPO_4	5 g
Distilled water	1000 ml
Final pH 6.9.	

Sterilize at 121°C for 15 min. Dispense in 0.5 ml amounts in 13 × 100 mm screw-capped tubes.

Although several modifications of the Clark and Lubs formula are available, the one given above has been used satisfactorily in this laboratory. See p. 201 for use of the medium.

Mueller-Hinton Agar

Beef, infusion from	300 g
Peptone (Acidicase or Bacto-Casamino Acids, Technical)	17.5 g
Starch	1.5 g
Agar	17 g
Distilled water	1000 ml
Final pH 7.4.	

Mix the medium thoroughly in distilled water, with heat and frequent agitation. Boil for approximately 1 min and dispense. Sterilize by autoclaving at 121°C for not more than 15 min.

This medium, which was designed for the primary isolation of *Neisseria gonorrhoeae* and *Neisseria meningitidis*, is most frequently used for the determination of antimicrobial susceptibility by the disk or agar-dilution method. Because it does not inhibit drug action, susceptiblity tests of sulfonamide and trimethoprim should be determined on this medium.

Mueller-Hinton Broth

This is similar to Mueller-Hinton agar in formulation, with the omission of agar. It is used primarily for antimicrobial susceptibility and should be supplemented with 50 mg of Ca^{2+} and 25^{2+} mg of Mg per liter of medium.

Nitrate Broth

Nutrient broth base	8 g
KNO_3	1 g
Distilled water	1000 ml
Final pH 7.0.	

Stir until dissolved. Bring to a boil. Dispense 2 ml amounts in 13 × 100 mm screw-capped tubes. Sterilize at 121°C for 15 min. See p. 221 for use of the medium.

Nalidixic Acid–Polymyxin–Crystal Violet (NPC) Broth

Todd-Hewitt broth	15 g
Distilled water	500 ml

Dissolve dehydrated medium in water and bring to a boil. Sterilize at 121°C for 15 min. Let medium cool to room temperature and aseptically add

Nalidixic acid, 0.75 ml at 10,000 μg/ml
Polymyxin B, 5 ml at 1000 U/ml
Crystal violet, 0.5 ml at 100 μg/ml prepared in methyl alcohol

Mix content of flask thoroughly. Adjust pH to 7.4. Aspetically dispense 3 ml amounts into 16 × 125 mm screw-capped tubes.

This is a selective medium used for the isolation of group B *Streptococcus* from contaminated sites.

Nutrient Agar

Beef extract	3 g
Peptone (Bacto or Gelysate)	5 g
Agar	15 g
Distilled water	1000 ml
Final pH 6.8.	

Dissolve dry ingredients in water with heat and frequent agitation. Dispense and sterilize at 121°C for 15 min.

Nutrient Broth

This is similar to nutrient agar with the omission of agar.

Oxidation-Fermentation (O-F) Medium (Hugh and Leifson)

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	2.5 g
Bromthymol blue	0.03 g
Distilled water	1000 ml
Final pH 7.1.	

Distribute basal medium in bottles and sterilize at 121°C for 15 min. Cool medium to 50°C and add appropriate filter-sterilized carbohydrate solutions to give a final concentration of 1%. Mix well and aseptically dispense 5 ml amounts into 16 × 125 mm screw-capped tubes. See p. 219 for use of the medium.

Phenylalanine Agar

Yeast extract	3 g
DL-Phenylalanine	2 g
(or L-phenylalanine)	(1 g)

K_2HPO_4	1 g
NaCl	5 g
Agar	12 g
Distilled water	1000 ml
Final pH 7.3.	

Dispense and sterilize at 121°C for 10 min. Allow to solidify as a long slant. See p. 203 for use of the medium.

Phenylethyl Alcohol (PEA) Blood Agar

Typticase peptone	15 g
Phytone peptone	5 g
NaCl	5 g
Phenylethyl alcohol	2.5 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.3.	

Sterilize at 121°C for 15 min and cool to 50°C. Aseptically add 50 ml of defibrinated sheep blood, mix well, and pour into petri dishes. PEA is used for the selective isolation of gram-positive cocci, either from clinical specimens or by subculture of primary plating media. *Pseudomonas aeruginosa,* however, is not inhibited. Hemolysis cannot be reliably determined on this medium; therefore, streptococci should be subcultured onto sheep blood agar.

Phosphate-Buffered Saline (PBS), pH 7.2, for Legionella

0.15 *M* KH_2PO_4 stock solution	
KH_2PO_4	20.41 g
Distilled water	1000 ml
0.15 *M* Na_2HPO_4 stock solution	
Na_2HPO_4, anhydrous	21.29 g
Distilled water	1000 ml
PBS, pH 7.2	
KH_2PO_4 stock solution	24 ml
Na_2HPO_4 stock solution	76 ml
NaCl	1.7 g
Distilled water	100 ml

Dispense 5 ml amounts into screw-capped tubes and sterilize at 121°C for 15 min.

This is a diluent used for preparing a suspension of lung tissue for isolation of *Legionella.*

Pigment Production Media

Pseudomonas agar P or Tech agar	
Peptone (Bacto or Gelysate)	20 g
Glycerol (C.P.)	10 ml
$MgCl_2$	1.4 g
K_2SO_4	10 g
Agar	13.6 g
Distilled water	1000 ml
Final pH 7.2.	

Pseudomonas agar F or Flo agar	
Peptone (Proteose no. 3 or Polypeptone)	20 g
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g
Agar	14 g
Distilled water	1000 ml
Final pH 7.2.	

Tube and sterilize at 121°C for 15 min. Slant tubes so as to obtain a deep butt. See p. 222 for use of these media.

Potassium Tellurite Agar

Heart infusion agar	20 g
Distilled water	500 ml
Final pH 6.0.	

Dissolve heart infusion agar in water with heat. Autoclave at 121°C for 15 min and maintain at 50°C. Aseptically add 25 ml of defibrinated sheep blood, and 75 ml of filter-sterilized potassium tellurite solution (0.25 g in 75 ml of water). Mix well and dispense into petri dishes.

This medium is used to distinguish between *Streptococcus faecalis* and other group D streptococci. *S. faecalis* is the only group D streptococcal species that can tolerate tellurite.

Purple Agar (Difco Laboratories)

Proteose peptone #3	10 g
Beef extract	1 g
NaCl	5 g
Agar	15 g
Brom cresol purple	0.02 g
Distilled water	1000 ml
Final pH 6.8.	

Dissolve 31 g of purple agar in water with heat. Autoclave at 121°C for 15 min.

This is a basal medium used in the carbohydrate fermentation test for speciation of staphylococci. Filter-sterilized carbohydrate solution is added to sterile medium maintained at 50°C to a final concentration of 1%.

Rogosa SL Agar

Tryptone	10 g
Yeast extract	5 g
Glucose	10 g
Arabinose	5 g
Sucrose	5 g
Sodium acetate	15 g
Ammonium citrate	2 g
KH_2PO_4	6 g
$MgSO_4 \cdot 7\ H_2O$	0.57 g
$MnSO_4$	0.12 g
$FeSO_4$	0.03 g
Sorbitan monooleate	1 g
Agar	15 g
Distilled water	1000 ml
Final pH 5.4.	

Add dry ingredients to distilled water and heat to boiling to dissolve the solids completely. Add 1.32 ml of glacial acetic acid and continue boiling for 2 to 3 min. *Do not autoclave.* Cool to 50°C and pour plates.

This selective medium is useful in the cultivation and presumptive identification of lactobacilli.

Salmonella-Shigella (SS) Agar

Beef extract	5 g
Peptone (Polypeptone or Proteose)	5 g
Lactose	10 g
Bile salts	8.5 g
Sodium citrate	8.5 g
Sodium thiosulfate	8.5 g
Ferric citrate	1.0 g
Agar	13.5 g
Brilliant green	0.33 mg
Neutral red	0.025 g
Distilled water	1000 ml
Final pH 7.0.	

Heat to boiling for 2 to 3 min to dissolve completely. Do not autoclave. Dispense medium into petri dishes after it has cooled to 50°C.

SS agar is an inhibitory medium used for the isolation of *Salmonella* and *Shigella.*

Selenite Broth

Peptone (Tryptone or Polypeptone	5 g
Lactose	4 g
Na_2HPO_4	10 g
Sodium acid selenite	4 g
Distilled water	1000 ml
Final pH 7.0.	

Dissolve ingredients in water and bring to a boil. Boil for 1 min. Do not autoclave. Dispense 5 ml into sterile screw-capped tubes. Store tubes at 4°C until use.

This is an enrichment medium for the isolation of *Salmonella* and *Shigella* (Table 3–6). Selenite and GN broths provide roughly comparable isolations of *Shigella* when subcultured onto a variety of plating media; however isolations of *Salmonella* tend to be greater from selenite broth.

SF Medium (Difco Laboratories)

Tryptone	20 g
Dextrose	5 g

K_2HPO_4	4 g
KH_2PO_4	1.5 g
NaCl	5 g
NaN_3	0.5 g
Brom cresol purple	0.032 g
Distilled water	1000 ml
Final pH 6.9.	

Dissolve 36 g of dehydrated medium in 1000 ml of water. Dispense 5-ml amounts in screw-capped tubes. Autoclave at 121°C for 15 min.

This is a selective medium for the identification of enterococci.

Sodium Bicarbonate Agar

Soybean-casein digest agar	90 ml
$NaHCO_3$, 7% aqueous solution	10 ml

Add 10 ml of filter-sterilized $NaHCO_3$ solution to 90 ml of sterile soybean-casein digest agar that is maintained at 50°C. Mix well and pour into petri dishes.

This medium is used to enhance the capsule production by *Bacillus anthracis.*

Sodium Chloride Broth, Modified[1]

Heart infusion broth (dehydrated)	25 g
NaCl	60 g
Glucose	10 g
Bromcresol purple, 1.6% in 95% ethyl alcohol	1 ml
Distilled water	1000 ml

Dispense in 5 ml aliquots in screw-capped tubes. Autoclave at 121°C for 15 min. See p. 150 for use of this medium.

Soybean–Casein Digest Agar (Tryptic Soy, Difco Laboratories, or Trypticase Soy, BBL Microbiology Systems)

Pancreatic digest of casein	15 g
Papaic digest of soy meal	5 g
NaCl	5 g

Agar	15 g
Distilled water	1000 ml
Final pH 7.3.	

Sterilize at 121°C for 15 min.

Soybean–Casein Digest Broth (Tryptic Soy, Difco Laboratories, or Trypticase Soy, BBL Microbiology Systems)

Pancreatic digest of casein	17 g
Papaic digest of soy meal	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Distilled water	1000 ml
Final pH 7.3.	

Dissolve, dispense in tubes, and sterilize at 121°C for 15 min.

This medium is a general purpose nutrient broth that is particularly useful for growing streptococci. It is also suitable for blood cultures and supports the growth of a variety of aerobic, facultatively anaerobic, and anaerobic bacteria.

Sucrose Agar

Heart infusion agar	40 g
Sucrose	50 g
Distilled water	1000 ml

Dissolve heart infusion agar in water with heat. Autoclave at 121°C for 15 min. Dispense in petri dishes.

This medium is used for the identification of streptococci.

Sucrose Broth

Basal medium

NIH thioglycollate broth (Difco Laboratories)	28.5 g
K_2HPO_4	10 g
Sodium acetate	12 g
Distilled water	500 ml

Sucrose solution	
Sucrose	50 g
Distilled water	500 ml

Autoclave basal medium and sucrose solution separately at 121°C for 15 min. Mix solutions when they are cooled to 50°C. Dispense 5-ml amounts into screw-capped tubes.

This medium is used for the identification of streptococci.

Thayer-Martin Medium, Modified[6,7,12]

GC agar base (double strength)	100 ml
Hemoglobin, 2% aqueous	100 ml
IsoVitaleX enrichment (BBL Microbiology Systems) or Supplement B (Difco Laboratories)	2 ml
Antimicrobials, to contain final concentration of	
Vancomycin	3 μg/ml
Colistin	7.5 μg/ml
Nystatin, *or*	1.25 U/ml
anisomycin	20 μg/ml
Trimethoprim lactate	5 μg/ml
Glucose	2 g

Add hemoglobin solution, supplement, and antimicrobials to sterile GC agar base that is maintained at 50°C. Mix well and pour plates.

This medium or one of its modifications is recommended for the selective isolation of *Neisseria gonorrhoeae* and *Neisseria meningitidis* from genital and/or respiratory tract sources.

TM2, TM4 Media

Two modifications of Thayer-Martin (TM) Medium are acceptable alternatives and can be conveniently prepared in a biplate. Incorporation of lincomycin instead of vancomycin in TM medium is especially useful for the isolation of vancomycin-susceptible strains of *N. gonorrhoeae.*

TM2 Medium

1. GC agar base	36 g
Agar	3 g
Distilled water	500 ml

Sterilize at 121°C for 15 min.

2. Hemoglobin (Difco Laboratories)	10 g
Distilled water	500 ml

Stir to dissolve on a magnetic stirrer. Sterilize at 121°C for 20 min.

3. Antimicrobials to contain final concentration of

Lincomycin	2 μg/ml
Colistin	7.5 μg/ml
Anisomycin	10 μg/ml

Mix sterile GC agar base with hemoglobin solution and cool to 50°C. Aseptically add 2 ml of IsoVitaleX enrichment and antimicrobials.

TM4 Medium

Basal medium is similar to TM2 medium except that 2.5 g of glucose and 7 g of agar are added, and the antimicrobial mixture contains final concentrations of vancomycin, 3 μg/ml; colistin, 7.5 μg/ml; anisomycin, 10 μg/ml; and trimethoprim, 5 μg/ml. Since anisomycin solution is unstable, it should be prepared the day it is to be incorporated in the medium.

Thioglycollate-135C (BBL Microbiology Systems)

Trypticase peptone	17 g
Phytone peptone	3 g
Glucose	6 g
NaCl	2.5 g
Sodium thioglycollate	0.5 g
Agar	0.7 g
L-Cystine	0.25 g
Na_2SO_3	0.1 g
Distilled water	1000 ml
Final pH 7.0.	

Dispense 10 ml in screw-capped test tubes and sterilize at 121°C for 15 min.

This thioglycollate medium does not contain an E_h indicator. It is able to support the growth of a wide variety of aerobic and anaerobic bacteria and is highly recommended for use as a general utility broth in the clinical laboratory.

Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar

Yeast extract	5 g
Polypeptone peptone	10 g
Sodium citrate	10 g
Sodium thiosulfate	10 g
Oxgall	5 g
Sodium cholate	3 g
Sucrose	20 g
NaCl	10 g
Ferric citrate	1 g
Bromthymol blue	0.04 g
Thymol blue	0.04 g
Agar	14 g
Distilled water	1000 ml

Final pH 8.6.

Dissolve dehydrated medium in distilled water by heating to boiling for 1 min; do not autoclave. Cool to 50°C, and pour plates.

This medium is recommended for the selective isolation of *Vibrio* (Table 3–6).

Todd-Hewitt Broth

Beef heart, infusion from	500 g
Neopeptone	20 g
NaCl	2 g
Na_2CO_3	2 g
Na_2HPO_4	0.4 g
Glucose	2 g

Final pH 7.8.

Bring ingredients to a slow boil and boil for 15 min. Dispense in tubes, and autoclave at 121°C for 15 min.

This is an enriched medium for the cultivation of streptococci. An additional 8 g/liter of glucose can be added to enhance growth of streptococci for antigen preparation in Lancefield's grouping (p. 144). Cysteine, 1 g/liter, can also be added to the basal medium for susceptibility testing of streptococci in broth.

Toxigenicity Test Agar

Bacto-KL Virulence Agar (Difco Laboratories)	37.5 g
Distilled water	1000 ml

Suspend the dehydrated medium in distilled water and dissolve by boiling. Dispense molten agar in 15 ml amounts in screw-capped tubes and sterilize at 121°C for 15 min. When ready for use, supplement each 15 ml of cooled (50°C) basal medium with 3 ml of Bacto-KL Virulence Enrichment and 0.75 ml of filter sterilized potassium tellurite, 1% solution. Mix and pour into petri dish.

This medium is used for the in vitro toxigenicity test of *Corynebacterium diphtheriae* (p. 169).

Triple Sugar–Iron Agar (TSIA)

Beef extract	3 g
Yeast extract	3 g
Peptone (Bacto)	15 g
Peptone (Proteose)	5 g
Lactose	10 g
Sucrose	10 g
Glucose	1 g
$FeSO_4$	0.2 g
NaCl	5 g
Sodium thiosulfate	0.3 g
Agar	15 g
Phenol red	0.024 g
Distilled water	1000 ml
Final pH 7.4.	

Sterilize at 121°C for 15 min. The medium is slanted with a deep butt. See p. 191 for use of the medium.

Urea Agar (Christensen)

a. Base Medium

Peptone (Bacto or Gelysate)	1 g
NaCl	5 g
Glucose	1 g
KH_2PO_4	2 g
Phenol red	0.012 g
Urea	20 g
Distilled water	100 ml

b. Agar

Agar	15 g
Distilled water	900 ml

c. Complete Medium

Dissolve 29 g of the base medium in 100 ml of distilled water. Adjust pH to 6.7–6.8. Filter sterilize. Dissolve 15 g of agar in 900 ml of distilled water. Sterilize at 121°C for 15 min. Cool to 50°C and aseptically add the base medium. Mix well and dispense into screw-capped tubes. Slant tubes with a deep butt. Final pH of the complete medium should be 6.8 to 6.9. See p. 194 for use of the medium.

Veal Infusion Broth

Veal heart, infusion from	500 g
Proteose peptone no. 3	10 g
NaCl	5 g
Distilled water	1000 ml
Final pH 7.4.	

Dispense 5 ml aliquots into screw-capped tubes. Autoclave at 121°C for 15 min.

This medium is used to grow fastidious bacteria.

"W" (Wisconsin) Medium[5,14]

To 200 ml of sterile heart infusion agar maintained at 50°C, aseptically add the following antimicrobials:

Actidione: 2 ml of 10 mg/ml stock solution; final concentration = 100 μg/ml of agar.
Bacitracin: 1 ml of 5000 U/ml stock solution; final concentration = 25 U/ml of agar.
Polymyxin B: 1.2 ml of 1000 U/ml stock solution; final concentration = 6 U/ml of agar.

Add 10 ml sterile sheep blood aseptically, mix well, and pour plates.

This is a selective medium for isolation of *Brucella* from contaminated surgical specimens.

Xylose-Lysine-Deoxycholate Agar (XLD)

Yeast extract	3 g
L-Lysine	5 g
Xylose	3.75 g

Lactose	7.5 g
Sucrose	7.5 g
NaCl	5 g
Phenol red	0.08 g
Agar	15 g
Distilled water	1000 ml

Heat mixture to boiling to dissolve the ingredients. Sterilize at 121°C for 15 min, cool to approximately 60°C, and aseptically add 20 ml of sterile solution containing:

Sodium thiosulfate	34 g
Ferric ammonium citrate	4 g
Distilled water	100 ml

Mix well, and then add 25 ml of 10% sterile solution of sodium deoxycholate per liter. Mix well and adjust pH to 6.9.

The commercially available "completed" XLD agar includes all the above ingredients.

This moderately selective medium is used for the isolation of enteric pathogens, especially *Shigella* (Table 3–6).

Yersinia Selective Agar (Difco Laboratories)

Yeast extract	2 g
Peptone	17 g
Proteose peptone	3 g
Mannitol	20 g
Sodium deoxycholate	0.5 g
Sodium cholate	0.5 g
Sodium chloride	1 g
Sodium pyruvate	2 g
$MgSO_4 \cdot 7H_2O$	10 mg
Agar	13.5 g
Neutral red	30 mg
Crystal violet	1 mg
Irgasan	4 mg
Distilled water	1000 ml

Final pH 7.4.

Dissolve dry ingredients in water, and heat to boiling. Sterilize at 121°C for 15 min. Cool to 50°C and aseptically add 10 ml of rehydrated Yersinia Antimicrobic Supplement CN, which contains 4 mg of cefsulodin and 2.5 mg of novobiocin. Mix thoroughly and pour into petri dishes.

This medium, which is a modification of the cefsulodin-irgasan-novobiocin (CIN) agar of Schiemann,[9] is recommended for use in the selective isolation of *Yersinia enterocolitica* from fecal specimens.

II. Media and Reagents for Anaerobic Bacteria

Anaerobic Media Supplements

a. Hemin Stock Solution, 5 mg/ml

Hemin	0.5 g
NaOH, 1 *M*	10 ml

Dissolve hemin in NaOH and adjust volume to 100 ml with distilled water. Store solution at 4°C. Add 1 ml to 1000 ml of medium to give 5 μg/ml, prior to autoclaving.

b. Vitamin K_1 Stock Solution for Solid Media, 10 mg/ml

Vitamin K_1	0.2 g
Ethyl alcohol, 95%	20 ml

Store solution in a sterile brown bottle at 4°C. Add 1 ml to 1000 ml of sterile agar to give 10 μg/ml.

c. Vitamin K_1 Stock Solution for Liquid Media, 0.1 mg/ml

Vitamin K_1 for solid media (10 mg/ml)	1 ml
Distilled water	100 ml

Store solution in a brown bottle at 4°C. Add 1 ml to 1000 ml of liquid medium to give 0.1 μg/ml, prior to autoclaving.

Bile-Deoxycholate

a. Broth

Thioglycollate-135C	30 g
Oxgall	20 g
Sodium deoxycholate	1 g
Hemin (5 mg/ml)	1 ml
Vitamin K_1 (0.1 mg/ml)	1 ml
Distilled water	1000 ml

Dissolve ingredients by heating and bring to a boil. Dispense 5 ml amounts into screw-capped tubes. Sterilize at 121°C for 15 min.

This medium is used for the identification of *Bacteroides*. Alternatively, oxgall-impregnated paper disks may be used (p. 337).

b. Disk

Oxgall	10 g
Distilled water	10 ml

Dissolve and sterilize at 121°C for 15 min. Also autoclave 6 mm paper disks in a glass petri dish at 121°C for 30 min in a dry cycle. Dry disks with dish cover ajar under a hood. Saturate each disk with one drop of oxgall solution and let dry overnight. Store disks with desiccant at 4°C.

Brucella Blood Agar

Brucella agar base	
Trypticase peptone	10 g
Thiotone peptone	10 g
Glucose	1 g
Yeast autolysate	2 g
NaCl	5 g
$NaHSO_3$	0.1 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.0.	

Suspend 43 g of dehydrated medium in distilled water and add 1 ml of hemin (5 mg/ml). Heat with agitation and boil for 1 min. Sterilize at 121°C for 15 min. Let agar cool to 50°C and add 50 ml of defibrinated rabbit or sheep blood and 1 ml of vitamin K_1 (10 mg/ml). Mix well and pour into petri dishes.

This medium is used for the primary isolation and subculturing of anaerobic bacteria.

Carbohydrate Fermentation Base for Anaerobic Bacteria

Base medium	
Thioglycollate medium without dextrose or indicator	24 g
Yeast extract	2 g
Hemin (5 mg/ml)	1 ml

Bromthymol blue, 1% solution	1 ml
Distilled water	1000 ml
Final pH 7.0.	

