Organization and practice in tuberculosis bacteriology

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Preface

The best way to prevent the spread of tuberculosis in any community is to identify those people who are excreting the tubercle bacillus and to render them non-infectious to others. At the same time the victims may themselves be restored to health.

These principles apply both in developing countries, where the disease still offers serious medical, social and economic problems, and in developed countries, where the incidence of tuberculosis is declining but not as rapidly as was hoped at the beginning of the chemotherapeutic era.

The tuberculosis laboratory has an essential role in the diagnosis of tuberculosis and other mycobacterial diseases, whether it concentrates, as a matter of economic necessity, on the detection of the most infectious patients, or also has facilities for finding the bacillus in those whose disease is not very far advanced, for monitoring treatment and providing health authorities with epidemiological data.

Advice on the organization and technical methods of tuberculosis bacteriology is distributed among many textbooks of general medical microbiology and in many journals. We have attempted to draw together in one book what we consider to be the most useful published material and added some from our own experience. We hope that the result will be of some value to those who work at all levels in diagnostic tuberculosis bacteriology.

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Chapter 1

Introduction: mycobacteria and mycobacterial disease

In ancient Hindu texts tuberculosis is referred to as *Rogaraj* – the king of disease, and *Rajayakshma* – the disease of kings. The first of these names, like John Bunyan's 'Captain of all of these Men of Death' emphasizes that this disease was, and in many countries still is, foremost among the causes of morbidity and mortality. The second name stresses that tuberculosis, being an infectious disease, strikes indiscriminately and affects monarch and peasant alike.

Another disease of kings is leprosy which numbers Robert Bruce, king of Scotland, among its victims. Both tuberculosis and leprosy are caused by members of the genus *Mycobacterium.* When originally named in 1896 in an Atlas of Bacteriology by Lehmann and Neumann, this genus contained the leprosy bacillus (*Mycobacterium leprae*) observed by Armauer Hansen in 1874 and the tubercle bacillus (*Mycobacterium tuberculosis*) observed and cultured by Robert Koch in 1882. The name *Mycobacterium* means 'fungus–bacterium' and arose from the characteristic fungus-like pellicle produced by the tubercle bacillus when grown on liquid media. The leprosy bacillus has never been grown convincingly *in vitro* but was included in the genus because of an important property that it shares with the tubercle bacillus: that of *acid fastness.* This is a characteristic staining reaction due to the ability of mycobacteria to resist decolorization in the presence of a weak mineral acid after staining with an arylmethane dye. A brief history of this staining method is given in Chapter 5. Nowadays the genus is defined more precisely on the basis of its antigenic structure and cell wall chemistry, particularly by the length of the carbon chains in long-chain fatty acids, termed mycolic acids.

As time went on, other bacilli with cultural and staining properties similar to those of Koch's tubercle bacillus were isolated from various animals with tuberculosis-like lesions and also from inanimate sources such as hay and compost.

Those acid-fast bacilli isolated from diseased animals were, not unreasonably, called tubercle bacilli and three main groups were described: mammalian, avian and 'cold-blooded'. The mammalian tubercle bacilli included Koch's original isolates but in 1898 Theobold Smith divided these into human and bovine types according to small but constant cultural differences. Later a third mammalian type – the vole bacillus – was added by Wells in 1946. These three types are often regarded as being three separate species and are named *Mycobacterium tuberculosis, M. bovis* and *M. microti* respectively. There is, however, strong evidence that these, together with a group of strains termed '*M. africanum*' are no

more than variants of a single species. In addition to scientific reasons, the use of the single name *M. tuberculosis* may avoid serious misunderstandings. A physician faced with a reported isolation of '*M. africanum*' may not appreciate that the patient is suffering from tuberculosis. Symmers (1983) tells the story of a surgeon whose son had enlarged cervical lymph nodes. When '*Mycobacterium bovis*' was isolated the father expressed delight that his son did not have a lymphadenoma but added that 'we are lucky it is not even TB'!