Dissolve ingredients by heating. Bring to a boil. Dispense 10 ml in screw-capped tubes. Sterilize at 121°C for 15 min. Cool medium to 50°C and aseptically add 1 ml of a 10% (5% for arabinose and trehalose) filter sterilized carbohydrate solution to give a final concentration of 1% (or 0.5%). The carbohydrate stock solution contains 1 μg of vitamin K_1 per ml so that the final concentration of vitamin K_1 in fermentation broth is 0.1 μg/ml.

Bromthymol blue indicator

Bromthymol blue	1 g
NaOH, 1 *M*	20 ml
Distilled water	80 ml

Dissolve indicator in alkali and add distilled water. See p. 341 for use of the medium.

Chopped Meat Medium

Ground beef (fat-free)	500 g
NaOH, 1 *M*	25 ml
Distilled water	1000 ml

Mix ingredients and bring to a boil. Cool and refrigerate overnight. Skim off any remaining fat. Filter through two layers of gauze and spread out meat particles to dry. Restore volume of broth to 1000 ml with distilled water and add:

Trypticase peptone	30 g
Yeast extract	5 g
K_2HPO_4	5 g
L-Cysteine hydrochloride·H_2O	0.5 g
Hemin (5 mg/ml)	1 ml
Agar	15 g

Heat to boiling, adjust pH between 7.4 and 7.8. Dispense 6 ml of medium into screw-capped tubes and add meat particles so that the final volume in each tube is approximately 8 ml. Sterilize at 121°C for 15 min. Let tubes cool in a slant position.

This medium is used to induce spore production in clostridia.

Cycloserine-Cefoxitin-Fructose-Egg Yolk Agar (CCFA)[4]

Egg yolk fructose agar base	
Proteose peptone no. 2	40 g
Na_2HPO_4	5 g
KH_2PO_4	1 g
NaCl	2 g
$MgSO_4$, anhydrous	0.1 g
Fructose	6 g
Agar	20 g
Neutral red, 1% solution in ethyl alcohol	3 ml
Distilled water	1000 ml

Final pH 7.28.

Dispense 100 ml amounts in screw-capped bottle. Sterilize at 121°C for 15 min. Store at 4°C until needed. Melt basal medium and maintain at 50°C. Aseptically add the following to each bottle:

Cycloserine base, final concentration of 500 μg/ml
Cefoxitin base, final concentration of 16 μg/ml
5 ml egg yolk (50% suspension in saline)

Mix well and dispense 20 ml per plate. This is a selective medium for *Clostridium difficile* (Table 3–6).

Egg Yolk Agar

Trypticase peptone	40 g
Na_2HPO_4	5 g
NaCl	2 g
$MgSO_4$, 5% aqueous	0.2 ml
Glucose	2 g
Agar	25 g
Distilled water	1000 ml

Dissolve ingredients by heating. Bring to a boil. Distribute into two 1 liter flasks. Adjust pH to 7.3 to 7.4. Sterilize at 121°C for 15 min. Cool medium to 60°C.

Soak two eggs in 95% ethyl alcohol for 30 to 45 min. With sterilized forceps, make an opening at one end of the egg. Pour out egg white and remove remaining traces with forceps. Beat egg yolk with forceps and transfer one egg yolk to each flask after flaming egg shell. Mix well and pour plates. See p. 362 for use of the medium.

Esculin Broth

Heart infusion broth	25 g
Esculin	1 g
Agar	1 g
Hemin (5 mg/ml)	1 ml
Vitamin K_1 (0.1 mg/ml)	1 ml
Distilled water	1000 ml
Final pH 7.0.	

Dissolve ingredients by heating. Bring to a boil. Dispense 5 ml amounts into screw-capped tubes. Sterilize at 121°C for 15 min. See p. 340 for use of the medium.

Gentamicin-Vancomycin Laked Blood Agar

Brain heart infusion agar base	
Calf brain, infusion from	200 g
Beef heart, infusion from	250 g
Polypeptone	10 g
Glucose	2 g
NaCl	5 g
Na_2HPO_4	2.5 g
Agar	15 g
Distilled water	1000 ml

Suspend 52 g of dehydrated medium in 1000 ml distilled water. Add 1 ml of hemin (5 mg/ml) and 5 ml of gentamicin (10 mg/ml). Heat to boiling to dissolve completely. Adjust pH to 7.6. Sterilize at 121°C for 15 min. Cool medium to 50°C and add the following:

0.75 ml of vancomycin (10 mg/ml)
1 ml of vitamin K_1 (10 mg/ml)
50 ml of laked rabbit blood*

Mix well and pour into petri dishes.

This is a selective medium for the isolation of anaerobic gram-negative bacilli (Table 3–2).

Indole-Nitrate Broth

Indole–nitrite medium	
Trypticase peptone	20 g

* Laked blood is prepared by freezing blood overnight and then thawing.

Na_2HPO_4	2 g
Glucose	1 g
Agar	1 g
KNO_3	1 g
Distilled water	1000 ml
Final pH 7.2.	

Suspend 25 g of dehydrated medium in water. Add 1 ml of hemin (5 mg/ml) and 1 ml of vitamin K_1 (0.1 mg/ml). Stir to dissolve. Bring to a boil. Dispense 2 ml amounts into screw-capped tubes. Sterilize at 121°C for 15 min. See p. 339 for use of the medium.

Motility Medium for Anaerobic Bacteria

Same as for aerobic bacteria (p. 785).

Peptone Yeast Glucose (PYG) Medium

Medium	
Peptone	10 g
Yeast extract	10 g
Glucose	10 g
Resazurin [one tablet (Allied Chemical, cat. no. 506) in 44 ml distilled water]	12 ml
Salt solution	40 ml
Hemin (5 mg/ml)	1 ml
L-Cysteine hydrochloride·H_2O	0.5 g
Vitamin K_1 (0.1 mg/ml)	1 ml
Distilled water	1000 ml
Salt solution	
$CaCl_2$, anhydrous	0.2 g
$MgSO_4$, anhydrous	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10 g
NaCl	2 g

Dissolve $CaCl_2$ and $MgSO_4$ in 300 ml of water. Add 500 ml of water and rest of the salt. Stir until solids have totally dissolved and add more water to give a total volume of 1 liter. Store at 4°C. If salts precipitate out during refrigeration, warm solution at 50°C in water bath before use.

Suspend ingredients in water except L-cysteine hydrochloride and vita-

min K_1. Heat to boiling to dissolve completely. Cool medium to 45°C. Add L-cysteine hydrochloride and vitamin K_1 solution. When dissolved, adjust pH to 6.8 with 0.1 *M* NaOH. Dispense 5 ml amounts into serum bottles (20 ml size, Wheaton Scientific) and reduce medium inside anaerobic glovebox. Apply stoppers (flange type, gray butyl, 20 mm, Wheaton Scientific) and secure with crimped three piece aluminum seals (20 mm, Wheaton Scientific). Sterilize at 121°C for 15 min.

This medium is used to prepare broth cultures of anaerobic bacteria for GLC (p. 342). Prepared bottles are available from Gibco Laboratories.

Phenylethyl Alcohol (PEA) Agar for Anaerobic Bacteria

PEA medium	42.5 g
Hemin (5 mg/ml)	1 ml
Distilled water	1000 ml
Final pH 7.3.	

Sterilize at 121°C for 15 min. Cool medium to 50°C and add 1 ml of vitamin K_1 (10 mg/ml) and 50 ml of sheep blood.

This medium is used as a primary isolation medium for anaerobic bacteria (Table 3–2).

Schaedler Broth

Trypticase peptone	5.6 g
Phytone peptone	1 g
Polypeptone peptone	5 g
NaCl	1.7 g
K_2HPO_4	0.82 g
Glucose	5.82 g
Yeast extract	5 g
Tris-(hydroxymethyl)-aminomethane	3 g
Hemin	0.01 g
L-Cystine	0.4 g
Distilled water	1000 ml
Final pH 7.6.	

Suspend 28.4 g of dehydrated medium in 1000 ml of water. Add 1 ml of vitaming K_1 solution (0.1 mg/ml). Mix and heat with frequent agitation. Boil for 1 min. Dispense 5 ml amounts in screw-capped tubes. Sterilize at 121°C for 15 min.

This medium is used for susceptibility testing of anaerobic bacteria by the microbroth dilution method (p. 375).

Starch Broth Medium

Soluble starch	5 g
Peptone yeast glucose (PYG) medium	1000 ml

Prepare medium as for PYG medium (p. 805) with added starch. Dispense 10 ml amount into screw-capped tubes and sterilize at 121°C for 15 min. See p. 362 for use of the medium.

Supplemented Thioglycollate for Anaerobic Bacteria

Medium	
Thioglycollate-135C	30 g
Hemin (5 mg/ml)	1 ml
Vitamin K_1 (0.1 mg/ml)	1 ml
Distilled water	1000 ml
Final pH 7.0	

Dissolve by boiling. Dispense 10 ml amounts into screw-capped tubes. Add one $CaCO_3$ chip to each tube. Sterilize at 121°C for 15 min. Before use, boil thioglycollate tubes for 10 min. When cooled, add 1 ml of rabbit serum that has been inactivated at 56°C for 30 min.

This medium is used for the primary isolation (Table 3–2) and subculturing of anaerobic bacteria.

Thiogel Medium

Trypticase peptone	17 g
Phytone peptone	3 g
Glucose	6 g
NaCl	2.5 g
Sodium thioglycollate	0.5 g
Agar	0.7 g
L-Cystine	0.25 g
Na_2SO_3	0.1 g
Gelatin	50 g
Distilled water	1000 ml
Final pH 7.0.	

Suspend 80 g of dehydrated medium in water. Add 1 ml of hemin (5 mg/ml) and 1 ml of vitamin K_1 solution (0.1 mg/ml). Heat to dissolve and bring to a boil. Dispense 8 ml amounts into screw-capped tubes. Sterilize at 121°C for 15 min. See p. 355 for use of the medium.

Transport Vial for Anaerobic Bacteria

Peptone	10 g
Yeast extract	10 g
Resazurin	12 ml
Salt solution	40 ml
Hemin (5 mg/ml)	1 ml
L-Cystine hydrochloride·H_2O	0.5 g
Vitamin K_1 (0.1 mg/ml)	1 ml
Distilled water	1000 ml
Final pH 6.8.	

Prepare and bottle as for PYG medium (p. 805), in aliquots of 1 ml per vial. This vial is used for transporting specimens for anaerobic culture (p. 21). It is available from Gibco Laboratories.

III. Media and Reagents for Mycobacteria and Nocardia

Bennett's Agar

Yeast extract	1 g
Beef extract	1 g
N-Z amine A (Sheffield Chemical)	2 g
Glucose	10 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.3.	

Dissolve by boiling. Sterilize at 121 °C for 15 min. Dispense 35 ml amounts into petri dishes. This medium is useful as an isolation medium to demonstrate the typical morphology of *Norcardia* and *Streptomyces.*

Decontamination and Digestion Solutions

Bromocresol Purple Indicator

Bromocresol purple	1.2 g
Ethyl alcohol, 95%	50 ml
Distilled water	50 ml

Dissolve bromocresol purple in alcohol, then add water.

Sodium Hydroxide Solution, 2%	
NaOH	40.8 g
Distilled water	2000 ml
Hydrochloric Acid Solution	
HCl	165 ml
Bromocresol purple	9 ml
Distilled water	1835 ml

Add acid to water, mix, then add indicator solution.

These reagents are used for the digestion and decontamination of specimens before culturing for mycobacteria (Table 3–4).

Lowenstein-Jensen Medium

Lowenstein medium base	37.2 g
Glycerol	12 ml
Homogenized eggs (fresh)	1000 ml
Distilled water	588 ml

Dissolve the Lowenstein medium base and glycerol in distilled water by boiling. Sterilize at 121°C for 15 min. Cool medium to 60°C. Add homogenized eggs and mix gently to obtain a uniform mixture. Dispense in sterile screw-capped tubes. Do not slant. Coagulate the medium in an inspissator, water bath, or autoclave at 85°C for 45 min. Store tubes in refrigerator until used. Prepared tubes are available from Difco Laboratories.

See p. 398 for use of this medium in the catalase test. By adding 5% NaCl, the medium can be used to test NaCl tolerance of mycobacteria (p. 400). The medium is also used for isolation of mycobacteria (Tables 3–2, 3–4).

Middlebrook 7H9 Broth

Ammonium sulfate	0.5 g
L-Glutamic acid (sodium salt)	0.5 g
Sodium citrate	0.1 g
Pyridoxine	0.001 g
Biotin	0.0005 g
Disodium phosphate	2.5 g
Monopotassium phosphate	1.0 g
Ferric ammonium citrate	0.04 g
Magnesium sulfate	0.05 g
Calcium chloride	0.0005 g
Zinc sulfate	0.001 g
Copper sulfate	0.001 g

Dissolve 4.7 g of dehydrated powder in 900 ml of distilled water, or 900 ml distilled water containing 0.5 g Tween-80 or 2 ml of glycerol if desired. Glycerol and Tween-80 should not be used together. Distribute in 180 ml amounts and sterilize at 121°C for 10 min. Aseptically add 20 ml of Middlebrook ADC enrichment to 180 ml of sterile medium maintained at 45°C and dispense into screwcapped tubes.

This is a basal medium used for the arylsulfatase (p. 397), tellurite (p. 402), and niacin (p. 403).

Middlebrook 7H10 Agar

Ammonium sulfate	0.5 g
Monopotassium phosphate	1.5 g
Dipotassium phosphate	1.5 g
Sodium citrate	0.4 g
Magnesium sulfate	0.025 g
Calcium chloride	0.0005 g
Zinc sulfate	0.001 g
Copper sulfate	0.001 g
L-Glutamic acid (sodium salt)	0.5 g
Ferric ammonium sulfate	0.04 g
Pyridoxine hydrochloride	0.001 g
Biotin	0.0005 g
Malachite green	0.00025 g
Agar	15 g
Distilled water	1000 ml
Final pH 6.6.	

Dissolve 19 g of dehydrated medium in 1000 ml distilled water containing 0.5% glycerol by boiling. Distribute in 200 ml amounts in dark bottles and sterilize at 121°C for 10 min. Aseptically add 20 ml Middlebrook OADC enrichment to each bottle of medium maintained at 50° to 55°C. Dispense 25 ml of medium into plates or 6 to 7 ml into 16 × 125 mm screw-capped tubes. *Keep prepared medium in the dark before and after inoculation.* Prepared plates are available from DiMed Corporation.

This medium is used for the isolation of mycobacteria (Tables 3–2, 3–4, and 3–5).

Middlebrook Selective S7H11 Agar

This is Middlebrook 7H10 medium to which have been added:

	Final concentration/ml of medium
Casein hydrolysate	1 mg
Amphotericin B	10 μg

Carbenicillin	50 μg
Polymyxin B	200 U
Trimethoprim	20 μg

Prepared plates are available from DiMed Corporation.

This medium is used for the selective isolation of mycobacteria from contaminated clinical material (Tables 3–2 and 3–4).

IV. Media for Fungi

Brain Heart Infusion Agar (BHIA)

Infusion from calf brains	200 g
Infusion from beef heart	250 g
Proteose peptone	10 g
Glucose	2 g
NaCl	5 g
Na_2HPO_4	2.5 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.4.	

Dissolve 52 g of the dehydrated medium in 1000 ml of distilled water by boiling. Sterilize at 121°C for 15 min. Pour 35 ml amount into petri dishes. Prepared plates are available from DiMed Corporation.

This medium is used for the isolation and subculture of fungi and *Nocardia* (Tables 3–2, 3–4, and 3–5).

BHIA-3	
Brain heart infusion agar	52 g
Gentamicin	5 mg
Chloramphenicol (solvent: 2 ml 95% ethanol)	16 mg
Distilled water	1000 ml
BHIA-4	
Brain heart infusion agar	52 g
Gentamicin	5 mg
Chloramphenicol	16 mg
Cycloheximide (solvent: 5 ml acetone)	500 mg
Distilled water	1000 ml

Prepare both media similarly to brain heart infusion agar. Cool to 50°C and aseptically add defibrinated sheep blood to a final concentration of 10%. Prepared plates are available from DiMed Corporation.

BHIA-3 and BHIA-4 are used for the selective isolation of fungi, excluding dermatophytes (Tables 3–2, 3–4, and 3–5).

Cottonseed Conversion Medium

Glucose	20 g
Agar	10 g
Pharmamedia (Traders Protein Division, Fort Worth, TX)	20 g
Distilled water	1000 ml

Suspend dry ingredients in water. Heat to dissolve and bring to a boil. Dispense into sterile screw-capped tubes. Sterilize at 121°C for 15 min. Let medium cool in a slant position. See p. 481 for use of the medium.

Czapek Solution Agar

Sucrose	30 g
Sodium nitrate	2 g
K_2HPO_4	1 g
$MgSO_4$	0.5 g
KCl	0.5 g
$FeSO_4$	0.01 g
Agar	15 g
Distilled water	1000 ml
Final pH 7.3.	

Suspend 49 g of dehydrated medium in 1000 ml of water. Dissolve by boiling. Sterilize at 121°C for 15 min. Dispense 35 ml amounts into petri dishes. See p. 448 for use of the medium.

Cystine Heart Agar

Cystine heart agar base (BBL)	
Beef heart, infusion from	500 g
Polypeptone peptone	10 g
Glucose	10 g
NaCl	5 g
L-Cystine	1 g
Agar	15 g
Distilled water	1000 ml
Final pH 6.8.	

Suspend 25.5 g of dehydrated medium in 250 ml of water to prepare a double strength base. Heat with frequent agitation and boil for one minute. Sterilize at 121°C for 15 min. Cool the basal medium to 50°C. Asepti-

cally add 250 ml of 2% hemoglobin solution that is maintained at 50°C. Mix well and dispense into screw-capped tubes. Let medium harden in slant position. This medium may be used for in vitro conversion of dimorphic hyaline molds (p. 481).

Inhibitory Mold Agar (IMA)

Tryptone	3 g
Beef extract	2 g
Yeast extract	5 g
Glucose	5 g
Starch (soluble)	2 g
Dextrin	1 g
Chloramphenicol	0.125 g
Salt A	10 ml
Salt C	20 ml
Agar	17 g
Distilled water	970 ml
Salt A	
NaH_2PO_4	25 g
Na_2HPO_4	25 g
H_2O	250 ml
Salt C	
$MgSO_4 \cdot 7H_2O$	10 g
$FeSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.5 g
$MnSO_4 \cdot H_2O$	1.2 g
H_2O	250 ml

Add one or two drops of concentrated HCl to salt C to solubilize components.

Suspend dry ingredients, except chloramphenicol, in water. Heat to dissolve and bring to a boil. Dissolve chloramphenicol in 2 ml of 95% of ethyl alcohol and add to the medium. Adjust pH to 6.7. Sterilize at 121°C for 15 min. Dispense 35 ml amounts into petri dishes. Prepared plate are available from DiMed Corporation.

This medium is used for isolation and subculturing of fungi (Tables 3-2, 3-4, and 3-5). Chloramphenicol should not be incorporated in the medium when culturing spinal fluid.

Mycosel Agar

Phytone peptone	10 g
Glucose	10 g

Cycloheximide	0.40 g
Chloramphenicol	0.05 g
Agar	15 g
Distilled water	1000 ml
Final pH 6.5.	

Suspend 36 g of dehydrated medium in 1000 ml of distilled water. Heat to dissolve and bring to a boil. Sterilize at 118°C for 15 min. Prepared plates are available from DiMed Corporation.

This medium is used for the isolation of dermatophytes.

Potato Dextrose Agar

Potato, infusion from	200 g
Glucose	20 g
Agar	15 g
Distilled water	1000 ml
Final pH 5.6.	

Suspend 39 g of dehydrated medium in 1000 ml of water. Bring to a boil to dissolve completely. Sterilize at 121°C for 15 min. Dispense into petri dishes.

This medium is used for demonstrating pigment production by *Trichophyton rubrum.*

Rice Grain Medium

Polished white rice (without added vitamins)	8 g
Distilled water	25 ml

Sterilize at 121°C for 15 min.

Microsporum audouinii, unlike other *Microsporum* species, is unable to grow on this medium.

Sabouraud Dextrose Agar (Emmons' modification)

Glucose	20 g
Neopeptone	10 g
Agar	17 g
Distilled water	1000 ml
Final pH 6.8–7.0	

Dissolve ingredients by heating and bring to a boil. Sterilize at 121°C for 15 min. Dispense 35 ml amounts into petri dishes. Prepared plates are available from DiMed Corporation.

This medium is used for isolation, subculturing, and identification of filamentous fungi (Tables 3–2, 3–4, and 3–5).

Trichophyton Agars (Difco Laboratories)

Bacto-Trichophyton Agar 1	
Bacto-vitamin free casamino acids	2.5 g
Bacto-dextrose	40 g
Monopotassium phosphate	1.8 g
Magnesium sulfate	0.1 g
Bacto-agar	15 g
Distilled water	1000 ml

Bacto-trichophyton agar 2 through 7 are composed of 59 g of trichophyton agar 1 with the following supplements per liter:

Trichophyton Agar 2	
Inositol	50 mg
Trichophyton Agar 3	
Inositol	50 mg
Thiamine	200 μg
Trichophyton Agar 4	
Thiamine	200 μg
Trichophyton Agar 5	
Nicotinic acid	2 mg
Trichophyton Agar 6	
Ammonium nitrate	1.5 g
Trichophyton Agar 7	
Ammonium nitrate	1.5 g
or histidine	3 mg

Prepare each medium according to the manufacturers' instructions. Sterilize at 121°C for 12 min. Dispense into petri dishes. See p. 470 for use of these media.

Yeast Extract Agar

Yeast extract	1 g
Buffer	2 ml
Agar	20 g
Distilled water	1000 ml

Heat to dissolve and sterilize at 121 °C for 15 min. Dispense 35 ml amounts into petri dishes.

Buffer	
Na_2HPO_4	40 g
KH_2PO_4	60 g
Distilled water	400 ml

Dissolve Na_2HPO_4 in 300 ml distilled H_2O; then add KH_2PO_4. The pH is 6.0. If necessary adjust with 1 *M* HCl or NaOH. Adjust the volume to 400 ml with distilled H_2O and store at 4°C. Final pH should be 6.0.

This medium is used for the identification of *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis.*

V. Media, Reagents, and Buffers for Chlamydiae, Mycoplasmas, and Viruses

A. Media

General Considerations

The work should be done in the clean hood area where the chances of airborne bacterial or myoctic contamination of the medium are minimal. All constitutents are equilibrated to room temperature. The contents of the flask are mixed well after addition of each solution. Constituted medium is transferred to sterile 100 ml milk dilution bottles which are labeled with the name of the medium and the dates of preparation and expiration. Medium is stored at 4°C for a maximum of 6 months except when stated otherwise.

Table B-1. Preparation of *Chlamydia* Medium

Component	Volume (ml)
Distilled H_2O	600
Eagle's dry medium 10× (MEM)	100
Sodium bicarbonate, 7.5%	7.5
Glucose[a]	20
Glutamine, 200 m*M*, 10× solution	10
Gentamicin[a]	0.2
Hepes buffer, 1 *M*[a]	20
Fetal bovine serum	100
Distilled water to make	1000

[a] See Solutions (p. 821–825).

Chlamydia Medium

See Table B-1 for preparation.

Chlamydia Transport and Storage Media

1. Phosphate Buffer

a. $NaH_2PO_4 \cdot H_2O$	0.552 g
Distilled water	200 ml
b. Na_2HPO_4	1.42 g
Distilled water	500 ml

Combine solutions a. and b. Adjust pH to 7.2. Sterilize by filtration.

2. Chlamydia Transport Medium (2 SP)

Sucrose	6.84 g
Phosphate buffer	100 ml
Nystatin[a]	0.25 ml

Add sucrose to phosphate buffer. Filter sterilize and aseptically add nystatin. Dispense in 1.8 ml aliquots and store at −20°C.

No other antibiotics are included when 2 SP is used for transport of both *Ureaplasma* and *Chlamydia;* when used for *Chlamydia* only, 2 SP medium should include gentamicin (50 mg/100 ml medium). Expiration date when frozen is 1 year.

3. Chlamydia Storage Medium (4 SP)

This medium is identical to 2 SP medium except that 13.68 g of sucrose and 0.2 ml of 0.5% phenol solution are added to the phosphate buffer.

Large Colony Mycoplasma Media

1. Unsupplemented Media

a. Agar

PPLO agar (Difco Laboratories)	35 g
Distilled water	1000 ml

[a] See Solutions, pp. 821–825.

Heat to dissolve the agar and dispense 80 ml amounts into 100 ml bottles. Sterilize at 121°C for 15 min. Store at room temperature. Expiration date is 6 months.

b. Broth

PPLO broth without crystal violet (Difco Laboratories)	21 g
Distilled water	1000 ml

Mix and distribute 80 amounts into 100 ml bottles. Sterilize at 121°C for 15 min. Store at room temperature. Expiration date is 6 months.

2. Supplemented (Complete) Medium

See Table B-2 for preparation. Melt PPLO agar prepared in 1,a. Aseptically add yeast extract. Let agar cool to 50°C, then add horse serum, antibiotics, and thallium acetate. Mix thoroughly and dispense 8 ml to 60 × 15 mm petri dish. Expiration date is 2 weeks. Diphasic medium is prepared by adding 3 ml amounts of PPLO agar to 16 × 125 mm screw-capped tubes, slanting the tubes at a 45° angle until the agar has solidified, and then adding 3 ml of the complete medium (Table A-2) to each tube. Store the tubes in an upright position.

3. Mycoplasma Medium with Arginine

See Table B-3 for preparation. Dispense in 10 ml amounts and store at −20°C.

Table B-2. Preparation of Complete *Mycoplasma* Medium

	Volum (ml)		
Component	Agar Plates	Diphasic Broth	Final Concentration per ml of medium
PPLO agar	70	—	—
PPLO broth	—	70	—
Horse serum	20	20	—
Yeast extract[a]	10	10	—
Penicillin G[a]	1	1	1000 U
Thallium acetate[a]	0.5	0.5	500 μg
Amphotericin B[a]	0.5	0.5	5 μg
Glucose[a]	—	2	10 mg
Phenol red[a]	—	1	20 μg

[a] See Solutions (pp. 821–827).

Table B-3. *Mycoplasma* Medium with Arginine

Component	Amount
PPLO broth	70.0 ml
Horse serum	20.0 ml
Yeast extract[a]	10.0 ml
Amphotericin B[a]	0.5 ml
Thallium acetate[a]	0.5 ml
Penicillin G[a]	1.0 ml
Glucose[a]	2.0 ml
Phenol red[a]	1.0 ml
Arginine (Calbiochem)	200.0 mg

[a] See Solutions (pp. 821–825).

Ureaplasma Media

1. U-9B Basal Broth

Tripticase soy broth powder (BBL Microbiology Systems) or Tryptic soy broth powder (Difco Laboratories)	0.75 g
NaCl	0.5 g
KH_2PO_4	0.02 g
Distilled water	100 ml

Dissolve ingredients and adjust pH to 5.5 with 2 *M* HCl. Sterilize at 121°C for 15 min.

2. U-9B Complete Medium

Sterile U-9 basal broth	95 ml
Unheated normal horse serum	4 ml
Urea solution[a]	0.5 ml
Phenol red solution[a]	0.1 ml
Penicillin G[a]	1 ml
L-Cysteine·HCl	
Final pH 6.0.	

Dispense in 1.8 ml aliquots. Store at −20°C.

3. Ureaplasma Urealyticum Agar (A-7 Agar Base)

Trypticase soy broth powder (BBL Microbiology Systems)	30 g

Distilled water	1000 ml

Adjust to pH 5.5 with 4 *M* HCl, then add:

Agar Ionagar No. 2 (Oxoid)	12.73 g

Dissolve the agar by bringing the solution to a boil three times. Dispense in 170 ml aliquots in 250 ml screwcapped bottles and sterilize at 121°C for 15 min.

4. Ureaplasma Urealyticum Complete (A-7B) Agar, pH 6.0

Sterile molten A-7 agar base	160 ml
Sterile normal horse serum with putrescein[a]	40 ml
Phenol red, 1% solution[a]	0.4 ml
CVA enrichment[a]	1 ml
Urea, 10% solution[a]	2 ml
Yeast extract[a]	2 ml
L-Cysteine·HCl[a]	0.5 ml
Penicillin G[a]	2 ml

Mix ingredients well and dispense 10- to 14-ml amounts to 60 × 15 mm petri dishes. Store at 4°C. Expiration date is 2 weeks.

Media for Viruses

1. Cell Culture Preservative, 10% Buffered Formalin

37–40% formaldehyde	100 ml
Distilled water	900 ml
$NaH_2PO_4 \cdot H_2O$	4 g
Na_2HPO_4	6.5 g
Phenol red, 0.5% solution[a]	2 ml

Solution is stable indefinitely at room temperature.

2. Growth and Maintenance Media

The constituents of these media are given in Table B-4. All constituents should be at room temperature. Dispense final formulations into 100 ml bottles and store at 4°C for a maximum of 6 months except when stated otherwise.

[a] See Solutions, pp. 821–825.

3. Serum-Free Medium for Extraction of Swabs

Eagle's serum-free maintenance medium for MK (Table B-4)	80 ml
Penicillin–streptomycin solution[a]	10 ml
Nystatin solution[a]	10 ml

The final medium is transferred to 2-dram vials in 2 ml amounts and stored at −20°C.

B. Solutions

1. Agarose

Agarose (Marine Colloids, Inc.)	1.5 g
Barbital buffer, 0.05 *M*	200 ml

Dissolve the agarose in buffer by heating with continual stirring on a hot plate. Dispense in 6 ml amounts into 16 × 125 mm screw-capped tubes. Store at 4°C. Expiration date is 6 months.

2. Alsever's Solution

Glucose	2.05 g
NaCl	0.42 g
Sodium citrate dihydrate	0.80 g
Citric acid	0.055 g
Distilled water to make	100 ml

Sterilize at 121°C for 15 min or by filtration; pH of solution should be 6.1. Solution is also available commercially. Expiration date is 3 years in unopened containers and 6 months in opened containers. Sheep red blood cells (10% packed cell volume) are suspended in solution for one week prior to use in complement fixation test.

3. Antibiotics

The antibiotic solutions described below are stable for one year when stored at −20°C; however, storage of antibiotic powders or solutions should not exceed the expiration date specified by the manufacturer.

a. Amphotericin B

Add 10-ml aliquots of sterile distilled water to a vial containing 50 mg of the powdered antibiotic. Mix, adjust the volume to 50 ml, and transfer

[a] See Solutions, pp. 823–825.

Table B-4. Growth and Maintenance Media for Virus Isolation

Component	Eagle's Basal Growth Medium (BME) for MRC-5 Cells	Eagle's Maintenance Medium for MRC-5 Cells	Eagle's Serum-Free Maintenance Medium for MK
Sterile distilled water	700 ml		1500 ml
Eagle's dry medium 10×[a]	100 ml		200 ml
Sodium bicarbonate, 7.5%	7.5 ml	1 ml/100 ml of BME	35 ml
Tris buffer, pH 7.6, 1 *M*[b]		1 ml/100 ml of BME	20 ml
Penicillin (10,000 U/ml)–streptomycin (10,000 μg/ml)[b]	10 ml		20 ml
Gentamicin (10 μg/ml)[b]	0.2 ml		0.4 ml
Fetal bovine serum	100 ml		

[a] Basal medium, with L-glutamine, without $NaHCO_3$ (Biological, Inc.).
[b] See Solutions, pp. 823–825.

to vials in 0.6-ml and 2.6-ml portions, and store at −20°C after wrapping in foil.

b. Cycloheximide

Add 50 ml sterile distilled water to a vial containing 10 mg of the crystalline antibiotic to yield a final concentration of 200 μg/ml. The solution is dispensed into 1-dram vials in 0.6-ml portions and stored at −20°C. Add 0.5 ml to 100 ml of Eagle's BME or MEM (Table A-4) for a final concentration of 1 μg/ml.

c. Gentamicin

Add 1 ml of 50 mg/ml solution in vial per 5,000 ml of Eagle's BME or MEM for a final concentration of 10 μg/ml.

d. Nystatin

Add 5 to 10 ml of sterile distilled water to a vial containing 500,000 units of the antibiotic. Mix, adjust the volume to 50 ml, transfer to vials in 5.2 and 10.2 ml portions, and store at 4°C after wrapping in foil.

e. Penicillin G

Prepare a stock solution of potassium penicillin G, USP (Calbiochem) by dissolving 0.63 gm of the powdered antibiotic in 10 ml of sterile distilled water. The final concentration of the penicillin G is 100,000 units/ml, based on the specific activity determined by the manufacturer. The antibiotic solution is distributed in vials and stored at −20°C.

f. Penicillin–Streptomycin Solution

Prepare a stock solution by dissolving the appropriate amounts of penicillin G and dihydrostreptomycin sulfate in about 70 ml of distilled water. From the specific activity of the antibiotic (determined by the manufacturer), calculate the amounts to use so that the final concentration of penicillin is 10,000 units/ml and of streptomycin, 10,000 μg/ml. Adjust volume to 200 ml with distilled water. Transfer to vials in 10.2 ml amounts, and store at −20°C.

g. Streptomycin and Vancomycin

Prepare a stock solution containing 1 mg/ml of each antibiotic, based on the specific activity determined by the manufacturer. The solution is dispensed in 1-dram vials in 0.5 and 1.0 ml portions and stored at

−20°C. The solution is used to treat contaminated specimens by adding 0.1 ml/0.5 ml of specimen.

4. Buffers

a. Dextrose–Gelatin–Veronal/Buffer

5,5′-Diethylbarbituric acid	0.58 g
Gelatin	0.6 g
Na 5,5′-diethylbarbiturate (sodium barbital)	0.38 g
$CaCl_2$ (anhydrous)	0.02 g
$MgSO_4 \cdot 7\,H_2O$	0.12 g
NaCl	8.5 g
Glucose	10 g
Bovine albumin	2 g

Dissolve the barbituric acid in 200 ml of distilled water by gentle heating. Then add, sequentially, dissolved gelatin and the other ingredients. Adjust volume to 1000 ml and reheat prior to sterilization by filtration. The buffer can be also obtained from commercial sources. The expiration date is 3 years for the buffer in unopened containers. When opened, the expiration date is 6 months.

b. HEPES, 1 M

This solution is obtained from commercial sources and should be prepared for use on the basis of 20 ml/liter of medium (20 m*M*/ml final concentration in medium). The expiration date is 6 months, but buffer in unopened containers can be kept up to 3 years.

c. HEPES Saline–Albumin–Gelatin Buffer (HSAG), 1 M, pH 6.2

This solution is obtained from commercial sources. Follow expiration date specified by manufacturer.

d. Tris, 1 M, for Eagle's Medium (Table B-4)

Tris-(hydroxymethyl)-aminomethane	24.3 g
HCl, 2 *M*	50 ml

Dissolve the Tris in approximately 50 ml of distilled water. With constant stirring, add 2 *M* HCl so that the pH is 7.6. Adjust the volume to 200 ml with distilled water, sterilize at 121°C for 15 min. Recheck the pH. This buffer is available commercially. Expiration date is 3 years for the

buffer in unopened containers. When opened, the expiration date is 6 months.

e. Tris Buffer, 0.2 *M*, for Counterimmunoelectrophoresis

See p. 270.
Stock solutions:

(1) Tris-(hydroxymethyl)-aminomethane	24.2 g
Distilled water	1000 ml
(2) 0.2 *M* HCl	

Prepare buffer, pH 8.0, by adding 50 ml of (1) to 26.8 ml of (2). Add distilled water to make 200 ml. Sterilize by autoclaving. Store at 4°C. Expiration date is 6 months.

f. Veronal Buffer for Complement Fixation Test

(1) $MgCl_2$–$CaCl_2$ Solution

$MgCl_2 \cdot 6\ H_2O$	20.3 g
$CaCl_2 \cdot 2\ H_2O$	4.4 g
Distilled water	100 ml

Mix and store at 4°C.

(2) Stock Buffer Solution

NaCl	83 g
Na-5, 5′-diethylbarbiturate	10.19 g
Distilled water	1500 ml

Mix by swirling until completely dissolved. Add 34.58 ml 1 *M* HCl, 5.0 ml of $MgCl_2$-$CaCl_2$ solution (1), and distilled water to make 2000 ml.

(3) Diluted Buffer Solution

Prepare 1:5 dilution by adding 1 ml of solution (2) to 4 ml of distilled water. The pH should be between 7.3 and 7.4; if not, discard, and prepare fresh solution. Store at 4°C. Expiration date is 6 months.

5. Calcium–Saline Solution for Receptor Destroying Enzyme

$CaCl_2 \cdot 2\ H_2O$	1 g
NaCl	9 g
H_3BO_3	1.203 g
$Na_2B_4O_7 \cdot 10\ H_2O$	.052 g
Distilled water	1000 ml

Mix the compounds in water in order listed. If used immediately, this solution need not be sterilized. If the solution is to be stored longer than 1 week, sterilize by autoclaving or filtering. Store at room temperature. Expiration date is 1 year.

6. Complement

Store the lyophilized guinea pig complement at 4°C until ready to use. When rehydrating complement, place distilled water, lyophilized complement, and 1 dram vials in a pan of crushed ice. Add 5 ml of water with a syringe to the lyophilized complement and mix on a Vortex mixer. Dispense 0.4 to 0.5 ml aliquots into the 1 dram vials and store at temperatures below −20°C. Expiration date is as specified by the manufacturer.

7. Gelatin, 0.125%

Gelatin	1 g
Distilled water	800 ml

Add gelatin to 100 ml water, boil, cool to 25°C, and add remaining water. Store at 4°C. Expiration date is one week.

8. Glucose for Chlamydia Medium (Table B-1)

Glucose	27 g
Distilled water	100 ml

Sterilize by filtration and store at −20°C. Expiration date is 1 year.

9. Glucose for Mycoplasma Medium

Glucose	50 g
Distilled water	100 ml

Dissolve glucose in water and store at −20°C. Expiration date is 1 year.

10. Kaolin

Kaolin	100 g
HCl, 1 *M*	400 ml

Mix kaolin in HCl for 1 hr. Filter through Buchner funnel. Wash filter paper with distilled water until no trace of acid is detected in the filtrate. Dry the kaolin overnight at room temperature, then grind to a fine powder with a mortar and pestle. Expiration date is 1 year.

For use in the hemagglutination test:

Kaolin, acid-washed	25 g
Borate-saline buffer, pH 9.0	100 ml

Stir the kaolin in buffer. Determine the pH. If less than 8.5, centrifuge at 250 g for 5 min, discard the supernatant, resuspend the kaolin in buffer, and redetermine the pH. If the pH is still less than 8.5, repeat the procedure until it is 8.5. Store at 4°C and recheck the pH prior to use. Expiration date is 1 year.

11. Phenol red

Table B-5.

	Media		
	Large Colony Mycoplasma	Ureaplasma	Trypsin-EDTA Solutions
Phenol red powder (Gibco Laboratories)	50 mg	100 mg	—
Distilled water	25 ml	10 ml	—
Phenol red solution, 0.5% (Flow Laboratories)	—	—	Commercially prepared

12. Putrescein

Putrescein (Sigma Chemical Company)	0.825 g
Sterile horse serum	100 ml

13. Receptor Destroying Enzyme (RDE)

Reconstitute RDE with 5 ml of sterile distilled water. Dispense in 1 ml amounts and store at −20°C. Expiration date is 1 year.

14. Thallium Acetate

Thallium acetate (Fisher Scientific Co.)	10 g
Distilled water	100 ml

Mix the thallium acetate in water. Sterilize by filtration and store at −20°C. Thallium acetate is extremely toxic and solutions must be transferred with mechanical pipettes. Expiration date is 1 year.

15. Trypsin-EDTA Solution

Disodium ethylenediaminetetraacetate (EDTA or Versene)	50 mg
Trypsin	50 mg
Sodium bicarbonate, 7.5%	1 ml
Tris buffer, 1 *M*[a]	1.6 ml
Penicillin–streptomycin solution[a]	1 ml
Nystatin solution[a]	0.5 ml
Phenol red, 0.5%[a]	022
10× special Earle's BSS for trypsin-EDTA solution	10 ml
NaCl	6.8 g
KCl	0.4 g
$NaH_2PO_4 \cdot H_2O$	0.14 g
Glucose	1 g
Distilled water	100 ml

Store at 4°C. Expiration date is 1 year.
Dissolve and add distilled water to make 100 ml. Sterilize by filtration. Distribute into vials in 3 and 5 ml amounts and store at −20°C. Expiration date is 1 year. Exclude penicillin-streptomycin solution when it is used with McCoy's cells.

16. Tryptose Phosphate Broth

Tryptose phosphate broth (Difco Laboratories)	29.5 g
Distilled water	1000 ml

Dissolve the powder in water and distribute in 100 ml bottles. Sterilize at 121° for 15 min and store at room temperature. Expiration date is 1 year.

17. Urea Solution for Ureaplasma Media

Urea	3 g
Distilled water	30 ml

Dissolve urea in water. Sterilize by filtration. Dispense in 0.7 ml amounts and store at −20°C. Expiration date is 1 year.

18. Yeast Extract

Purchased from Flow Laboratories. For use in *Ureaplasma urealyticum* medium, adjust pH to 5.5 with 2 M HCl. Expiration date is 1 year.

[a] See Solutions, pp. 281–287.

References

1. Facklam, R. R., and Moody, M. D. Presumptive identification of group D streptococci: The bile-esculin test. *Appl. Microbiol.* **20**:245, 1970.
2. Feeley, J. C., Gibson, R. J., Gorman, G. W., Langford, N. C., Rasheed, J. K., Mackel, D. C., and Baine, W. B. Charcoal-yeast extract agar: Primary isolation medium for *Legionella pneumophila. J. Clin. Microbiol.* **10**:437, 1979.
3. Flynn, J., and Waitkins, S. A. A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae. J. Clin. Pathol.* **25**:525, 1972.
4. George, W. L., Sutter, V. L., Citron, D., and Finegold, S. M. Selective and differential medium for isolation of *Clostridium difficile. J. Clin. Microbiol.* **9**:214, 1979.
5. Kuzdas, C. D., and Morse, E. V. A selective medium for the isolation of brucellae from contaminated materials. *J. Bacteriol.* **66**:502, 1953.
6. Martin, J. E., Armstrong, J. H., and Smith, P. B. New system for cultivation of *Neisseria gonorrhoeae. Appl. Microbiol.* **27**:802, 1974.
7. Martin, J. E., Jr., and Lewis, J. S. Anisomycin: Improved antimycotic activity in modified Thayer-Martin medium. *Public Health Rep.* **35**:53, 1977.
8. Pasculle, A. W., Feeley, J. C., Gibson, R. J., Cordes, L. G., Meyerowitz, R. L., Patton, C. M., Gorman, G. W., Carmack, C. L., Ezzell, J. W., and Dowling, J. N. Pittsburgh pneumonia agent: Direct isolation from human lung tissue. *J. Infect. Dis.* **141**:727, 1980.
9. Schiemann, D. A. *Yersinia enterocolitica:* Observation of some growth characteristics and response to selective agents. *Can. J. Microbiol.* **26**:1232, 1980.
10. Sutcliffe, E. M., and Abbott, J. D. Selective medium for the isolation of *Bordetella pertussis* and *parapertussis. J. Clin. Pathol.* **25**:732, 1972.
11. Swan, A. The use of a bile-aesculin medium and of Maxted's technique of Lancefield grouping in the identification of enterococci (group D streptococci). *J. Clin. Pathol.* **7**:160, 1954.
12. Thayer, J. D., and Martin, J. E., Jr. An improved medium selective for cultivation of *N. gonorrhoeae* and *N. meningitidis. Public Health Rep.* **81**:559, 1966.
13. Totten, P. A., Amsel, R., Hale, J., Piot, P., and Holmes, K. K. Selective differential human blood bilayer media for isolation of *Gardnerella* (*Haemophilus*) *vaginalis. J. Clin. Microbiol.* **15**:141, 1982.
14. Weed, L. A. Use of a selective medium for isolation of *Brucella* from contaminated surgical specimens. *Am. J. Clin. Pathol.* **27**:482, 1957.

APPENDIX C

Quality Control

John P. Anhalt, Ph.D., M.D. *Pauline K. W. Yu, M.S.*

I. Overview

A. General Considerations

The quality control program in Clinical Microbiology at the Mayo Clinic relies heavily on performance testing and good communication between technologists, laboratory directors, and the clinical staff to ensure accurate and meaningful results. To this end, we have implemented a systematic program with reasonable limits for routine characterizations and operational checks. Good communication serves not only to detect when the inevitable errors occur, but also to decrease the likelihood, we hope, that an error will lead to an adverse result for the patient.

Laboratory problems that can lead to errors are of several types, and the actions taken in response should be appropriate for the problem and the medical significance of the resulting error. For example, the underlying cause of a rare or sporadic problem is difficult to determine. Unless the error resulting from such a problem is highly significant from the medical standpoint, the value derived may not justify the costs and time required to solve the problem. In contrast, a systematic or frequent problem is relatively easily solved and usually requires a change in procedure or implementation of additional control procedures.

Continuing education of laboratory personnel, including laboratory directors, is an integral part of quality control. Procedures can become outdated or gradually changed, and education helps to ensure use of optimal procedures, awareness of problems that can occur, and performance of procedures according to design. Laboratory safety and the working environment (Chapter 13) also are important factors that can affect performance.

B. Laboratory Manual

A complete and updated laboratory manual is the starting point for accurate laboratory results. Each new technologist is provided with a copy of the manual and is expected to become thoroughly familiar with its contents. Any revisions of procedures are entered, dated, and signed by the laboratory director in a master copy of the manual located in a central area within the laboratory. Technologists are asked to record revisions in their personal copies of the manual. Yearly, the manual is reviewed in detail in a series of meetings with all technologists. Following this review, a new edition of the manual is printed and distributed to all technologists. To prevent older editions from being used, technologists must exchange old copies for new editions.

C. Education

New technologists are instructed in the methods for proper collection, identification, and transport of specimens, aseptic technique, inoculation of appropriate media, and laboratory safety and policies. They are given a rotating assignment in the laboratory, during which time they work under the immediate and close supervision of a senior, or "lead," technologist until their performance is considered to be sufficiently reliable and accurate to allow more independent work. Technologists do not usually assume full responsibilities, including on-call work, until they have been in training for 6 months.

Weekly meetings of all technologists are held to discuss and to announce new or revised procedures and policies. These announcements are then posted in the laboratory for 1 week. Daily laboratory rounds are held at which senior or lead technologists, postdoctoral trainees, and the staff microbiologists, pathologists, and infectious disease clinicians discuss interesting or unusual cases and all cases involving isolation of an organism from normally sterile body fluids or tissues. In addition, at various times during the year, lecture series are presented by the staff for technologists.

Lastly, attendance and active participation through scientific presentations at national and local meetings and in workshops is expected of senior and lead technologists.

D. Proficiency Testing

1. Internal

Simulated or real clinical specimens are submitted to the laboratory using a conventional request form bearing the name and registration number

of a deceased patient. Isolation and identification techniques are tested with simulated specimens containing stock organisms. Serological procedures and antimicrobial assays are tested by using serum remaining from previous tests that has been stored at −60°C, and results are compared with those obtained initially. At least once every 6 months, a proficiency specimen is submitted for each serological procedure. A variety of safeguards are used to ensure that the technologist doing the testing does not know a particular specimen is for proficiency testing and to protect against confusion caused by inadvertent reporting of results outside of the laboratory. The results from these internal proficiency specimens are used as a check on adequacy of procedures and as an indicator for additional technologist training. Causes of problems are investigated, and after appropriate action (e.g., a change in procedure or additional training), a similar specimen is resubmitted to test whether improvement has occurred.

2. External

The laboratory participates in or is a referee for various state and federal programs, including those of the College of American Pathologists (7400 North Skokie Boulevard, Skokie, IL 60077) and the Centers for Disease Control, Atlanta, GA 30333.

E. Records and Review

All results from proficiency tests, instrument function tests, major items of instrument maintenance, and performance tests of media and reagents are recorded by the quality control technologist and reviewed monthly by the staff director of quality control. Results that indicate a failure in procedures, media, reagents, or equipment are recorded in a separate log along with the remedial action taken. Each entry in the log is initialed and dated by the director of quality control.

II. Media and Reagents

A. General Considerations

1. Selection

Media and reagents should be selected to provide efficient recovery and rapid identification of a wide variety of organisms. Where appropriate, selective media should be used for isolation of common pathogens; however, these media should be used together with nonselective media, because the selective media may inhibit some pathogens. Media and re-

agents should be purchased from reliable sources that, on request, can provide information regarding quality control of their products. It is also desirable to have alternative sources readily identified in case an unexpected problem with supply develops.

2. Preparation

Media and reagents must be prepared according to directions of the manufacturer or from well-documented sources. Dehydrated media should be reconstituted in distilled water or deionized water of proved chemical and microbiological quality. Apparently minor variations in procedure (e.g., the order of addition and mixing of components) can have effects on the performance of media. It is essential, therefore, that procedures be monitored to ensure that the intended directions are followed. Sterilization of media must be controlled to ensure sterility without degradation of labile constituents such as carbohydrates. The pH of each batch of media must be checked to ensure that it is within the proper range. The pH of broth and agar media changes during autoclaving and is, therefore, usually checked after autoclaving rather than before. The media must be equilibrated to room temperature before pH can be measured. The pH of agar media can be measured using a surface electrode. Alternatively, a small portion of the molten agar can be placed in a beaker and allowed to solidify around a standard pH electrode, or the pH electrode can be immersed in solid media that has been minced with a few drops of water. In any case, the electrode must be supported securely to avoid unreliable readings resulting from pressure on the glass tip. The pH of media can usually be adjusted to the proper range by adding dilute NaOH or HCl solutions.

3. Sterility Tests

To avoid confusing results, the sterility of media must be ensured. A variety of methods can be used to accomplish this goal, depending on the nature of the medium and its intended use. Generally, the incidence of contamination is greater in media to which a heat-labile substance (e.g., sugars or blood) is added or which are dispensed into tubes or plates after autoclaving than in media that are terminally autoclaved. For most media, incubation of each tube or plate to detect contamination before use is impractical, and only a representative sample of each batch can be tested. The size of the sample that should be tested depends on the maximum incidence of contamination that is acceptable. For example, if the sample consists of 300 tubes or plates from a very large batch, one would expect to detect, based on sampling statistics,[4] an incidence of contamination of 1% with about 95% certainty. Our experience with commercially prepared media supports this prediction (J. P. Anhalt, un-

published data). One manufacturer that supplies media to the Mayo Clinic tests 10% of each lot of 4000 to 5000 plates (i.e., a sample of 400 to 500 plates), and discards the entire lot if a single plate is contaminated.

After shipment without refrigeration to our laboratory, each plate is visually inspected, and plates showing contamination are discarded. (Credit is received for discarded plates and the remainder of each lot is used.) Over a period of 6 months, we detected contamination in an average of 0.4% of 165,000 sheep blood agar plates examined and the incidence never exceeded 1.5% per lot of 5000 plates. These findings are in accord with the sensitivity expected for the sample size tested by the manufacturer. Obviously, attempting to detect 1 or 2% contamination is impractical for clinical laboratories unless each tube, bottle, or plate is tested. Testing a sample of 5 or 10 plates or tubes from a batch has been recommended[1,2]; however, this sample size would be expected only to detect reliably a contamination rate of 25 to 40%. In practice, such high rates of contamination are not found except when there has been a gross failure of normal aseptic procedures. When such failures occur, contamination is usually detected during routine performance testing or by casual inspection of the media before inoculation and does not require separate sterility testing per se. In part because of the requirements for inspection and accreditation (failure to check all media for sterility is a phase II deficiency in the inspection and accreditation program of the College of American Pathologists[3]) and in part to detect failure of aseptic procedures, we have continued to test sterility by incubation of a single, uninoculated sample from each batch of media prepared in-house. These tests are performed at the same time as performance tests, and the period and conditions of incubation are the same as those used for the performance tests. For most commercially prepared media, sterility tests involve either incubation of a single sample at the same time as performance tests or careful inspection of each plate or tube prior to use. In the latter case, which is used principally for primary isolation media, media are inspected also for drying, discoloration, or other evidence of deterioration. Only those tubes or plates that fail the inspection are discarded and the remainder of the lot or shipment is used, unless there is evidence that the problem is characteristic of the entire lot. Commercially prepared blood culture bottles are not checked for sterility prior to use, because experience has shown the rate of contamination in these bottles to be low. Instead, a daily record of the isolation of presumed contaminants (*Bacillus* sp., *Propionibacterium acnes*, *Corynebacterium* sp., *Staphylococcus* coagulase-negative, *Aerococcus viridans*, and gram-negative, nonfermenters other than *Pseudomonas* sp. present in single cultures) is maintained. An incidence of these isolates greater than 2% is considered evidence of a problem with contamination that must be investigated. Finally, media may contain nonviable organisms that may, nevertheless, cause confusion when a Gram-stained smear

is performed in the course of normal procedures. Broth media and growth supplements that are used to culture normally sterile fluids (Schaedler broth, transport media, thioglycollate broth, nutrient broth, and rabbit serum) are checked, therefore, by Gram-stained smear before use to ensure that nonviable organisms are not present.

4. Dates

All media and reagents are dated when received in the laboratory and when prepared or first put into use. All media and many reagents should also have an expiration date. The expiration date provided by the manufacturer must be followed. When an expiration date is not provided, published guidelines[1,5] should be consulted. For most media and working solutions of reagents, an expiration date 6 months following preparation is a useful guideline. However, because dehydration is the principal determinant of expiration for most media, it is frequently desirable to adjust preparation schedules to allow media to be used within 3 months. Simple chemical solutions (e.g., acid–base indicators) and stock buffer solutions can often be stored up to 3 years if there is no evidence of microbial growth and performance is satisfactory. Pure chemicals (e.g., salts) and dehydrated media are generally stable and have unlimited shelf life unless otherwise specified by the manufacturer. To minimize space requirements and inventory, it is usually desirable to purchase quantities of media that can be used within 3 years. Sterile materials used in collection and processing of specimens, such as glass tubes, glass tubes with needles, and sand for grinding tissues, should have an expiration date of 3 to 6 months. These materials are not discarded, but are simply resterilized at their expiration date.

5. Storage

Media and reagents should be stored according to directions of the manufacturer. In most instances, a cool, dry area is satisfactory for storage of dehydrated media and reagents. Prepared media should be stored in sealed tubes or bottles or by wrapping plates in plastic. In general, prepared media should be stored at 2 to 8°C, which greatly reduces evaporation of water from plates and tubes with loosely fitting caps. In tightly sealed containers, however, many media may be stored up to 6 months at room temperature.[1] Media should be allowed to equilibrate to room temperature prior to inoculation. Stocks of media and reagents should be rotated so that older supplies are used first.

6. Performance Testing

Media and reagents should be tested using controls of known positive and negative reactivity. Specific guidelines for performance tests are

given in the following sections as appropriate for each division of Clinical Microbiology. The frequency of testing varies with the stability of the medium or reagent and the necessity in some tests to have a positive and a negative control for comparison. In general, each batch or lot of media or reagents must be checked for performance. If one lot is received in separate shipments, each shipment is checked. Because the aim of performance testing is to identify whether an entire lot or batch is satisfactory, a minimum number of plates (each plate can be divided into sections) or tubes of media should be used to accomplish the required tests. If a medium or reagent does not perform satisfactorily, the entire lot or batch should be discarded. The manufacturer should be notified and consulted before action is taken with commercial materials, because it may be necessary to return them to receive credit.

B. Bacteriology

1. Maintenance of Stock Cultures

a. Aerobic and Facultatively Anaerobic Bacteria

Two vials of all isolates used for performance testing are stored frozen. Following isolation on appropriate agar, suspend the bacteria in sterile, defibrinated sheep blood that contains no preservatives or anticoagulants. Place two to three drops in a ½ dram, screw-cap vial, and store at −60°C or lower. Vials should be thoroughly cleaned and sterilized. To recover bacteria, remove a vial from the freezer and allow the contents to thaw. Streak a loopful or a drop of the suspension on blood agar or other suitable, nonselective medium and incubate overnight at 35°C. The frozen suspensions can be stored indefinitely and are not adversely affected by alternate freezing and thawing. When bacteria in one vial are no longer viable, the second vial is used and a replacement vial is prepared.

Frequently used isolates are maintained on plain agar slants (subculture every 6 months) or on appropriate agar plates (subculture every week). *Neisseria* and *Haemophilus* may not survive repeated subculturing and are routinely stored frozen.

b. Anaerobic Bacteria

To store anaerobic bacteria for long periods, add 10 drops of a turbid broth culture to 0.5 ml of double-strength skim milk medium. Dispense into ½ dram vials and store at −60°C or lower. Frequently used strains are maintained in supplemented thioglycollate broth and are subcultured weekly to fresh broth. Because these cultures often become contaminated

with other bacteria, they are subcultured monthly to sheep blood agar, and fresh broth cultures are made from isolated colonies.

2. Stock Organisms Required for Testing Media and Reagents

Tables C-1 and C-2 list stock organisms that are used for performance testing of media and reagents. Most of these organisms are clinical isolates obtained at the Mayo Clinic, although other isolates with appropriate biochemical reactions should be satisfactory. In particular, isolates from the American Type Culture Collection should be used for susceptibility testing (Section 4.4).

3. Performance Testing of Media and Reagents

Tables C-3 through C-9 list the organisms and expected reactions for performance testing of media and reagents used in bacteriology. Each batch of medium prepared in-house and each lot of prepared medium

Table C-1. Stock Cultures Used for Testing of Media and Reagents Used in Anaerobic Bacteriology

Organism
Bacteroides fragilis
Bacteroides vulgatus
Bacteroides melaninogenicus
Bacteroides thetaiotaomicron
Bifidobacterium adolescentis
Clostridium b. bifermentans
Clostridium difficile
Clostridium perfringens
Clostridium sp. (urea positive)
Clostridium sporogenes
Escherichia coli
Fusobacterium mortiferum
Fusobacterium necrophorum
Fusobacterium nucleatum
Fusobacterium symbiosum
Peptostreptococcus anaerobius
Peptostreptococcus prevotil
Streptococcus, group D
Veillonella parvula

Table C-2. Stock Cultures Used for Testing of Media and Reagents Used for Aerobic and Facultatively Anaerobic Bacteria

Organism	Organism
Acinetobacter calcoaceticus	*Neisseria meningitidis,* group Y
Aeromonas hydrophila	*Proteus mirabilis*
Alcaligenes sp.	*Providencia rettgeri*
Bacillus subtilis	*Pseudomonas aeruginosa*
Bacteroides fragilis	*Pseudomonas cepacia*
Bordetella pertussis	*Salmonella,* group B
Branhamella catarrhalis	*Sarcina lutea* (ATCC 9341)
Brucella suis	*Serratia marcescens*
Campylobacter jejuni	*Shigella flexneri*
Candida albicans	*Staphylococcus aureus* (ATCC 25923)
Citrobacter freundii	*Staphylococcus epidermidis* (ATCC 27626)
Corynebacterium diphtheriae	*Staphylococcus epidermidis* (ATCC 14990)
Edwardsiella tarda	*Staphylococcus saprophyticus*
Enterobacter aerogenes	*Staphylococcus sciuri* (ATCC 29060)
Enterobacter cloacae	*Staphylococcus simulans* (ATCC 27851)
Escherichia coli	*Staphylococcus xylosus*
Gardnerella vaginalis	*Streptococcus* group A
Group II-F	*Streptococcus* group B
Haemophilus influenzae	*Streptococcus* group D
Haemophilus parainfluenzae	*Streptococcus bovis*
Klebsiella pneumoniae	*Streptococcus durans*
Lactobacillus sp.	*Streptococcus equinus*
Legionella pneumophila	*Streptococcus faecalis*
Leptospira sp.	*Streptococcus faecium*
Listeria monocytogenes	*Streptococcus mutans*
Neisseria sp. (sucrose positive)	*Streptococcus pneumoniae*
Neisseria gonorrhoeae	*Streptococcus sanguis* I
Neisseria lactamica	*Streptococcus sanguis* II
Neisseria meningitidis, group B	*Streptococcus,* viridans group
	Vibrio parahaemolyticus
	Yersinia enterocolitica

purchased is checked before being used for diagnostic purposes. For all media, each test also includes incubation of an uninoculated tube or plate to determine sterility. These samples are incubated 24 hr at 35°C for routine bacteriology and 48 hr at 35°C in an anaerobic chamber for anaerobic bacteriology. For anaerobic bacteria, biochemical tests are read after 48 hr or good growth has appeared, whichever is later. For other bacteria, results are read generally after 24 hr of incubation.

Table C-3. Performance Tests for Media and Reagents Used for Anaerobic Bacteria

Medium or reagent	Test or test organism	Acceptable result
Carbohydrate fermentations[a]		
Arabinose	*Veillonella parvula*	No color change (−)
	Bacteroides vulgatus	Yellow (+)
Cellobiose	*Veillonella parvula*	−
	Bifidobacterium adolescentis	+
Fructose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Glucose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Inositol	*Veillonella parvula*	−
	Clostridium perfringens	+
Lactose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Maltose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Mannitol	*Veillonella parvula*	−
	Bifidobacterium adolescentis	+
Mannose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Raffinose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Rhamnose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Sorbitol	*Veillonella parvula*	−
	Bifidobacterium adolescentis	+
Starch	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Sucrose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Trehalose	*Veillonella parvula*	−
	Bacteroides thetaiotaomicron	+
Xylose	*Veillonella parvula*	−
	Bacteroides vulgatus	+
Chopped meat slant	*Clostridium sporogenes*	Spores after 72 hr
Cycloserine-cefoxitin fructose-egg yolk agar (CCFA)	*Clostridium difficile*	Growth, yellow, flat, irregular edged colonies
	Clostridium sporogenes	No growth
	Peptostreptococcus anaerobius	No growth
	Escherichia coli	No growth
	Streptococcus group D	No growth

Table C-3 (*continued*)

Medium or reagent	Test or test organism	Acceptable result	
Disk identification tests for *Bacteroides fragilis*[b]		*Bacteroides fragilis*	*Fusobacterium nucleatum*
Bile		Growth	No growth
Colistin		R	S
Erythromycin		S	S
Kanamycin		R	S
Penicillin		R	S
Rifampin		S	S
Vancomycin		R	R or S
Egg yolk agar	*Clostridium perfringens*	Lecithinase production (opacity around growth)	
	Clostridium sporogenes	Lipase production ("waxy" colonies)	
Esculin broth[a] (add ferric ammonium citrate or Minitek disk)	*Fusobacterium nucleatum*	*No color*	
	Bacteroides vulgatus	Black	
Indole/nitrate broth[a]	*Fusobacterium nucleatum*	Indole positive Nitrate negative	
	Veillonella parvula	Indole negative Nitrate positive	
Kovacs' or Erhlich's reagents[a]	*Veillonella parvula*	No color	
	Fusobacterium nucleatum	Red	
Nitrate A and B reagents[a]	*Fusobacterium nucleatum*	Nitrate negative	
	Veillonella parvula	Nitrate positive	
Nitrocefin[c]	*Bacteroides fragilis*	Pink	
	Bacteroides melaninogenicus	No color	
Oxgall or bile disk	*Fusobacterium nucleatum*	No growth	
	Bacteroides fragilis	Stimulated growth	
Peptone-yeast (PY) broth	Indicator (vent)	Color change	
	Fusobacterium necrophorum *Fusobacterium mortiferum* *Fusobacterium symbiosum*	Baseline amounts of propionate and lactate for comparison with PYL and PYT	
Peptone-yeast-glucose (PYG) broth	Indicator (vent)	Color change	
	GLC	Record amounts of succinic and acetic acids present	

Table C-3 (*continued*)

Medium or reagent	Test or test organism	Acceptable result
Peptone-yeast-lactate (PYL) broth	Indicator (vent)	Color change
	Fusobacterium necrophorum	Increased propionate and decreased lactate
	Fusobacterium mortiferum	No change (GLC)
Peptone-yeast-threonine (PYT) broth	Indicator (vent)	Color change
	Fusobacterium necrophorum	Increased propionate
	Fusobacterium symbosium	No change (GLC)
Phenylethyl alcohol agar	*Escherichia coli*	Inhibited
	Peptococcus prevotii	growth
Rabbit blood agar	*Bacteroides fragilis*	Growth within 48 hr
	Clostridium perfringens	Hemolysis with double zone
	Peptostreptococcus prevotii	Growth within 48 hr
Rabbit blood agar (laked) with gentamicin and vancomycin (RGV)	*Bacteroides fragilis*	Growth
	Peptostreptococcus prevotii	No growth
	Bacteroides melaninogenicus	Black pigment within 3 to 5 days
Reversed CAMP	*Clostridium perfringens*	Enhanced hemolysis
	Clostridium bifermentans	No enhanced hemolysis
Sheep blood agar (Brucella agar base)	*Bacteroides fragilis*	Growth within 48 hr
	Clostridium perfringens	Hemolysis with double zone
	Peptostreptococcus prevotii	Growth within 48 hr
Supplemented thioglycollate broth	All quality control organisms	Growth within 48 hr
Thiogel	*Clostridium perfringens*	Not solidified after refrigeration
	Bacteroides fragilis	Solidified after refrigeration
Transport vial	Pigment	No color
	Introduce air	Pink
	Peptostreptococcus anaerobius	Survival for 3 hr at room temperature[d]
	Fusobacterium nucleatum	Survival for 3 hr at room temperature[d]

Table C-3 (*continued*)

Medium or reagent	Test or test organism	Acceptable result
Urease[a]	*Clostridium perfringens*	No color
	Clostridium sp. (urease positive)	Purple
X-ray film	*Bacteroides fragilis*	No removal of emulsion
	Clostridium perfringens	Removal of emulsion

[a] These tests apply equally to prepared media in tubes or to the Minitek system (BBL Microbiology Systems). Each batch of tubes is tested. Each shipment (single lot numbers) of Minitek supplies is tested. Minitek disks are checked for sterility by incubating in uninoculated broth.

[b] Test a complete set of disks weekly.

[c] Cefinase™ disk (BBL Microbiology Systems).

[d] Prepare inoculum by diluting an overnight thioglycollate broth culture to a density equal to that of a No. 0.5 McFarland standard with indole broth. Further dilute 1:10 with the same broth. Mix a 1 μl loopful of the diluted suspension in 1.0 ml of lactated Ringers solution and inject the whole amount into a vial (final inoculum approximately 0.5×10^4 CFU/ml). After 3 hr, remove 0.1 ml and inoculate onto sheep blood agar (Brucella agar base). Incubate for 48 hr under anaerobic conditions to determine survival.

Table C-4. Performance Tests for Media Used for the Isolation of Aerobic and Facultatively Anaerobic Bacteria

Medium	Test organism	Acceptable result
Brucella agar[a]	*Brucella suis*	Growth
Buffered charcoal yeast extract agar (BCYEα)[a]	*Legionella pneumophila*	Growth
Buffered charcoal yeast extract agar with antimicrobials (BVPAα)[a]	*Legionella pneumophila*	Growth
Campylobacter medium (C5)	*Campylobacter jejuni*	Growth
	Escherichia coli	No growth
Cefsulodin-irgasan-novobiocin agar (CIN)[a]	*Escherichia coli*	No growth
	Yersinia enterocolitica	Growth, clear colonies with dark pink centers
Chocolate blood agar[a]	*Haemophilus influenzae*	Growth
	Neisseria gonorrhoeae	Growth
Charcoal agar[a]	*Bordetella pertussis*	Growth
Colistin–nalidixic acid agar (CNA)[a]	*Escherichia coli*	No growth
	Streptococcus, group D	Growth
	Gardnerella vaginalis	Growth
Cystine tellurite agar[a]	*Corynebacterium diphtheriae*	Growth
Eosin–methylene blue (EMB) agar	*Shigella flexneri*	Growth (no color)
	Escherichia coli	Growth (green metallic sheen)

Table C-4 (*continued*)

Medium	Test organism	Acceptable result
Hektoen enteric (HE) agar	*Salmonella,* group B	Green colonies with black centers
	Shigella flexneri	Green colonies
	Escherichia coli	Yellow colonies
Human blood Tween-80 agar (HBT)[a]	*Escherichia coli*	No growth
	Gardnerella vaginalis	Growth with β-hemolysis
Leptospira medium (Fletcher)	*Leptospira*	Growth
Loeffler agar	*Corynebacterium diphtheriae*	Growth
MacConkey agar	*Escherichia coli*	Pink colonies
	Yersinia enterocolitica	Growth (no color)
Mueller–Hinton broth	*Neisseria meningitidis*	Growth
Nalidixic acid–polymyxin–crystal violet broth (NPC)	*Streptococcus,* group B	Growth
Rogosa medium	*Lactobacillus*	Growth
Selenite broth	*Salmonella,* group B	Growth
	Escherichia coli	Inhibition
Septi-Chek™ blood culture bottle[b]	*Candida albicans*	Growth
	Haemophilus influenzae	Growth
	Pseudomonas aeruginosa	Growth
	Streptococcus pneumoniae	Growth
Septi-Chek slide[b]	*Candida albicans*	Growth
	Haemophilus influenzae	Growth
	Pseudomonas aeruginosa	Growth
	Streptococcus pneumoniae	Growth
Sheep blood agar[a]	*Listeria monocytogenes*	Growth with β-hemolysis
	Streptococcus, group A	Growth with β-hemolysis
	Streptococcus, viridans group	Growth with α-hemolysis
	Streptococcus, group D	Growth without hemolysis
Thiosulfate citrate bile salts sucrose (TCBS) agar	*Vibrio parahaemolyticus*	Green colonies
Thioglycollate broth	*Bacteroides fragilis*	Growth
Thayer–Martin biplate (TM2, TM4)[a]	*Escherichia coli*	No growth
	Staphylococcus epidermidis	No growth on TM4
	Neisseria gonorrhoeae	Growth
	Neisseria meningitidis	Growth

Table C-4 (*continued*)

Medium	Test organism	Acceptable result
Tryptic soy broth blood culture bottles[b]	*Bacteroides fragilis*	Growth
	Klebsiella pneumoniae	Growth
	Pseudomonas aeruginosa	Growth
	Streptococcus, viridans group	Growth

[a] Prepare a suspension of organisms to a density equal to that of a No. 0.5 to 1.0 McFarland standard and dilute 1:10 twice with broth. Apply 1 μl of diluted suspension to test medium using a calibrated loop.

[b] Prepare a suspension of organisms to a density equal to that of a No. 0.5 to 1.0 McFarland standard. Add 0.5 ml of a 1:10^4 dilution of this suspension. For Septi-Chek™ bottle and slide, attach slide to bottle and tip bottle to inoculate slide.

Table C-5. Performance Tests for Media Used for the Identification of Aerobic and Facultatively Anaerobic Bacteria

Medium	Test organism	Acceptable result[a]
Acetate agar	*Escherichia coli*	+
	Edwardsiella tarda	−
Bile-esculin agar	*Streptococcus,* group D	Growth with blackening of area around colonies within 24 hr
	Streptococcus, viridans group	No color change (usually no growth)
Carbohydrate fermentation broth, Andrade's		
Adonitol	*Enterobacter aerogenes*	+
	Citrobacter freundii	−
Arabinose	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Dulcitol	*Citrobacter freundii*	+
	Enterobacter aerogenes	−
Fructose	*Citrobacter freundii*	+
	Pseudomonas aeruginosa	−
Glucose	*Citrobacter freundii*	+
	Pseudomonas aeruginosa	−
Inositol	*Enterobacter aerogenes*	+
	Citrobacter freundii	−
Lactose	*Citrobacter freundii*	+
	Providencia rettgeri	−
Maltose	*Citrobacter freundii*	+
	Providencia rettgeri	−
Mannitol	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Raffinose	*Citrobacter freundii*	+
	Edwardsiella tarda	−

Table C-5. (*continued*)

Medium	Test organism	Acceptable result[a]
Rhamnose	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Salicin	*Enterobacter aerogenes*	+
	Citrobacter freundii	−
Sorbitol	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Sucrose	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Trehalose	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Xylose	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Carbohydrate utilization media for *Neisseria*		
Glucose	*Neisseria gonorrhoeae*	+
	Branhamella catarrhalis	−
Maltose	*Neisseria meningitidis*	+
	Neisseria gonorrhoeae	−
Sucrose	*Neisseria* sp. (sucrose positive)	+
	Neisseria gonorrhoeae	−
Cetrimide	*Pseudomonas cepacia*	Growth
	Acinetobacter calcoaceticus	No growth
Citrate agar	*Citrobacter freundii*	+
	Edwardsiella tarda	−
Decarboxylase media		
Arginine	*Aeromonas hydrophila*	+
	Edwardsiella tarda	−
Lysine	*Edwardsiella tarda*	+
	Citrobacter freundii	−
Ornithine	*Edwardsiella tarda*	+
	Klebsiella pneumoniae	−
DNase agar	*Enterobacter aerogenes*	Growth with no pink zone
	Serratia marcescens	Growth with pink zone
Esculin	*Enterobacter aerogenes*	+
	Pseudomonas cepacia	−
Indole broth	*Providencia rettgeri*	+
	Enterobacter aerogenes	−
Lysine iron agar (LIA)	*Citrobacter freundii*	Alkaline slant/acid butt, H_2S
	Edwardsiella tarda	Alkaline slant/alkaline butt, H_2S
	Providencia rettgeri	Red slant/acid butt, no H_2S
MacConkey agar	*Pseudomonas cepacia*	Growth
	Group II-F	No growth

Table C-5. (*continued*)

Medium	Test organism	Acceptable result[a]
Malonate broth	*Enterobacter aerogenes*	+
	Edwardsiella tarda	−
Methyl red–Voges Proskauer (MR-VP) broth	*Citrobacter freundii*	MR, +; VP, −
	Enterobacter aerogenes	MR, −; VP, +
Motility agar	*Edwardsiella tarda*	+
	Klebsiella pneumoniae	−
Nitrate broth	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Oxidation-fermentation (OF) media		
Fructose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Glucose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Lactose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Maltose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Mannitol	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Sucrose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Xylose	*Pseudomonas cepacia*	+
	Acinetobacter calcoaceticus	−
Phenylalanine deaminase (PAD) agar	*Providencia rettgeri*	+
	Enterobacter aerogenes	−
Pigment F and P agar	*Pseudomonas aeruginosa*	+
	Pseudomonas cepacia	−
Salmonella-Shigella (SS) agar	*Pseudomonas aeruginosa*	Growth
	Acinetobacter calcoaceticus	No growth
Triple sugar iron agar (TSIA)	*Citrobacter freundii*	Acid slant/acid butt, gas, H_2S
	Pseudomonas cepacia	Alkaline slant/alkaline butt
	Providencia rettgeri	Alkaline slant/acid butt, no H_2S
Urea agar	*Providencia rettgeri*	4+
	Edwardsiella tarda	−
Veal infusion broth	*Streptococcus pneumoniae*	Growth and lysis with 4% Dreft
Xylose-purple agar	*Staphylococcus xylosus*	Yellow color
	Staphylococcus epidermidis	No color change

[a] Reactions: +, positive reaction as appropriate (e.g., acid production); −, negative or no reaction.

Table C-6. Performance Tests and Acceptable Profiles for Media Used with Replica Agar Testing

	Test Organism[b]			
Medium[a]	*Pseudomonas aeruginosa* (Mayo Clinic Strain)	*Escherichia coli* (ATCC 25922)	*Serratia marcescens*	*Proteus mirabilis*
Citrate	+	−	+	+ or −
Lysine decarboxylase	+	+	+	−
Ornithine decarboxylase	+ or −	+	+	−
Urease	−	−	−	+
DNase	−	−	+	−
Colistin	−	−	+	+
Cefazolin	+	−	+	−
H_2S	−	−	−	+
Bile-esculin	−	−	+	−
Arginine decarboxylase (dihydrolase)	+	+ or −	−	−
Glucose	−	+	+	+
Lactose	−	+	−	−
Sucrose	−	−	+	−
Mannitol	−	+	+	−
Inositol	−	−	+	−
Arabinose	−	+	−	−

[a] Media are listed in the order in which results are entered.
[b] Acceptable profiles: *Pseudomonas aeruginosa,* E240 or C40; *Escherichia coli,* 6035 or 6075; *Serratia marcescens,* EEAE; *Proteus mirabilis,* 1520 or 9520. Reactions: +, positive reaction or growth as appropriate; −, no reaction or no growth as appropriate.

Table C-7. Performance Tests for Reagents Used for the Identification of Aerobic and Facultatively Anaerobic Bacteria

Reagent or item	Test organism	Acceptable result
Sodium deoxycholate, 10% solution	*Streptococcus,* viridans group	No lysis
	Streptococcus penumoniae	Lysis
Ferric chloride reagent ($FeCl_3$)	*Providencia rettgeri*	Blue green color on phenylalanine deaminase agar (PAD)
	Enterobacter aerogenes	No color change on PAD
α-Glucosidase test substrate	*Gardnerella vaginalis*	Yellow color
	Enterobacter cloacae	No color

Table C-7. (*continued*)

Reagent or item	Test organism	Acceptable result
β-Glucosidase test substrate	*Gardnerella vaginalis*	No color
	Enterobacter cloacae	Yellow color
Gram stain	*Escherichia coli*	Pink to red rod shaped organism
	Staphylococcus aureus	Purple to blue coccal shaped organism
Hydrogen peroxide (H_2O_2) solution, 3%	*Aeromonas hydrophila*	Weak production of gas
	Streptococcus pneumoniae	No gas
Indole reagent (Kovacs')	*Providencia rettgeri*	Red in indole broth
	Enterobacter aerogenes	No color change in indole broth
KCl/HCl buffer	*Legionella penumophila*	Growth
Methyl red indicator	*Citrobacter freundii*	Red color in MR-VP broth
	Enterobacter aerogenes	No color change in MR-VP broth
Nalidixic acid disk, 30 μg	*Campylobacter jejuni*	Susceptible
Nitrate reagents	*Pseudomonas cepacia*	Positive
	Acinetobacter calcoaceticus	Negative
Novobiocin disk, 30 μg	*Staphylococcus aureus* (ATCC 25923)	Zone size 22–28 mm
ONPG test disks	*Neisseria lactamica*	Yellow color
	Neisseria gonorrhoeae	No color
Oxidase reagent and oxidase strips	*Aeromonas hydrophila*	Blue color
	Escherichia coli	No color change
PGUA test substrate	*Escherichia coli*	Yellow color
	Proteus mirabilis	No color
Porphyrin test (δ-amino levulinic acid substrate)	*Haemophilus parainfluenzae*	Red fluorescence
	Haemophilus influenzae	No red fluorescence
Rabbit plasma (slide and tube coagulase tests)	*Staphylococcus aureus*	Agglutination and coagulation
	Staphylococcus epidermidis	No agglutination and coagulation
Spot indole reagent	*Escherichia coli*	Turquoise color
	Proteus mirabilis	Pink color
SeroSTAT Streptococcus group D test kit	*Streptococcus faecalis*	Agglutination
	Streptococcus, viridans group	No agglutination

Table C-7. (*continued*)

Reagent or item	Test organism	Acceptable result
Staphyloslide™	*Staphylococcus aureus* with sensitized sheep red cells	Agglutination
	Staphylococcus aureus with negative control sheep red cells	No agglutination
	Staphylococcus, coagulase negative with sensitized sheep red cells	No agglutination
Voges-Proskauer reagents	*Enterobacter aerogenes*	Red color in MR-VP broth
	Citrobacter freundii	No color change in MR-VP broth
X and V strips	*Haemophilus influenzae*	Growth between strips

Table C-8. Performance Tests and Acceptable Results of API Staph-Ident™ Strip

	Test organism[a]			
Test	*S. aureus* (ATCC 25923)	*S. sciuri* (ATCC 29060)	*S. simulans* (ATCC 27851)	*S. epidermidis* (ATCC 14990)
Phosphatase	+	+ or −	−	+ or −
Urea utilization	+	−	+	+
β-Glucosidase	+	+	−	−
Mannose utilization	+	+ or −	−	− or +
Mannitol utilization	+	+	−	−
Trehalose utilization	+	+	+ or −	−
Salicin utilization	−	+	−	−
β-Glucuronidase	−	−	+	−
Arginine utilization	−	−	+	+
β-Galactosidase	−	−	+	−

[a] Reactions: +, positive reaction as appropriate; −, negative or no reaction.

Table C-9. Performance Tests for Media Used to Identify Streptococci

Medium	Test organism	Acceptable result
Carbohydrate fermentation broth[a]		
Arabinose	*Streptococcus faecium*	Yellow color
	Streptococcus faecalis	No color change
Lactose	*Streptococcus faecalis*	Yellow color
	Streptococcus equinus	No color change
Mannitol	*Streptococcus faecalis*	Yellow color
	Streptococcus sanguis I	No color change
Raffinose	*Streptococcus bovis*	Yellow color
	Streptococcus faecalis	No color change
Sorbitol	*Streptococcus faecalis*	Yellow color
	Streptococcus bovis	No color change
Hippurate hydrolysis	*Streptococcus,* group B	Color with ninhydrin (hydrolysis)
	Streptococcus, group A	No color with ninhydrin
1% Inulin	*Streptococcus sanguis* I	Yellow color
	Streptococcus, group D	No color change
	Streptococcus, group D	Acid with clot
	Streptococcus equinus	No color change
NaCl 6.5% broth	*Streptococcus faecalis*	Growth
	Streptococcus, viridans group	No growth
Potassium tellurite agar	*Streptococcus faecalis*	Growth
	Streptococcus, viridans group	No growth
SF broth	*Streptococcus faecalis*	Growth
	Streptococcus bovis	No growth
Sucrose agar	*Streptococcus,* viridans group	Growth
Sucrose broth, 5%	*Streptococcus sanguis* II	Growth
	Streptococcus mutans	Crystalline deposit at 48 hr
	Streptococcus sanguis I	Gel effect
Todd-Hewitt broth with glucose	*Streptococcus,* group A	Growth
Todd-Hewitt broth without glucose	*Streptococcus,* viridans group	Growth

[a] Heart infusion broth base with bromcresol purple indicator and final carbohydrate concentration of 1%.

Table C-10. Performance Tests for Fluorescent Antibody and Serological Tests Used in Bacteriology

Item[a]	Test organism or material	Acceptable result
Fluorescent conjugates		
Streptococcus, group A	*Streptococcus,* group A	Fluorescence
	Streptococcus, group C	No fluorescence
	Streptococcus, group G	No fluorescence
	Streptococcus aureus	No fluorescence
Listeria monocytogenes	Listeria types 1 to 4	Fluorescence
	Normal rabbit globulin	No fluorescence
Neisseria gonorrhoeae	*Neisseria gonorrhoeae*	Fluorescence
	Boiled suspension of *Enterobacter cloacae*	No fluorescence
Neisseria meningitidis	*Neisseria meningitidis*	Fluorescence
	Normal rabbit serum	No fluorescence
Streptococcus pneumoniae	*Streptococcus pneumoniae*	Fluorescence
	Normal rabbit globulin	No fluorescence
Haemophilus influenzae, type b	*Haemophilus influenzae,* type b	Fluorescence
	Normal rabbit globulin	No fluorescence
Brucella	Homologous *Brucella* strain	Fluorescence
	Normal rabbit globulin	No fluorescence
Bordetella pertussis	*Bordetella pertussis*	Fluorescence
	Normal rabbit globulin	No fluorescence
Legionella pneumophila (direct)	Positive smear	Fluorescence
	Negative control	No fluorescence
Legionella pneumophila (indirect)	Positive control serum	Positive at $\geq$1:512
	Negative control	Negative
Streptococcal antisera	Homologous strain	Precipitate
Antisera for counterimmunoelectrophoresis		
Haemophilus influenzae, type b	*H. influenzae,* type b antigen, 0.04 μg/ml	Precipitin line
Streptococcus pneumoniae polyvalent	*S. pneumoniae,* type 3 antigen, 0.1 μg/ml	Precipitin line
Neisseria meningitidis, group A	*N. meningitidis,* group A antigen, 0.1 μg/ml	Precipitin line
Neisseria meningitidis, group B	*N. meningitidis,* group B culture supernatant, 1:2 dilution	Precipitin line
Neisseria meningitidis, group C	*N. meningitidis,* group C antigen, 0.05 μg/ml	Precipitin line
Neisseria meningitidis, polyvalent groups X–Z	*N. meningitidis,* group Y control dilution	Precipitin line
Streptococcus, group B	*Streptococcus,* group B antigen (Difco)	Precipitin line

[a] All turbid antisera are filtered or centrifuged to remove denatured proteins or contaminating organisms.

4. Performance Testing of Reagents and Sera Used for Antimicrobial Susceptibility Tests and Assays

Horse Serum

Purpose:
To detect antimicrobial activity and microbial contamination of horse serum. Each lot number of horse serum is tested for antimicrobial activity and each bottle is tested for sterility.

Procedure:

1. Saturate a paper disk, 12.7 mm in diameter (Schleicher and Schuell, cat. no. 740E), with the serum to be tested.
2. Place the saturated disk on a penicillin assay plate containing *Sarcina lutea* ATCC 9341 as the test organism (Section 12.1).
3. Incubate the plate for 18 hr at 30°C.
4. Check plate for zone of inhibition.
5. To test for sterility, remove 1 ml from each bottle of serum as it is to be used and inoculate it into 10 ml of thioglycollate broth. Incubate the broth for 3 days at 35°C.
6. If zone of inhibition is present, or if growth is detected in the thioglycollate broth, either discard the entire lot or bottle or return to the company for credit.
7. Record results and action taken, if any.

Human Serum

Purpose:
To test human serum for the presence of hepatitis B antigen (HB_sAg), antimicrobial activity, sterility, and the presence of inhibitors or inactivators of penicillin or cephalosporins.

Procedure:

1. Test each lot number for HB_sAg by an approved, third generation test.
2. Test each lot number for antimicrobial activity and each bottle for sterility as described above for horse serum.
3. To test for inhibitors or inactivators of penicillins or cephalosporins, prepare the assay reference concentration of penicillin and cefazolin (Table 12.1–4) in the serum to be tested. Assay these solutions according to the appropriate method (Section 12.1). If there is a loss in activity

of either the penicillin or cefazolin, discard the entire lot or return to the company for credit.

$CaCl_2$ Assay Plates

Purpose:
To test for complete inhibition of aminoglycoside activity. Each batch of plates is tested.

Procedure:

1. Impregnate a paper disk, 6.25 mm in diameter (Schleicher and Schuell, cat. no. 740E) with 20 μl of a solution containing 100 μg/ml of streptomycin. Impregnate a second disk with a solution containing 20 μg/ml of gentamicin.
2. Apply both disks to a penicillin assay plate containing $CaCl_2$ (Table 12.1–6).
3. Incubate for 16 hr at 30°C and examine for zones of inhibition.
4. In any zone of inhibition is present, discard the entire batch of agar.
5. Record results.

β-Lactamases

Purpose:
To test for sterility and for penicillinase and cephalosporinase activities. Test each lot of enzyme for activity and each bottle for sterility.

Procedure:

1. Mix 1.6 ml of a solution containing 100 μg/ml of penicillin G in water or buffer with 0.4 ml of a penicillinase solution containing 10×10^6 kinetic (Kersey) units per ml (BBL Microbiology Systems, cat. no. 11898).
2. Incubate the mixture for 20 min at 35°C.
3. Impregnate a paper disk, 6.25 mm in diameter, with 20 μl of the mixture and place on a penicillin assay plate (Table 12.1–6).
4. Incubate the plate for 16 hr at 30°C.
5. After incubation, check for a zone of inhibition.
6. To test for sterility, inoculate 10 ml of thioglycollate broth with 1 ml of penicillinase solution. Incubate for 3 days at 35°C and check for growth.
7. If microbial growth or a zone of inhibition is detected, discard or return the penicillinase to the company for credit.

8. The broad spectrum of β-lactamase from *Enterobacter cloacae* (p. 706) is tested for sterility and activity against cefazolin in an analogous manner to the test for penicillinase. To test sterility, inoculate 0.1 ml of the β-lactamase into 10 ml of thioglycollate broth and incubate for 3 days at 35°C.

C. Mycology and Mycobacteriology

1. Maintenance of Stock Cultures and Serum Controls

Organisms are stored as suspensions in sterile water, on agar slants, or frozen. The water suspensions are contained in tubes sealed with paraffin and are stored at room temperature. This method has been shown to be reliable for storing fungi. Our experience with mycobacteria is limited to only 1 year, during which time no problems have occurred; however, a back-up method should still be used. Organisms are stored frozen at −50°C or lower in a mixture of equal parts of horse serum and Trypticase soy broth (BBL Microbiology Systems). Serum controls are stored at −50°C or lower.

2. Stock Organisms Required for Testing Media and Reagents

Stock cultures required for performance testing of media and reagents used in mycology are listed in Table C-11.

3. Performance Testing of Media and Reagents

Tables C-12 through C-14 list the organisms and expected reactions for performance tests of media and reagents used in mycology and mycobac-

Table C-11. Stock Cultures Used for Testing Media and Reagents Used in Mycology

Organism	Organism
Aspergillus fumigatus	*Nocardia asteroides*
Aspergillus versicolor	*Saccharomyces cerevisiae*
Candida albicans	*Staphylococcus aureus*
Candida glabrata	*Streptomyces* sp.
Candida parapsilosis	*Trichosporon beigelii*
Candida tropicalis	*Trichophyton mentagrophytes*
Cryptococcus albidus	*Trichophyton rubrum*
Cryptococcus neoformans	*Trichophyton tonsurans*
Klebsiella pneumoniae	

Table C-12. Performance Tests for Media and Reagents Used for the Isolation and Identification of Aerobic Actinomycetes and Fungi

Medium or reagent	Test organism	Acceptable result
Acid-fast stain	*Nocardia asteroides*	Acid-fast positive
	Streptomyces sp.	Acid-fast negative
Amphotericin B	*Saccharomyces cerevisiae* (ATCC 9763)	No growth at 0.2 or 0.4 μg/ml
Ascospore agar	*Saccharomyces cerevisiae*	Ascospore formation
	Candida albicans	No ascospore formation
Bennett's medium	*Nocardia asteroides*	Growth
	Streptomyces sp.	Growth
Bird seed agar	*Cryptococcus neoformans*	Growth with brown pigment
	Cryptococcus albidus	Growth with no pigment
Brain heart infusion agar	*Candida albicans*	Growth
	Cryptococcus neoformans	Growth
	Aspergillus fumigatus	Growth
	Nocardia asteroides	Growth
Brain heart infusion agar with blood and antibiotics (chloramphenicol, gentamicin, with or without cycloheximide)[a]	*Candida albicans*	Growth
	Cryptococcus neoformans	Growth (except on agar with cycloheximide)
	Aspergillus fumigatus	Growth (may be only partially inhibited with cycloheximide)
	Klebsiella pneumoniae	No growth
	Staphylococcus aureus	No growth
Casein agar	*Nocardia asteroides*	No hydrolysis
	Streptomyces sp.	Hydrolysis
Cornmeal agar	*Trichophyton rubrum*	Growth
Cornmeal Tween-80 agar	*Candida albicans*	Chlamydospore production and hyphae
Czapek agar	*Aspergillus fumigatus*	Conidial heads typical for *Aspergillus fumigatus*
L-Dopa disk	*Cryptococcus neformans*	Pigment
	Cryptococcus albidus	No pigment
Flucytosine	*Saccharomyces cerevisiae* (ATCC 9763)	No growth at 0.02 μg/ml
Inhibitory mold agar (plate)[a]	*Candida albicans*	Growth
	Cryptococcus neoformans	Growth
	Aspergillus fumigatus	Growth
	Klebsiella pneumoniae	No growth
	Staphylococcus aureus	No growth
Inhibitory mold agar (slant)	*Aspergillus fumigatus*	Growth at 45°C
	Aspergillus versicolor	No growth at 45°C
Mycobiotic agar[a]	*Cryptococcus neoformans*	No growth
	Staphylococcus aureus	No growth
	Trichophyton rubrum	Growth

Table C-12. (*continued*)

Medium or reagent	Test organism	Acceptable result
Nigrosin	*Cryptococcus neoformans*	Capsules
	Candida albicans	No capsules
Nitrate broth	*Cryptococcus albidus*	Nitrate positive
	Cryptococcus neoformans	Nitrate negative
Potato dextrose agar	*Trichophyton rubrum*	Red pigment
	Trichophyton mentagrophytes	No pigment
Sabouraud's dextrose agar[a]	*Candida albicans*	Growth
	Cryptococcus neoformans	Growth
	Aspergillus fumigatus	Growth
	Nocardia asteroides	Growth
Sheep serum	*Candida albicans*	Germ tube formation
	Candida tropicalis	No germ tube formation
Trichophyton agars #1, #2, #3, #4	*Trichophyton tonsurans*	#1, #2: 1+growth #3, #4: 4+growth
	Trichophyton rubrum	Growth on all four media
Tryosine agar	*Nocardia asteroides*	No hydrolysis
	Streptomyces sp.	Hydrolysis
Urea R broth	*Cryptococcus neoformans*	Pink within 4 hr (positive)
	Candida albicans	No color change in 4 hr
Xanthine agar	*Nocardia asteroides*	No hydrolysis
	Streptomyces sp.	Hydrolysis

[a] Prepare a suspension of organisms to a density equal to that of a No. 0.5 to 1.0 McFarland standard and dilute 1:10 twice. Apply 1 μl of diluted suspension to test medium using a calibrated loop. Inoculate media with *Trichophyton rubrum, Nocardia asteroides,* and *Aspergillus fumigatus* without dilution in the usual manner.

teriology. Generally, each batch or lot of media or reagents used in mycology is tested before use. Sheep serum, which is used for detecting germ tube formation, is tested daily with each use. For reagents used in mycobacteriology, a positive and negative control (Table C-14) is tested with each clinical specimen. Positive and negative controls for serological tests in mycology are also done concurrently with each specimen or batch of specimens.

D. Parasitology

Performance tests for reagents and stains used in parasitology are described in Table C-15. A culture of *Entamoeba histolytica* is maintained on a charcoal agar slant containing rice starch and is transferred weekly. Clinical specimens that contain protozoa are fixed in formalin or polyvinyl

Table C–13. Performance Tests and Acceptable Profiles for the API 20C Yeast Test Strip

Test	Test organism[a]		
	Candida parapsilosis	*Candida glabrata*	*Trichosporon beigelii*
Glucose (GLU)	+	+	+
Glycerol (GLY)	+	−	+
2-Keto-D-gluconate (2KG)	+	−	+
Arabinose (ARA)	+	−	+
Xylose (XYL)	+	−	+
Adonitol (ADO)	+	−	+
Xylitol (XLT)	−	−	+
Galactose (GAL)	+	−	+
Inositol (INO)	−	−	+
Sorbitol (SOR)	+	−	+
Methyl-D-glucoside (MDG)	+	−	+
N-Acetyl-D-glucosamine (NAG)	+	−	+
Cellobiose (CEL)	−	−	+
Lactose (LAC)	−	−	+
Maltose (MAL)	+	−	+
Sucrose (SAC)	+	−	+
Trehalose (TRE)	+	+	+
Melezitose (MLZ)	+	−	+
Raffinose (RAF)	−	−	+

[a] Acceptable or usual profile numbers: *Candida parapsilosis,* 6–756–171; *Candida glabrata,* 2–000–040; *Trichosporon beigelii,* 6–777–773.

alcohol (PVA) and are stored in a refrigerator until needed. Blood smears from known cases of malaria usually are not available to test the performance of the Giemsa stain. This stain, which is obtained commercially prepared, is tested using a routine thin blood smear.

E. Virology

Performance tests for media and cell cultures used to isolate or identify viruses, *Mycoplasma,* and *Chlamydia* are described in Table C–16. Generally, these tests are performed with each new batch or lot of media or cell cultures. The test for *Chlamydia* isolation with McCoy cells is performed with each run, and tests with MRC-5 and primary rhesus monkey kidney cells for virus isolation are performed monthly. Known

Table C–14. Performance Tests for Media and Reagents Used for the Isolation and Identification of Mycobacteria

Medium, Reagent, or Test	Test organism	Acceptable result
Arysulfatase	*M. avium-intracellulare*	Negative
	M. fortuitum	Positive (pink to red color)
Catalase (68°C)	*M. tuberculosis* H37RA	Negative
	M. fortuitum	Positive (bubble production)
Fluorochrome stain	*Streptomyces* sp.	No fluorescence
	M. tuberculosis H37RA	Fluorescence
Kinyoun acid-fast stain	*Streptomyces* sp.	Blue branching or bacilli forms
	M. tuberculosis H37RA	Red bacilli
Light exposure	*M. avium-intracellulare*	No pigment production
	M. kansasii	Pigment production (yellow to orange)
Lowenstein medium	*M. tuberculosis* H37RA	Growth
Middlebrook S7H11	*M. tuberculosis* H37RA	Growth
	Escherichia coli	No growth
	Pseudomonas aeruginosa	No growth
Middlebrook 7H9 medium	*M. avium-intracellulare*	Homogeneous growth
	M. tuberculosis H37RA	Rough growth with clumping of cells
Middlebrook 7H10 agar	*M. tuberculosis* H37RA	Growth
Niacin	*M. avium-intracellulare*	Negative
	M. tuberculosis H37RA	Positive (yellow pigment)
Nitrate reduction	*M. avium-intracellulare*	Negative
	M. kansasii	Positive
Semiquantitative catalase	*M. tuberculosis* H37RA	Weak positive
	M. fortuitum	Positive
Sodium chloride tolerance test	*M. avium-intracellulare*	Growth of <50 colonies
	M. fortuitum	Growth of >50 colonies
Tellurite reduction	*M. kansasii*	Negative
	M. avium-intracellulare	Dark pigment
Thiophene-2-carboxylic acid hydrazide (TCH) susceptibility	*M. tuberculosis* H37RA	Growth at ≤ 1 μg/ml
	M. bovis	No growth at ≤ 1 μg/ml
Tween-80 hydrolysis	*M. avium-intracellulare*	Negative
	M. kansasii	Positive
Urease	*M. avium-intracellulare*	Negative
	M. kansasii	Positive (pink color)

Table C–15. Performance Tests for Reagents and Stains Used in Parasitology

Stain or reagent	Test or test organism	Acceptable result
Eosin-saline solution	*Entamoeba histolytica* (trophozoites)	Background staining with viable amoeba
Giemsa stain	Blood smear	Clear and distinct staining of white blood cells and red blood cells
Iodine solution	Formalin-treated specimen with protozoan cysts	Cyst nuclei should stain and become readily visible
Modified Kinyoun stain	*Cryptosporidium*	Purple staining bodies 2–6 μm in size
Polyvinyl alcohol (PVA) fixative	Physical appearance	Colorless and fluid
	Entamoeba histolytica (trophozoites)	Good preservation
Trichrome stain	PVA-fixed specimen positive for amoeba	Nuclear characteristics clearly stained

Table C–16. Performance Tests for Media and Cell Cultures Used for the Isolation of Chlamydiae, Mycoplasmas, and Viruses

Medium or cell culture	Test or test organism	Acceptable result
Chlamydia isolation		
Eagle's MEM (*Chlamydia*)	Sterility	No growth
McCoy's cells	*Mycoplasma* contamination	No growth
	Chlamydia trachomatis	Growth
Mycoplasma isolation		
Large colony agar	Sterility (incubate 1 week at 36°C)	No growth
	Mycoplasma pneumoniae	Growth
Ureaplasma urealyticum agar	Sterility	No growth
	Ureaplasma urealyticum	Growth
Virus isolation:		
Eagle's BME (virus)	Sterility	No growth
MRC-5	*Mycoplasma* contamination	No growth
	Herpes simplex virus	Characteristic cytopathic effects
Primary rhesus monkey (primary cynomolgus monkey) kidney	Parainfluenza virus	Characteristic cytopathic effects and hemadsorption
	Adenovirus	Characteristic cytopathic effects
	Hemadsorption (not infected)	No hemadsorption

positive and negative control serums are run concurrently with each serological test.

III. Equipment

A. General Considerations

A complete quality control program for laboratory equipment must include preventive maintenance and performance checks on a regular schedule. Records must be maintained to enable one to determine readily when and by whom equipment was checked last and whether performance was satisfactory. The records also provide maintenance and performance histories that are often useful in solving problems when they arise. Each person using a piece of equipment must be familiar with how to determine that performance is satisfactory. For major equipment, e.g., chromatographs and spectrophotometers, the records should also include an operator's manual, a service manual with a list of replacement parts, calibration procedures, serial number and other identifying numbers, date installed, date of next scheduled service, and a telephone number to call for service.

B. Incubators

Incubator temperature must be checked and recorded daily, and acceptable ranges established. If CO_2 is added, the concentration should be checked every other day. A Fyrite analyzer (Bacharach Instrument Co.) can be used to measure the CO_2 concentration. A record should also be made of the cylinder pressure and date of installation to help predict when replacement will be necessary and to detect abnormally high use, which may be caused by leaks. Incubators should be cleaned periodically (e.g., quarterly), and servicing of circulation fans and humidity controls should be included in maintenance procedures.

C. Refrigerators and Freezers

Temperature must be checked and recorded daily and acceptable ranges established. Refrigerators and freezers should be cleaned periodically. If specimens are being stored, there should be an established procedure for decontamination. Freezers should not be self-defrosting.

D. Autoclaves

Autoclaves must have accurate temperature and pressure gauges for control of sterilization procedures. Performance should be checked weekly with spore strips or ampules (Kilit, BBL Microbiology Systems) and results recorded. Heat-sensitive tape should be used with every run.

E. Hot-Air Ovens

Operating temperatures should be checked weekly to confirm the function of the thermostat control.

F. Water Baths and Heating Blocks

Water baths must be kept scrupulously clean. Before routine use, the temperature of a heating block should be measured in each corner and in the center to ensure that there are no hot spots. Temperature should be monitored daily.

G. Thermometers

All new thermometers used for critical temperature measurements (e.g., refrigerators, incubators, water baths, heating blocks) must be calibrated against a reference thermometer that has calibration traceable to a National Bureau of Standards reference. The deviation from the reference thermometer should be recorded and generally should be within ± 1°C. Calibration should be repeated yearly. A common source of error, often undetected, is separation of the mercury column. To correct this, the mercury column should be retracted fully into the bulb by placing the thermometer in an ice-salt or Dry Ice-acetone bath. Calibration should be repeated after this procedure.

H. pH Meters

Before each use, standardized buffer solutions (pH 4.0 and 7.0) should be used to calibrate the instrument. A standard at pH 10.0 should be used when the instrument is going to be used to measure alkaline solutions. When not in use, place electrodes in saturated KCl or distilled water, depending on the recommendation of the manufacturer. To prevent polarization of electrodes, they should never be removed from a solution when the instrument is in the measuring mode. The electrodes

should be cleaned monthly. Protein deposits may be removed by alternately dipping electrodes in 0.1 mol/liter NaOH and 0.1 mol/liter HCl followed by a thorough rinse with distilled water. A 5% solution of NaOCl (Clorox) works well for removing deposits of blood. Never place electrodes in an autoclave. When necessary, electrodes can be sterilized with a 5% solution of phenol or NaOCl. Yearly, the instrument should be thoroughly cleaned, and an antistatic solution should be applied to the face of the indicating meter, if appropriate.

I. Centrifuges

The centrifugal speed should be checked annually with an accurate tachometer, and other maintenance appropriate to the type of motor and braking system should be done. Centrifuges that are used for specimens should be disinfected with a 5% solution of phenol or with Amphyl at least weekly.

J. Balances

Balances must be kept clean and level, and should be protected against temperature variations, vibration, and high humidity. Preventive maintenance should be performed annually and should include a check of accuracy using National Bureau of Standards Class S weights.

K. Biological Safety Cabinets

Specific instructions of the manufacturer should be followed in regard to operation and maintenance, including correct air velocity, frequency of and methods for changing filters, and decontamination. Generally, daily disinfection is recommended.

L. Microscopes

Daily, clean all lenses with lens paper. If the oil immersion lens was used, clean the lens with lens paper that has been lightly moistened with xylene, then wipe dry with a clean piece of lens paper. Preventive maintenance should be performed every 6 months and should include an overall inspection with adjustment and lubrication of all necessary parts.

M. Pipettes and Dilutors

The appropriate pipette for the type of work being performed must be used. For example, a volumetric pipette, rather than a serological one, should be used for delivering relatively large volumes accurately. Pipettes of the Eppendorf type should be calibrated monthly by a gravimetric method. Dilutors should be tested by gravimetric method or other method appropriate to the volume delivered

References

1. Barlett, R. C., Allen, V. D., Blazevic, D. J., Dolan, C. T., Dowell, V. R., Gavan, T. L., Inhorn, S. L., Lombard, G. L., Matsen, J. M., Melvin, D. M., Sommers, H. M., Suggs, M. T., and West, B. S. Clinical Microbiology. *In* Inhorn, S. L. (ed.), *Quality Assurance Practices for Health Laboratories.* Washington, D.C., American Public Health Association, 1978, p. 871.
2. Blazevic, D. J., Hall, C. T., and Wilson, M. E. *Cumitech 3: Practical Quality Control Procedures for the Clinical Microbiology Laboratory.* Balows, A., (coordinating ed.). Washington, D.C., American Society for Microbiology, 1976.
3. College of American Pathologists Commission on Inspection and Accreditation: Inspection Checklist, Section IV, Microbiology. Skokie, IL, College of American Pathologists, 1983, checklist item no 04.1590.
4. Moroney, M. J. *Facts from Figures.* Baltimore, Penguin Books, 1951, p. 82.
5. Prier, J. E., Bartola, J. T., and Friedman, H. (eds.). *Quality Control in Microbiology.* Baltimore, University Park Press, 1975.

Index

A

Abscess, 35, 40, 56, 57, 74, 75, 98–99, 102
Absidia, 451, 452
Acetate agar, 770
Acetate utilization test, 205–206
Achromobacter xylosoxidans, 224, 225
Acid-fast stain, 76–77, 390–393, 412–415
Acidaminococcus fermentans, 319
Acidimetric test, 310
Acinetobacter calcoaceticus, 224, 225
Acquired immune deficiency syndrome, 762
Acremonium, 420, 456
Actinobacillus actinomycetemcomitans, 224, 226
Actinomyces, 329, 369–371
Actinomycetales, 379, 390
Actinomycetes, 411–417
Adenovirus, 604–610
Aerobic bacteria
  antimicrobial susceptibility tests of, 281–310
  gram-negative bacilli, 179–241
  gram-negative cocci, 157–164
  gram-positive bacilli, 164–179
  gram-positive cocci, 131–156
  identification of, 131–241
  media for, 770–800, 837
Aerococcus viridans, 156
Aeromonas, 211, 214
Agarose solutions, 821
Albert stain, 89–90
Alcaligenes, 225
Alsever's solution, 821
Alternaria, 420, 482
Amebae, 630, 640–641
Aminoglycoside assay, 710–720
Amphotericin B susceptibility test, 492–494
ANIDENT system, 372
Anaerobic bacteria
  antimicrobial susceptibility tests of, 373–377
  Bacteroides bivius, 346–347, 348–349
  Bacteroides disiens, 346–347, 348–349
  Bacteroides fragilis group, 331–341
    antibiotic disk identification of, 335–336
    bile tolerance test of, 337
    carbohydrate fermentation test of, 341
    catalase test of, 338–339
    esculin hydrolysis of, 340
    growth characteristics of, 331
    identification of, 346
    indole test of, 339–340
    lactamase test of, 335
  Bacteroides melaninogenicus group, 341–344
  Bacteroides ruminicola, 346–347, 348
  Bacteroides ureolyticus, 345–347

Anaerobic bacteria (*cont.*)
 Capnocytophaga, 352–353
 Clostridium, 357–369
  disk antibiogram of, 362
  fecal isolation and assay of, 363–364, 369
  growth characteristics of, 359
  identification of, 358, 359–369
  lecithinase vs non-lecithinase producing, 362–363
  spore detection of, 359–362
 cocci, 318–319, 353–356
 cultures for, 21, 98
 facultatively anaerobic bacteria, *see* Aerobic bacteria
 Fusobacterium, 349–352
 general considerations, 315–321
 gram-negative bacilli, 317–318
  Bacteroides, see Anaerobic bacteria, *Bacteroides*
  Capnocytophaga, 352–353
  Fusobacterium, 349–352
 gram-positive bacilli, 317
  Clostridium, 357–369
  non-spore forming, 369–371
 identification of, 330–373
  ANIDENT system for, 372
  adequate growth for, 330
  flow chart for, 334
  reporting results for, 330–331
  tests required for, 331, 332–333
 infections caused by, 319–321
 isolation of, 321–329
  Actinomyces cultures for, 329
  aerotolerance determination in, 328
  anaerobic glove box for, 324–325
  capnophilic streptococci subcultures in, 328–329
  clues to, 321–322
  culture examination for, 324
  culture incubation for, 322–325
  jars for, 322–324
  laboratory workcard for, 325–328
  PRAS media for, 325
  procedures for, 325–329
 media for, 325, 800–808, 837
 specimen transport for, 21
 taxonomy and description of, 316–319
Anaerobic glove box, 324–325
Anaerobic jars, 323–324
Anaerobic transport, 21
Animal inoculation test, 175
Antibiotic disk identification test, 335–336
Antibiotic solutions, 821–824
Antimicrobial susceptibility tests
 of aerobic and facultatively anaerobic bacteria, 281–310
  acidimetric test, 310
  antimicrobial selection for, 285–287
  antimicrobial solution preparation for, 288–291
  β-lactamase detection, 305–310
  chromogenic cephalosporin test, 308
  dilution tests, agar, 293–303
  dilution tests, broth, 303
  disk-diffusion tests, 303–305
  disk-elution tests, 305
  general considerations, 281–282
  incubation for, 285
  indications for, 283
  inoculum for, 285
  iodometric slide, 309
  media for, 283–284
  procedures for, 291–310
  quality control for, 287–288
  susceptibility definitions, 282–283
  variables in, 283–287
 of anaerobic bacteria, 373–377
  broth-disk elution, 374–375
  indications for, 373
  methods for, 373–379
  microbroth dilution, 375–377
 of *Chlamydia,* 513–514
 of fungi, 487–497
 of mycobacteria, 408–411
 of mycoplasmas, 532
Antisepsis, 4, 26
Arboviruses, 571
Arylsulfatase production tests, 397–398
Ascaris, 637
Aseptic meningitis, 14, 34
Aspergillus, 80, 420, 456–457
Assays, antimicrobial
 aminoglycoside, 710–720
 bioassays, 695–709
  broth-dilution, 707–709

disk-plate, 697–707
general considerations, 695–697
inactivation of beta-lactam antibiotics for, 704–707
chloramphenicol, 723–726
indications for, 691–692
liquid chromatographic, 709–726
methodology, 695
specimen collection and handling for, 693
specimen timing for, 692–693
vancomycin, 720–723
Auramine-rhodamine stain, 85–86
Aureobasidium pullulans, 482
Autoclaves, 752, 862
Autopsy tissue specimen, 64

B

Babesia, 31, 659–665
Bacilli
gram-negative, aerobic and facultatively anaerobic, 179–241
gram-negative, anaerobic, 316, 317–318
gram-positive, aerobic and facultatively anaerobic, 164–179
gram-positive, anaerobic, 316–317
Bacillus anthracis, 177, 178
Bacillus cereus, 176, 178
Bacitracin inhibition test, 143–144
Bacteremia
anaerobic, 321, 357, 372
etiology of, 24
specimen collection with, 25–30
Bacteria
aerobic, *see* Aerobic bacteria
anaerobic, *see* Anaerobic bacteria
facultatively anaerobic, *see* Aerobic bacteria
general classification of, general, 127–130
growth characteristics of, 127, 128
Bacterial cultures
anaerobic, 321–329
of blood, 24–30
of body fluids, tissue, wounds, and abscesses, 98–99, 100, 101–102, 103
of feces, 113–114
of genital tract infections, 116–119
of intravascular catheter tips, 102
quality control on, 837–854
quantitative, 101–102
respiratory tract infections, 103, 104–105, 106, 107, 110
streaking plates for, 100
from urine, 110, 111
Bacterial vaginosis, 59
Bactericidal tests, 731–743
combination studies, 739–743
minimal bactericidal concentration, 732–735
serum inhibitory titer and bactericidal titer, 735–739
Bacteriuria, detection of, 82
Bacteroides bivius, 346–347, 348–349
Bacteroides disiens, 346–347, 348–349
Bacteroides fragilis group
antibiotic disk identification of, 335–336
β-lactamase tests of, 335
bile tolerance test of, 337
carbohydrate fermentation test of, 341
catalase test of, 338–339
esculin hydrolysis of, 340
general considerations, 317, 331
growth characteristics of, 331
indole test of, 339–340
infections caused by, 320, 321
microscopic appearance of, 321
Bacteroides melaninogenicus group
gas liquid chromotography of, 342–344
general considerations, 317–318, 341–342
growth characteristics of, 342
identification tests for, 342
microscopic appearance of, 322
Bacteroides ruminicola, 346–347, 348
Bacteroides ureolyticus, 345–347
Baerman digestion technique, 676
Balantidium coli, 632, 644
Beaver egg-counting technique, 677
Bennett's agar, 808
β-lactamase tests
acidimetric test, 310
as antimicrobial susceptibility test, 305–310

β-lactamase tests (*cont.*)
of *Bacteroides* groups, 335
chromogenic cephalosporin test, 308
iodometric slide test, 309
organisms identified by, 305, 335
Bifidobacterium, 369–371
Bile solubility test, 151–152
Bile tolerance test, 337
Bile-deoxycholate medium, 800–801
Bile-esculin agar, 770
Bile-esculin test, 148–149
Bioassays, *see* Assays, antimicrobial, bioassays
Biopsy
lung, 43, 77–82, 106
for parasite examination, 671–672
for specimen contamination prevention, 4–5
Blastomyces dermatitidis
colonial morphology of, 448
exoantigen microimmunodiffusion test of, 472
growth rate of, 446
infections associated with, 420
mold form of, 473, 481
serologic tests for, 487
yeast form of, 473, 481
Blood agar, 771
Brucella, 801
Columbia colistin-nalidixic acid, 777–778
cystine tellurite, 778
gentamicin-vancomycin laked, 804
phenylethyl alcohol, 788
Blood cultures, 24–30, 95–97
Blood or blood products, 121
Blood smears, 32–34, 633–634, 636, 637, 666–669
Blood specimen collection, 6, 26–27
Bordetella, 37, 104, 225, 234–235, 267
Brain abscess, 34–35, 74, 319
Brain heart infusion agar, 811
Branhamella catarrhalis, 157–164, 204
Broth-dilution bioassays, 707–709
Brucella, 6, 98, 233, 267
Brucella blood agar, 801
Brucella broth, 771
Brugia malayi, 661, 668
Buffered charcoal yeast extract agar, 771–772

C

Calcium chloride assay plates, 854
California encephalitis virus, 571, 572, 579–580
CAMP test, 146–147
Campylobacter, 145, 215–217, 772–773
Candida, 116
germ tube production of, 440–441
identification of, 427
infections associated with, 83, 424, 425
morphology of, on inhibitory mold agar, 430
urease production by, 428
Capnocytophaga, 317, 352–353
Capsule stain, 86
Carbohydrate fermentation broth, 773–774
Carbohydrate fermentation tests
of *Bacteroides fragilis* group, 341
of *Corynebacterium,* 168–169
of Enterobacteriaceae, 207–208
media for, 801–802
of yeast, 443
Carbohydrate utilization tests
of gram-negative cocci, 161–163
media for, 774–775
of yeast, 441–443
Carbol-fuchsin stain, 651–654
Cardiobacterium hominis, 224, 226
CARP system, 184
Casoni skin test, 677–678
Catalase test
of *Bacteroides fragilis* group, 338–339
of mycobacteria, 398–400
of streptococci, 134–135
Catheters, 6–7, 102
Cedecea, 181, 188
Cellulitis, 75
Central nervous system infections, 14, 34–35, 73–74
Centrifuges, 751, 753, 863
Cerebrospinal fluid, 35, 271–276
Cetrimide medium, 775–776
Chancroid, 83
Charcoal agar, 776
Chilomastix mesnili, 632, 643, 650

Chlamydia
collection and transport of, 10, 18–19, 23
cultures for, 109, 116, 503–514
description of group, 501–503
general considerations, 503–504
growth characteristics of, 505
identification tests for, 505–514
infections associated with, 501, 502
media and reagents for, 816–817
source and clinical importance of, 503
specimen examination for, 18–19, 42, 58, 84
Chloramphenicol assay, 723–726
Chloroform test, 552–553
Chocolate agar, 776–777
Chopped meat medium, 802
Chromogenic cephalosporin test, 308
Chrysosporium, 458
Citrate agar, 777
Citrate utilization test, 193–194
Citrobacter, 181, 185
Cladosporium, 421, 483
Clostridium
general considerations, 316–317, 357–359
growth characteristics of, 359
identification tests for, 359–369
cytotoxicity assay, 364–366
disk antibiogram, 362
neutralization assay, 366–369
spore detection via microscopy, 359
starch-heat, 362
lecithinase-negative, 360, 363
lecithinase-positive, 358, 362–363
microscopic appearance of, 322
Clostridium botulinum, 319, 357, 363, 369
Clostridium difficile, 114, 319, 359, 363–369
Clostridium perfringens, 322, 359, 363
Clostridium tetani, 319, 357, 363
Coagulase test, 135–136
Cocci
gram-negative, aerobic and facultatively anaerobic, 157–164
gram-negative, anaerobic, 316, 319, 353–356
gram-positive, aerobic and facultatively anaerobic, 131–156
gram-positive, anaerobic, 316, 318–319, 328–329, 353–356
Coccidioides immitis
colonial morphology of, 448
exoantigen microimmunodiffusion test of, 472
growth rate of, 446
infections associated with, 421
microscopic morphology of, 78
mold form of, 474, 481
serologic tests for, 487
spherule form of, 475
yeast form of, 481
Columbia colistin-nalidixic acid blood agar, 777–778
Congenital viral infections, 15
Conjunctivitis, 59–60, 62
Corynebacterium, 164–172
carbohydrate fermentation test of, 168–169
characteristics of, 164–167
collection and transport of, 10
diagnostic features of, 168
direct examination for, 75
growth characteristics of, 166
identification of, 166–172
toxigenicity test of, 169–172
Corynebacterium diphtheriae, 105, 166, 167, 168–172
Corynebacterium haemolyticum, 166, 167
Corynebacterium minutissimum, 166
Corynebacterium pseudotuberculosis, 167, 169
Corynebacterium pyogenes, 166, 167
Corynebacterium ulcerans, 166, 167, 168
Corynebacterium xerosis, 166, 167
Cottonseed conversion medium, 812
Counterimmunoelectrophoresis
for bacterial isolate identification, 278
for California virus antibody detection, 579–580
for cerebrospinal fluid examination, 271–276
comparative tests to, 252–253
fluorescent antibody technique vs, 251–252

Counterimmunoelectrophoresis (*cont.*)
  general considerations, 270
  indications for, 251–252
  for urine examination, 276–277
Coxsackievirus A, 545, 553
Creutzfeld-Jakob disease, 761–762
Cryptococcus
  identification of, 427
  infections associated with, 425
  morphology of, microscopic, 74, 79
  morphology of, on inhibitory mold agar, 430
  pigment production by, 444
  serologic tests for, 487
  urease production by, 428
Cryptosporidium, 50, 645, 651–653
Cultures
  abscess, 98–99, 102
  for antimicrobial susceptibility testing, 287–288
  bacterial, *see* Bacterial cultures
  blood, 27–30, 95–97
  of body fluids, normally sterile, 97–101
  chlamydial, 116, 503–514
  dropped or spilled, 750
  for epidemiological purposes, 119–121
  fecal, 113–115
  fungal, *see* Fungal cultures
  of genital tract infections, 115–119
  hookworm, 679–680
  of integumentary infections, 119
  laboratory safety with, 757
  maintenance of, 837–838
  mycobacteria, *see* Mycobacteria cultures
  processing of, initial, 95–121
  quantitation, 4, 101
  of respiratory tract specimens, 103–110
  tissue, 98–99, 101–102
  urine, *see* Urine cultures
  viral, *see* Viral cultures
  wound, 98–99, 102
Cunninghamella, 451, 452
Curvularia, 483
Cycloserine-cefoxitin-fructose-egg yolk agar, 803
Cynomolgus kidney cell cultures
  of adenovirus, 607
  of cytomegalovirus, 591–592
  description and source, 541–542
  of enteroviruses, 545
  of herpes simplex virus, 589
  of influenza virus, 558
  of measles virus, 560
  of mumps virus, 559
  of parainfluenza virus, 559
  preparation of, 542
  of respiratory syncytial virus, 562
  of rhinovirus, 546
  of varicella-zoster virus, 590
Cystine heart agar, 812–813
Cystine tellurite blood agar, 778
Cytomegalovirus, 15, 17, 586, 587–588, 591–594
Czapek solution agar, 812

D

Decarboxylase medium, 778–779
Deoxyribonuclease test, 204–205
Dermatophytes, 455, 464–471, 472
Dientamoeba fragilis, 632, 642, 650
Dilution tests, 292–303
Dipetalonema perstans, 661, 668
Dipetalonema streptocerca, 661, 668
Disinfection, 755–756
Disk antibiogram, 362
Disk-diffusion tests, 303–304
Disk-elution tests, 305
Disk-plate bioassays, 697–704
DNase test agar, 779
Drechslera, 421, 484
Dreft test, 152–153

E

Echinocuccus, 37
Echoviruses, 544, 550–551
Edwardsiella, 181, 186
Egg yolk agar, 803
Eikenella corrodens, 224, 226
Encephalitis, 14, 34, 74
Endolimax nana, 630, 631, 640, 649
Entamoeba coli, 630, 631, 640, 648
Entamoeba hartmanni, 630, 631
Entamoeba histolytica, 630, 633, 640, 648, 671

Enterobacter, 181, 187, 201, 202, 203, 204, 206
Enterobacteriaceae, 180–211
differential characteristics of, 185–190
diseases frequently associated with, 181
general considerations, 180–182
identification of, 182–211
acetate utilization test, 205–206
agglutination tests, 209–210
beta-glucuronidase (PGUA) test, 200–201
carbohydrate fermentation test, 207–208
citrate utilization test, 193–194
deoxyribonuclease test, 204–205
indole test, 197–200
lysine iron agar test, 192–193
malonate utilization test, 206–207
methyl red test, 201–202
ornithine-motility test, 196–197
phenylalanine deaminase test, 203–204
triple sugar iron agar test, 191–192
urease test, 194–195
Voges-Proskauer test, 202–203
Enteromonas hominis, 643, 650
Enteroviruses, 543–545, 551–552, 553
Enzyme-linked immunoassay test, 580–585
Eosin-methylene blue agar, 779–780
Epicoccum, 484
Epidermophyton, 421, 455, 466
Epstein-Barr virus, 17, 586, 588, 598–604, 605
Erwinia-Pectobacterium, 181
Erysipelothrix, 173, 175–176
Escherichia
acetate utilization test of, 205
in antimicrobial susceptibility testing, 300
citrate utilization test of, 193
differential characteristics of, 185
diseases associated with, 181
glucuronidase test of, 200
indole test of, 197, 198
methyl red test of, 201
Voges-Proskauer test of, 202
Esculin agar, 780
Esculin broth, 804
Esculin hydrolysis, 340–341
Ether, 755
Eubacterium, 369–371
Ewingella, 181, 185
Exanthem, 15, 16
Exoantigen microimmunodiffusion test, 472, 479–481
Exophiala, 485
Exudates, 7, 20

F

Fairley test, 46
Feces specimens
collection and transport from, 7–8, 21–22, 634–635
cultures of, 113–115
examination of, 627, 631, 633
appropriate intervals between, 636
with carbol-fuchsin stain, 651–653
with delayed delivery to laboratory, 658–659
with eosin-saline wet preparation, 638–639
formalin-ether concentration for, 646–647, 651
gross, 637
with immediate delivery to laboratory, 637–658
with iron hematoxylin staining, 657–658
methods of, 635–636
with methylene blue stain, 646
microscopic, 638
number of specimens for, 636
with trichome staining, 655–657, 659
protozoa found in, 630, 632
Filariae, 32, 661, 666–669, 670
Flagella stain, 86–87
Flavobacterium, 224, 225
Fletcher's medium, 780
Flucytosine susceptibility test, 494–497
Fluorescent antibody procedures
counterimmunoelectrophoresis vs, 251–252
definitions, 253
direct, 72–73, 104, 253, 257–266
for group A streptococci, 257–260

Fluorescent antibody procedures (*cont.*)
indications for, 251–252
indirect, 253, 266–270
interpretative criteria, 254
for *Legionella*, 262–270
microscope for, 253–254
for parainfluenza identification, 564–566
stained specimen examination, 254
titration of conjugates, 255–257
for *Yersinia pestis*, 260–262
Francisella, 237–238
Fungal cultures
of blood, 24–30
of body fluids, tissue, wounds, and abscesses, 99, 100–101
dropped or spilled, 750
of genital tract infections, 116
preparation of, 449–451
microculture, 451
mounting media for, 449–450
scotch tape, 450
wet mount, 450
quality control on, 855–857
of respiratory tract infections, 105, 108–109
streaking plates for, 100–101
from urine, 110, 112
Fungi
antimicrobial susceptibility tests of, 487–497
body sites with, 423
description of, 419
filamentous
colonial morphology of, 448
common aseptate hyaline molds (Zygomycetes), 451, 452–454
common laboratory, 447
common septate hyaline monomorphic molds, 451, 455, 456–463
dematiaceous molds, 481–486
dermatophytes, 455, 464, 465–469, 470–472
dimorphic hyaline molds, 471–481
exoantigen microimmunodiffusion test of, 472–481
general considerations, 446–449
growth rate of, 446
identification of, 446–486
infections associated with, 420–422
microscopic morphology of, 78–80, 448–449
rice grain medium growth of, 470
trichophyton agars growth of, 470–471
urease test of, 464–470
identification of, 426–486
infections associated with, 420–422, 424–425
media for, 27–30, 811–816
serodiagnosis of infections of, 487
specimen collection of, 6, 11–13, 25–30, 41–43, 62–64, 67
source and clinical importance of, 419–426
yeast, *see* Yeast
Fusarium, 421, 458
Fusobacterium
general considerations, 317, 318, 349
growth characteristics of, 349–350
identification tests for, 350–352
microscopic appearance of, 321–322

G

Gardnerella vaginalis, 59, 117, 145, 231, 232
Gas liquid chromotography
of anaerobic bacteria, 342–344, 372
of mycobacteria, 384, 405–408
Gastroenteritis, 47–56, 84
Gelatin liquefaction test, 355–356
Genital tract infections, 57–59, 82–84, 115–119
Gentamicin-vancomycin laked blood agar, 804
Geotrichium, 421, 459
Germ tube production tests, 440–441
Giardia lamblia, 632, 643, 650, 671
Giemsa stain, 87–88
Gliocladium, 459
Glucose cysteine agar, 780–781
Glucosidase tests, 232
Glucuronidase (PGUA) test, 200–201
Gram-negative broth, 781
Gram stain, 76, 88–89

H

Haemophilus, 227–231
Haemophilus aphrophilus, 224, 226
Haemophilus ducreyi, 83, 117, 227, 228, 229
Haemophilus influenzae
  in antimicrobial susceptibility testing, 301, 302
  counterimmunoelectrophoresis of, 251, 271, 273, 274, 275
  differential characteristics of, 227–228
  fluorescent antibody technique for detection of, 251, 267
  growth characteristics of, 228
  lactamase elaboration by, 305
  porphyrin test of, 229–231
Haemophilus parainfluenzae, 227–228, 229
Hafnia, 181
Hanging drop test for motility, 173–174
Heart infusion agar and broth, 781–782, 811
Hektoen enteric agar, 782
Hemadsorption tests, 526–527, 562–564
Hemagglutination tests, 566–568, 573–576
Hemagglutination-inhibition test, 568–571, 576–578
Hepatitis, 761, 766
Herpes simplex virus, 116, 586–589, 594–598
Herpesviruses
  description of, 585–586
  growth characteristics of, 588–592
  identification tests for, 592–604
  source and clinical importance of, 586–588
  specimen collection of, 14–17, 58, 62
Hippurate hydrolysis test, 145–146
Histoplasma capsulatum
  colonial morphology of, 448
  exoantigen microimmunodiffusion test of, 472
  growth rate of, 446
  infections associated with, 421
  microscopic morphology of, 449
  mold form of, 475, 481
  serologic tests for, 487
  yeast form of, 476, 481
Hookworm culture, 679–680
Horse serum, 853
Human embryonic kidney cell cultures, 542–543, 589, 608

I

Immunofluorescence test, direct
  of adenovirus, 608–610
  for cytomegalovirus, 592–594
  for Epstein-Barr virus, 598–604, 605
  for herpes simplex virus, 594–598
Incubators, 753–754, 861
Indole broth, 783, 804–805
Indole test, 197–200, 339–340
Influenza viruses, 555, 556, 557, 558
Inhibitory mold agar, 813
Integumentary infections, 12–13, 65–67, 119
Intravascular catheter tips, 6, 102
Iodamoeba butschlii, 630, 631, 640, 649
Iodometric slide test, 309
Iron hematoxylin stain, 657–658
Isospora, 632, 645

J

JEMBEC, 9, 22–23, 115

K

Kaolin, 826–827
Kidney specimens, 64–65
Kingella kingae, 224, 226
Kinyoun stain, 390–393, 412–415
Klebsiella, 181, 187, 202, 206
Kluyvera, 181, 185

L

Laboratory safety, 747–767
  acquired immune deficiency syndrome and, 762
  for aerosols, 748, 756–757
  attire and, 757–758
  autoclaves, 752
  bench tops, 752
  blood tests for, 766
  centrifuges, 753
  chemicals, 749, 755
  disinfection and sterilization, 755–756
  environmental control and, 763–765
  equipment and facilities, 752–754

Laboratory safety *(cont.)*
floors, 753
freezers, 754
fungal serology for, 765
hepatitis and, 761, 766
incubators, 753–754
with ingestion, 748, 758
leaking specimen containers, 754
occupational health and, 765–766
physical examinations for, 766
pneumatic tube systems for specimen transport, 754
procedures after exposure or accident, 750–752
roentgenograms for, 765
routes of laboratory-acquired infections and, 748–749
for shipment and distribution of etiologic agents, 758–761
transmissible virus dementia and, 761–762
tuberculin skin testing for, 765
waste products and, 758
Laboratory tests
acetate utilization, 205–206
animal inoculation, 175
antibiotic disk identification, 335–336
arylsulfatase production, 397–398
ascospore production, 444–445
assays, *see* Assays, antimicrobial
bacitracin inhibition test, 143–144
bactericidal, 731–743
Baerman digestion technique for, 676
Beaver egg counting technique for, 677
beta-glucuronidase (PGUA), 200–201
β-lactamase, 305–310, 335
bile-esculin, 148–149
bile solubility, 151–152
bile tolerance, 337
CAMP test, 146–147
carbohydrate fermentation, 168–169, 207–208, 341, 443
carbohydrate utilization, 161–163, 441–443
Casoni skin test, 677–678
catalase test, 134–135, 338–339, 398–400
chloroform, 552–553
citrate utilization, 193–194
coagulase test, 135–136
deoxyribonuclase, 204–205
enzyme-linked immunoassay, 580–585
for epidemiological purposes of culturing, 120–121
esculin hydrolysis, 340
exoantigen microimmunodiffusion, 472, 479–481
fungal serologic, 487, 488–491
gas liquid chromotography, 342–344
gelatin liquefaction, 355–356
germ tube production of yeast, 440–441
glucosidase, 232
hanging drop test for motility, 173–174
hemadsorption, 526–527, 562–564
hemagglutination, 566–568, 573–578
hemagglutination-inhibition test, 568–571
hippurate hydrolysis, 145–146
immunofluorescence, 592–604, 605, 608–610
indole, 197–200, 339–340
inoculation of suckling mice, 553–555
latex agglutination test, 149–150, 252, 253
levodopa-ferric citrate test, 437–438
lysine iron agar, 192–193
malonate utilization, 206–207
manganous chloride-urea, 529–531
methyl red, 201–202
motility test in semisolid agar, 174–175
Neufeld Quellung reaction, 155–156
niacin production (Konno-Runyon method), 403–404
nitrate reduction, 221–222, 394–395, 430–436
novobiocin inhibition test, 138
ONPG test, 163–164
optochin sensitivity, 154
ornithine-motility, 196–197
oxidase test, 160–161
oxidation-fermentation, 219–220
pH 3, 551–552
phenylalanine deaminase, 203–204
pigment production, 222–223, 444

porphyrin, 229–231
quality control of, 831–864
education for, 832
regarding equipment, 861–864
general considerations, 831
laboratory manual for, 832
regarding media and reagents, 833–861
proficiency testing for, 832–833
records and review for, 833
rice grain medium growth, 470
salt tolerance test, 150–151, 400
schistosomiasis hatching test, 678–679
sodium chloride tolerance, 150–151, 400
sodium dodecylsulfate solubility (Dreft test), 152–153
of Staphylococcus, 134–138
staphyloslide test, 137–138
stoll egg counting technique for, 680–681
tapeworm recovery, 681–683
tellurite reduction, 402–403
tetrazolium reduction, 527–529
thiophene-2-carboxylic acid hydrazide susceptibility, 404–405
of threonine or lactate conversion to propionate, 352
trichophyton agar growth, 470–471, 472
triple sugar iron agar, 191–192
Tween–80 hydrolysis, 395–397
urease, 194–195, 401–402, 416–417, 428–430, 464, 470
virus neutralization, 547–551
Voges-Proskauer, 202–203
worm fixation, 683–685
yeast morphology on cornmeal Tween–80 agar, 431, 432–433, 438–440
Lactobacillus, 178–179, 369
Latex agglutination test, 149–150, 252, 253
Legionella
cultures of, 107
diagnostic features of, 241
differential characteristics of, 240
fluorescent antibody technique for detection of, 262–270
general considerations, 238–239
growth characteristics of, 239
identification of, 239–241
media for cultures of, 788–789
microscopic appearance of, 241
specimen collection with, 42, 43
Leifson stain, 86–87
Leishmania, 661, 672
Leptospirosis, 6, 30, 112
Leukocytes, in feces, 84
Levodopa-ferric citrate test, 437–438
Liquid chromatographic assays, 706–726
aminoglycoside, 710–720
chloramphenicol, 723–726
general considerations, 709
vancomycin, 720–723
Listeria, 172–175, 267
Liver specimens, 64–65
Loa loa, 661, 668
Localization studies, of urinary tract, 45–46
Loeffler medium, 783
Lowenstein-Jensen medium, 809
Lungs
abscess of, 40, 319
biopsy of, 43, 77–82, 106
tissue specimens from, 64–65
Lyme disease, 31
Lysine iron agar, 192–193, 783–784

M

MacConkey agar, 784
Malaria, 659–665
thick and thin films, 6, 32–34
Malonate broth, 784–785
Malonate utilization test, 206–207
Manganous chloride-urea test, 529–531
Mansonella ozzardi, 661, 668
McCoy's cell cultures, 109, 504, 505, 545–546
Measles virus, 555, 557–558, 560–561
Media and reagents
acetate agar, 770
for aerobic and facultatively anaerobic bacteria, 770–800
for anaerobic bacteria, 325, 328, 800–808
antibiotic, 821–824

Media and reagents (*cont.*)
for antimicrobial susceptibility testing, 283–284
Bennett's agar, 808
bile-deoxycholate, 800–801
bile-esculin agar, 770
blood agar, *see* Blood agar
for blood cultures, 27–30, 96
for body fluids, normally sterile, 97, 98, 99
brain heart infusion agar, 811
Brucella blood agar, 801
Brucella broth, 771
buffered charcoal yeast extract agar, 771–772
Campylobacter, 772–773
carbohydrate fermentation base, 801–802
carbohydrate fermentation broth, 773–774
carbohydrate utilization media for *Neisseria*, 774–775
cetrimide (pseudosel agar), 775–776
charcoal agar, 776
for *Chlamydia*, 816–817
chocolate agar, 776–777
chopped meat, 802
citrate agar (Simmons), 777
Columbia colistin-nalidixic acid blood agar, 777–778
cottonseed conversion, 812
cycloserine-cefoxitin-fructose-egg yolk agar, 803
cystine heart agar, 812–813
cystine tellurite blood agar, 778
Czapek solution agar, 812
DNase test agar, 779
dates on, 836
decarboxylase (Moeller), 778–779
egg yolk agar, 803
eosin-methylene blue agar, Levine, 779–780
esculin agar, 780
esculin broth, 804
for fecal cultures, 114
for filamentous fungal cultures, 448
Fletcher's, 780
for fungi, 811–816
gentamicin-vancomycin laked blood agar, 804
glucose cysteine agar, 780–781
gram-negative broth, 781
heart infusion agar and broth, 781–782
Hektoen enteric agar, 782
human blood Tween 80, 782–783
indole broth, 783
indole-nitrate broth, 804–805
inhibitory mold agar, 813
Loeffler, 783
Lowenstein-Jensen, 809
lysine-iron agar, 783–784
MR-VP broth, 785
MacConkey agar, 784
maintenance of, 837–838
malonate broth, Ewing modified, 784–785
Middlebrook 7H10 agar, 810
Middlebrook 7H9 broth, 809–810
Middlebrook selective S7H11 agar, 810–811
motility, 785
Mueller-Hinton agar and broth, 785–786
for mycobacteria and Nocardia, 808–811
for mycoplasmas, 816, 817–820
mycosel agar, 813–814
nalidixic acid-polymyxin-crystal violet broth, 786–787
nitrate broth, 786
nutrient agar and broth, 787
oxidation-fermentation, 787
PRAS, 325
for parasite cultures, 685
peptone yeast glucose, 805–806
performance testing of
for bacterial cultures, 836–837, 838–855
for fungal and mycobacterial culture, 855–857
for parasite cultures, 857–858
stock organisms for, 838
for viral cultures, 858–861
phenylalanine agar, 787–788
phenylethyl alcohol agar, 788, 806
phosphate-buffered saline, pH7.2, for *Legionella*, 788–789

pigment production, 789
potassium tellurite agar, 789
potato dextrose agar, 814
preparation of, 834
purple agar, 790
quality control of, 833–861
for respiratory tract infection cultures, 103, 104–105, 107–109
rice grain, 814
Rogosa SL agar, 790
SF, 791–792
Sabouraud dextrose agar (Emmon's modification), 814–815
Salmonella-Shigella agar, 791
Schaedler broth, 806
selection of, 833–834
selenite broth, 791
for sexually transmitted disease specimen cultures, 116–117
sodium bicarbonate agar, 792
sodium chloride broth, modified, 792
soybean-casein digest agar and broth, 792–793
for specimen collection in bacteremia, 27–30
for specimen collection in spirochetemia, 31
starch broth, 807
sterility tests for, 834–836
storage of, 836
sucrose agar and broth, 793–794
TM2, TM4, 794–795
Thayer-Martin, 794
thiogel, 807
thioglycollate, 795, 807
thiosulfate citrate bile salts sucrose agar, 796
Todd-Hewitt broth, 796
toxigenicity test agar, 796–797
Trichophyton agars, 815
triple sugar iron agar, 797
urea agar (Christensen), 797–798
for urine cultures, 111–112
veal infusion broth, 798
for viruses, 816, 820–828
W (Wisconsin), 798
xylose-lysine-deoxycholate agar, 798–799
yeast extract agar, 815–816
Yersinia selective agar, 799–800
Meningitis, 14, 34, 73–74
Methyl red test, 201–202
Methylene blue stain, 84, 89–90, 646
Microbial flora, indigenous, 1–5
Micrococcaceae, 131–139
Micrococcus, 139
Microfilariae, 32, 659–669
Micrometer, ocular, 625–627
Microscope slides, 32–34
Microscopes, 863
Microsporum, 421, 455, 466–467, 470, 472
Middlebrook 7H10 agar, 810
Middlebrook 7H9 broth, 809–810
Middlebrook selective S7H11 agar, 810–811
Minimal bactericidal concentration, 732–735
Minimal inhibitory concentration, 282, 283, 293, 294, 300, 303
Moellerella, 181, 189
Moraxella, 225
Morganella, 181, 189, 194
Motility medium, 785
Motility test in semisolid agar, 174–175
MR-VP broth, 785
MRC–5 cell cultures
of adenovirus, 606
of cytomegalovirus, 591
description and source of, 539
of herpes simplex virus, 588
incubation and maintenance of tubes, 541
of influenza virus, 558
of measles virus, 560
of mumps virus, 559
of parainfluenza virus, 559
of respiratory syncytial virus, 561
trypsinization for subculturing, 540
of varicella-zoster virus, 589
Mucor, 80, 421, 451, 453
Mueller-Hinton agar and broth, 283–284, 785–786
Mumps virus, 555, 557, 559
Mycobacteria
antimicrobial susceptibility testing of, 408–411
description of, 379

identification of, 381–408
acid-fast stains for, 85–86, 390–393
arylsulfatase production in, 397–398
catalase tests for, 398–400
gas liquid chromatographic, 405–408
general considerations, 381–384
niacin production in, 403–404
nitrate reduction in, 394–395
pigment production in, 393–394
presumptive, tests for, 382–383
sodium chloride tolerance test for, 400
tellurite reduction for, 402–403
thiophene-2-carboxylic acid hydrazide susceptibility in, 404–405
Tween–80 hydrolysis in, 395–397
urease production in, 401–402
infections from, 380–381
media for, 808–811
quality control on cultures of, 855–857
source and clinical importance of, 379
Mycobacteria cultures
of body fluids, tissue, wounds, and abscesses, 99, 101, 102–103
of respiratory tract infections, 108
specimen collection for, 11, 20–21
from urine, 13, 111–112
Mycobacteriaceae, 379
Mycobacterium avium-intracellulare, 380, 382, 384, 389, 402, 408
Mycobacterium bovis, 380, 382, 385, 389, 404, 408
Mycobacterium chelonei, 380, 382, 385, 389, 408
Mycobacterium fortuitum, 380, 382, 386, 389, 408
Mycobacterium gastri, 380, 382, 386, 389, 408
Mycobacterium gordonae, 380, 382, 386, 389
Mycobacterium haemophilum, 380, 382, 387, 389
Mycobacterium kansasii, 380, 383, 387, 389
Mycobacterium marinum, 380, 383, 388, 389, 408
Mycobacterium scrofulaceum, 380, 383, 388, 389, 408
Mycobacterium simiae, 380, 383, 389, 390
Mycobacterium smegmatis, 380, 383, 389, 390, 408
Mycobacterium szulgai, 381, 383, 389, 390, 408
Mycobacterium tuberculosis, 381, 383, 389, 390, 403, 404, 407, 408
Mycobacterium xenopi, 381, 383, 389, 390, 408
Mycoplasma hominis, 521
Mycoplasma pneumoniae, 105, 519, 520–521, 523, 526–529
Mycoplasmas
description of group, 519
growth characteristics of, 523–526
identification of, 521–532
antimicrobial susceptibility test for, 532
hemadsorption test for, 526–527
manganous chloride-urea test for, 529–531
serologic tests for, 531–532
tetrazolium reduction test for, 527–529
large colony, 817–818
media and reagents for, 816, 817–820
source and clinical importance of, 520–521
specimen collection with, 18, 42
Mycosel agar, 813–814

N

Nalidixic acid-polymyxin-crystal violet broth, 786–787
Neisseria gonorrhoeae
β-lactamase elaborated by, 305
carbohydrate utilization media for, 774–775
collection and transport of, 9–10, 22–23
cultures of, 105, 116
direct examination for, 82
diseases caused by, 58–59, 157
fluorescent antibody technique for detection of, 267
growth characteristics of, 158
identification of, 158–164
Neisseria lactamica, 157, 163

Neisseria meningitidis
in antimicrobial susceptibility testing, 302
collection and transport of, 10
counterimmunoelectrophoresis exam for, 271, 273, 275, 276
cultures of, 105
fluorescent antibody technique for detection of, 267
general considerations, 157
growth characteristics of, 158
ONPG test of, 163
Neufeld Quellung reaction, 155–156
Niacin production tests, 403–404
Nigrospora, 485
Nitrate broth, 786
Nitrate reduction test, 221–222, 394–395, 430–436
Nocardia, 77, 81, 107, 411–417, 808–811
Nonspecific vaginitis, 59, 83
Novobiocin inhibition test, 138
Nutrient agar and broth, 787

O

Ocular infections, 59–63, 84–85
ONPG test, 163–164
Onchocerca volvulus, 661, 668
Open lung biopsy, 43, 77–82, 106
Optochin sensitivity test, 154
Ornithine motility test, 196–197
Otitis media, 37
Oxidase test, 160–161
Oxidation-fermentation medium, 787
Oxidation-fermentation test, 219–220

P

Paecilomyces, 460
Paracoccidioides brasiliensis, 476–477
Paragonimus westermarri, 37
Parainfluenza virus, 555, 557, 559, 562, 564–566
Parasitemia, 31–34
Parasites
anal swab, 8, 22, 670
Babesia, exam for, 31–34, 659–665
bile, 674
biopsies for detection of, 671–673
blood and tissue, 659–669
blood films of, 31–34, 633–634, 636–637
drainage material, aspirates and spinal fluid with, 673
filaria, 31–34, 661, 659–669, 670
infections from, 31–32, 49–54
lymph nodes, 674
malaria, exam for, 6, 31–34, 659–665
ocular micrometer for detection of, 625–627
quality control on cultures of, 857–858
Schistosoma haematobium, 669–670, 671
serologic tests for, 637, 674–675
skin, 672–673
sputum, 673
stool specimens of, 627–633
collection of, 47, 55–56, 634–635
examination methods for, 635–636
necessary number of, 47, 636
processing of, 637–659
Trichomonas vaginalis, 10, 83, 670
trypanosomes, exam for, 659–669
in urine, 669–670
vaginal swab, 670–671
Parenteral fluids, 119–121
Pasteurella, 235–237
Penicillin G assay, 698–704
Penicillinase, 30, 305–310
Penicillium, 422, 460
Peptococcus and Peptostreptococcus, 318, 320, 353–356
Peptone yeast glucose medium, 805–806
PGUA test, 200–201
pH 3 test, 551–552
pH meters, 862–863
Phase microscopy, 84
Phenol red, 827
Phenylalanine agar, 787–788
Phenylalanine deaminase test, 203–204
Phenylethyl alcohol agar, 788, 806
Phialophora, 422, 486
Phosphate-buffered saline for *Legionella*, 788–789
Picornaviruses
description of, 543
growth characteristics of, 545–546
identification tests for, 547–555
chloroform test, 552–553

inoculation of suckling mice, 553–555
neutralization of infectivity, 547–551
pH 3 test, 551–552
source and clinical importance of, 543–545
Pigment production media, 789
Pigment production test, 222–223
Pipettes, 864
Plasmodium, 31, 634, 665
Plesiomonas, 211, 215
Pneumatic tube systems, 754
Pneumocystis carinii, 42, 43, 81, 88, 91
Polioviruses, 545
Polymyxin broth-dilution assay, 707–709
Polyvinyl alcohol fixative, 22
Porphyrin test, 229–231
Postmortem tissue specimen, 64
Potassium hydroxide wet mount preparation, 77, 90
Potassium tellurite agar, 789
Potato dextrose agar, 814
Poxvirus, 610
Propionibacterium, 317, 369–371
Prostatitis, 45–46, 58
Protected catheter brush, 12, 41
Proteus
differential characteristics of, 189
diseases associated with, 181
indole test of, 197, 198
phenylalanine deaminase test of, 203
urease test of, 194, 195
Providencia, 181, 189, 194, 203
Pseudoallescheria, 422, 461
Pseudomonas
in antimicrobial susceptibility testing, 300
general considerations, 217
identification of, 218–224
nitrate reduction test, 221–222
oxidation-fermentation test, 219–220
pigment production test, 222–223
Pseudosel agar, 775–776
Purple agar, 790
Pus, 20
Putrescein, 827

R

Receptor destroying enzyme, 827
Respiratory syncytial virus, 17, 555, 558, 561–562
Respiratory tract infections
cultures of specimens from, 103–110
examination of, 106, 110
with lung biopsy, 106
procedures, 103–106
etiology of, 37, 38–40
specimen collection with, 10–12, 17–19, 37–43
specimen examination for, 75–82
Retortamonas intestinalis, 643, 650
Rhesus monkey kidney cell cultures, 541–542
Rhinoviruses, 543, 545–546, 551–552
Rhizopus, 422, 451, 453
Rhodotorula, 424, 427, 430
Rice grain medium, 814
Rice grain medium growth tests, 470
Roentgenograms, 765
Rogosa SL agar, 790
Rotaviruses, 17, 583–585
Rubella virus, 15, 571, 572, 573, 580–583

S

Sabouraud dextrose agar, 814–815
Saccharomyces, 424, 430, 444
Salmonella
agglutination tests of, 209, 210
classifications within, 182
culture of, 114
differential characteristics of, 186
diseases associated with, 181
identification of, 184
malonate utilization test of, 206
Salmonella-Shigella agar, 791
Salt tolerance test, 150–151, 400
Schaedler broth, 806
Schistosoma haematobium, 669–670, 671
Schistosomiasis hatching test, 678–679
Scopulariopsis, 422, 462
Selenite broth, 791
Sepedonium, 462
Septicemia, 24–34
Serologic tests

for *Chlamydia* identification, 511–513
fungal, 765
for mycoplasma identification, 531–532
for parasites, 637, 674–675
for viruses, 14–17, 611–616
Serratia, 181, 188, 202, 204
Sexually transmitted diseases
cultures of specimens from, 115, 116–117
etiology of, 57
specimen collection and transport with, 9–10, 22–23, 58–59
specimen examination for, 82–84
SF medium, 791–792
Shigella
acetate utilization test of, 205
agglutination tests of, 209, 210
citrate utilization test of, 193
culture of, 114
diseases associated with, 181
glucuronidase test of, 200
identification of, 184
Sinusitis, 37
Sodium bicarbonate agar, 792
Sodium chloride broth, 792
Sodium chloride tolerance tests, 150–151, 400
Sodium dodecylsulfate solubility test, 152–153
Sodium polyanetholsulfonate, 27, 30
Sorbitol, 30
Soybean-casein digest agar and broth, 792–793
Specimens
acquired immune deficiency syndrome, 43, 77–82, 762
for antimicrobial assays, 692–693
collection and transport of, 5–67
for antimicrobial assays, 693
with bacteremia and fungemia, 24–30
in blood, 6, 65
in body fluids, 8–9, 65
by catheter, 6–7
with central nervous system infections, 34–35
Chlamydia, 10, 18–19, 23
in exudates, 7, 20
from fecal material, 7–8, 21–22
with gastroenteritis, 47–56
with genital tract infections, 9–10, 22–23, 57–59
with integumentary infections, 65–67
with intraabdominal infections, 56–57
from kidney, 64–65
Legionella, 18
from liver, 64–65
from lungs, 64–65
from lymph nodes, 65
Mycoplasma pneumoniae, 18
Neisseria gonorrhoeae, 9–10
with ocular infections, 7, 59–63
pinworms, 22
with parasitemia, 31–34
patient identification with, 23
from pericardial fluids, 65
from peritoneal fluids, 65
from pleural fluids, 65
pneumatic tube systems for, 754
postmortem, 64
Q-fever, 18
with respiratory tract infections, 10–12, 37–43
with septicemia, 24–34
in skin and nail scrapings, 12–13, 67
specimen identification with, 5, 23
swabs, use of, 5, 20–21
with spirochetemia, 30–31
from spleen, 64–65
test specification with, 24
time and date specification with, 24
from tissue, 13, 63–65
Trichomonas vaginalis, 10
Ureaplasma, 23
with urinary infections, 9–10, 44–47
from urine, 13, 44–47, 65
viral, 14–17
from wounds, 13, 36–37
examination of, direct, 71–92
for central nervous system infections, 73–74
for gastroenteritis, 84
for genitourinary tract infections, 82–84

Specimens
  examinations of, direct (*cont.*)
    from tissue, 13, 63–65
    rapid tests for, summary of, 72–73
    for respiratory tract infections, 75–82
    stains and wet mount preparations for, 85–92
    for wound, abscess, cellulitis, 75
  hepatitis, 761
  identification of, 5, 23
  indigenous flora contaminating, 1–5
  leaking containers for, 754
  transmissible virus dementia, 761–762
  transport containers, devices, and media for, 5–23
Spirochetemia, 30–31
Spleen specimens, 64–65
Spore stain, 90–91
Sporobolomyces, 427
Sporothrix, 422, 446, 477–478, 481, 673
Sputum, 37, 41, 76–77
St. Louis encephalitis virus, 14, 572, 573–578
Stains
  acid-fast (Kinyoun and Ziehl-Neelsen), 76–77, 390–393, 412–415
  auramine-rhodamine (Truant), 85–86
  capsule, 86
  carbol-fuchsin, 651–654
  flagella (Leifson), 86–87
  Giemsa, 87–88
  Gram, 88–89
  iodine, 508–511
  iron hematoxylin, 657–658
  methylene blue (Albert), 84, 89–90, 646
  spore, 90–91
  toluidine blue O, 91–92
  trichrome, 655–657, 659
  for worm, 684–685
Stamey test, 46
Staphylococcal agglutination test, 252–253
Staphylococcus
  catalase test of, 134–135
  coagulase test of, 135–137
  general considerations, 131–133
  growth characteristics of, 133–134
  identification of, 133–139
  Micrococcus vs, 139
  novobiocin inhibition test of, 138
  speciation of, 134
  Staphyloslide test of, 137–138
Staphylococcus aureus
  in antimicrobial susceptibility testing, 300
  coagulase test of, 135, 136
  deoxyribonuclease test of, 204
  general considerations, 131–133
  growth characteristics of, 133–134
  minimal bactericidal concentration for, 732
  Staphyloslide test of, 137
Staphylococcus epidermidis, 133
Staphylococcus haemolyticus, 133
Staphylococcus hominis, 133
Staphylococcus saprophyticus, 133, 138
Staphyloslide test, 137–138
Starch broth medium, 807
Sterile body fluids, 8, 64–65, 97–101
Sterilization, 755–756
Stoll egg counting technique, 680–681
Stool specimens, *see* Feces specimens
Streaking of plates, 100, 101
Streptococcaceae, 139–156
Streptococcus
  bacitracin inhibition test of, 143–144
  bactericidal tests for, 734, 735
  bile-esculin test of, 148–149
  bile solubility test of, 151–152
  CAMP test of, 146–147
  capnophilic, 328–329
  collection and transport of, 11
  general considerations, 139–140
  growth characteristics of, 142
  hippurate hydrolysis of, 145–146
  identification of, 140–156
    counterimmunoelectrophoresis for, 271, 273, 275
    fluorescent antibody techniques for, 257–260
  latex agglutination test of, 149–150
  microscopic appearance of, 142
  Neufeld Quellung reaction test of, 155–156
  optochin sensitivity test of, 154
  salt tolerance test of, 150–151

sodium dodecylsulfate solubility test of, 152–153
Streptococcus agalactiae (group B), 118, 139
Streptococcus faecalis, 142, 300
Streptococcus mutans, 148
Streptococcus pneumoniae, 140, 141
bile solubility test of, 151, 152
counterimmunoelectrophoresis exam for, 271
growth characteristics of, 142
Neufeld Quellung reaction to, 156
optochin sensitivity test of, 154
sodium dodecylsulfate solubility test of, 152, 153
Streptococcus pyogenes (group A), 103–104, 139, 141
Streptomyces, 411–412
Sucrose agar and broth, 30, 793–794
Suprapubic aspirate, 44–45
Swabs, 5, 20–21, 670–671
SWUBE disposable paddle, 22
Syncephalastrum, 451, 454
Syphilis, 59
Syringe, 21

T

Tapeworm, 637, 681–683
Tatumella, 181, 203
Tellurite production tests, 402–403
Tetrazolium reduction tests, 527–529
Thallium acetate, 827
Thayer-Martin medium, 794–795
Thermometers, 862
Thiogel medium, 807
Thioglycollate medium, 795, 807
Thiophene-2-carboxylic acid hydrazide susceptibility tests, 404–405
Thiosulfate citrate bile salts sucrose agar, 796
Tissue, 63–64, 101–102
Todd-Hewitt broth, 796
Togaviruses
clinical importance of, 572–573
description of, 571
identification tests of, 573–583
counterimmunoelectrophoresis, 579–580
enzyme-linked immunoassay, 580–583
hemagglutination, 573–576
hemagglutination-inhibition, 576–578
source and growth characteristics of, 571–572
Toluidine blue O stain, 43, 91–92
Toxic shock syndrome, 118
Toxigenicity test agar, 796–797
Transgrow, 22, 115
Transtracheal aspirate, 12, 41, 77
Trichoderma, 463, 642, 650
Trichomonas hominis, 632
Trichomonas vaginalis, 10, 83, 116, 670
Trichophyton, 422, 455, 464, 468–469, 470, 472
Trichophyton agar, 815
Trichophyton agar growth tests, 470–471, 472
Trichosporon, 424, 428, 430
Trichrome staining 655–657, 659
Triple sugar iron agar, 191–192, 797
Truant stain, 85–86
Trypanosomes, 32, 659–669
Trypsin-EDTA solution, 828
Tryptose phosphate broth, 828
Tuberculin skin testing, 765
Tween–80 agar, 431, 432–433, 438–440
Tween–80 hydrolysis, 395–397
Tween–80 medium, human blood, 782–783

U

Urea agar, 797–798
Ureaplasma urealyticum, 23, 116, 519, 520–526
identification tests for, 529–531
media for, 819–820
Urease production tests
for Actinomycetes, 416–417
for aerobic and facultatively anaerobic bacteria, 194–195
for filamentous fungi, 464, 470
for mycobacteria, 401–402
for *Ureaplasma,* 529–531
for yeast, 428–430
Urinary infection, 44–47, 82–84

Urine counterimmunoelectrophoresis, 276–277
Urine cultures
  examination of, 82, 110–113
  procedures for, 110, 111–112
  specimen collection and transport for, 13, 44–47, 65

V

Vaccinia virus, 610
Vancomycin assay, 720–723
Varicella-zoster virus, 586, 587, 589–590
Veal infusion broth, 798
Veillonella, 319
Vibrio, 114, 211–214
Vibrionaceae, 211–215
Viral cultures
  of body fluids, tissue, wounds, and abscesses, 14–17, 99, 101
  CPE pattern in, 614–615
  cynomolgus kidney cell, *see* Cynomolgus kidney cell cultures
  of genital tract infections, 116
  human embryonic kidney cell, 542–543, 589, 608
  MRC–5 cell, *see* MRC–5 cell cultures
  processing of, 539
  quality control on, 858–861
  of respiratory tract infections, 17, 105, 109
  from urine, 15, 112
Virtanen method, 394–395
Viruses
  adenoviruses, 604–610
cultures of, 99, 105, 112
  DNA-containing, 585–616
  description of, 537–538
  herpesviruses, 585–604
  identification tests for
    chloroform, 552–553
    counterimmunoelectrophoresis, 579–580
    enzyme-linked immunoassay, 580–585
    fluorescent antibody, 564–566
    hemadsorption, 562–564
    hemagglutination, 566–568, 573–576
    hemagglutination-inhibition, 568–570, 576–578
    immunofluorescence, 592–604, 608–610
    inoculation of suckling mice, 553–555
    neutraliztion of infectivity, 547–551, 608
    pH 3, 551–552
    serologic, 611–616
  media for, 816, 820–828
  metamyxoviruses, 555–570
  orthomyxovirus, 555–571
  paramyxoviruses, 555–570
  picornaviruses, 543–555
  poxviruses, 610
  RNA-containing, 543–585
  reporting of isolates of, 611
  rotaviruses, 583–585
  source and clinical importance of, 538–539
  togaviruses, 571–583
Voges-Proskauer test, 202–203

W

W (Wisconsin) medium, 798
Wangiella, 486
Western equine encephalitis virus, 572, 573–578
Wet mount preparations, 85–92
  eosin-saline, 638–639
  for fungal cultures, 450
  phase microscopy of, 84
  potassium hydroxide, 77–81, 90
  stains with,*see* Stains
Wounds, 13, 36–37, 75, 98–99, 102, 750–751
Wuchereria bancrofti, 661, 668

X

Xylose-lysine deoxycholate agar, 798–799

Y

Yeast extract agar, 815–816
Yeasts
  ascospore production by, 444–445
  carbohydrate fermentation by, 443
  carbohydrate utilization by, 441–443
  on cornmeal Tween–80 agar, morphology of, 438–440

frequencies of, on various body sites, 423
general considerations, 426–427
germ tube production test of, 440–441
identification of, 426–445
infections associated with, 424
on inhibitory mold agar, morphology of, 430
levodopa-ferric citrate test of, 437–438
pigment production of, on niger seed agar, 444
rapid nitrate reduction test of, 430–436
source and clinical importance of, 420, 425–426
urease production tests of, 428–430
Yersinia, 114, 181, 184, 190, 260–262
Yersinia selective agar, 799–800

Z

Ziehl-Neelsen stain, 390, 392–393
Zygomycetes, 446, 448, 451, 452–454