For the above practical reasons, as well as the taxonomic considerations discussed in Chapter 8, we regard all four 'species' as being variants of *M. tuberculosis*, but divide those causing disease in man into human, bovine and African types (Collins, Yates and Grange, 1982). The human type consists of two important variants: the original type described by Koch which is virulent in the guinea pig, and a more recently described variant which is attenuated in this animal. The former is found worldwide while the latter is particularly prevalent in South India and among ethnic Asian patients living in other countries. Accordingly we term these variants the classical and Asian types respectively. The heterogeneous strains comprising the African type appear superficially to bridge the gap between the human and bovine types. Those resembling the bovine type are found principally in West Africa (Ghana, Mauritania, Nigeria) while those resembling the human type are found in more easterly countries, notably Burundi and Rwanda. Both types are, however, found in other countries including Great Britain. For epidemiological purposes we divide these into two types – African I and II according to their nitratase activity (*see* Chapter 8), the former being negative and the latter positive. It should be stressed that such a subdivision of *M. tuberculosis* is purely for epidemiological purposes: the management of the patient is identical for all the above types.

The other 'tubercle bacilli', the avian and 'cold-blooded', are species quite distinct from *M. tuberculosis*. The avian tubercle bacilli belong to the species *M. avium*, which is very closely related to *M. intracellulare*. Indeed many bacteriologists refer to them as the *M. avium–intracellulare* group, while others, especially workers in the USA, add the distinct species *M. scrofulaceum* and call the resulting cluster the MAIS complex. Also closely related to *M. avium* are two pathogens of animals, namely *M. paratuberculosis* – the cause of hypertrophic enteritis or Johne's disease of cattle and *M. lepraemurium* – the cause of rat leprosy.

The group originally termed the 'cold-blooded' tubercle bacilli includes three distinct species *M. marinum, M. chelonei* and *M. fortuitum* (formerly *M. ranae*), corresponding to the fish, turtle and frog tubercle bacilli. All of these may cause disease in man.

There are, at present, 41 approved mycobacterial species – approved, that is, by 'experts' but not by all of those bacteriologists who actually work with the mycobacteria. The mycobacterial species fall into two main clusters – the slow growers and the rapid growers – which appear to have arisen as a result of a major split early in the evolution of the genus.

From the purely clinical point of view, mycobacteria are divisible into three groups (Grange and Collins, 1983). These are

(1) the obligate pathogens *M. tuberculosis* and *M. leprae*;
(2) species that normally live freely in the environment but also cause 'opportunist' infections in man; and

(3) species that never, or with extreme rarity, cause disease.

To this a fourth group may be added to include the specific animal pathogens *M. paratuberculosis* and *M. lepraemurium*.

The species in the second group have been referred to by a number of rather unsatisfactory collective epithets including 'anonymous', 'atypical', 'tuberculoid', 'non-tuberculous' and 'mycobacteria other than tubercle (MOTT)' bacilli. To this list we have added the more picturesque term 'nyrocine mycobacteria' (Grange and Collins, 1983). A history of these names is given in Chapter 9. The organisms in this group differ from *M. tuberculosis* in several important ways. They are rarely transmitted directly from host to host: they are almost always acquired directly from the environment. Consequently a major factor determining the occurrence of infections due to the various species is their distribution in the environment. This is influenced by many factors and varies considerably from region to region. As these species live freely in the environment they may occur as harmless residents of the skin, gut or upper respiratory tract of man and animals. As a result, the isolation of such a bacillus from a clinical specimen does not *per se* imply that it is responsible for disease.

Tuberculosis

Tuberculosis is by far the most frequently encountered mycobacterial disease in the world and, although its incidence has diminished significantly in the industrially more developed countries, it remains a major public health problem in most developing nations. Between 0.1 and 0.3 per cent of the population become infected each year in the former countries and this infection rate is declining by about 12 per cent annually. In the latter the annual infection rate is 20–50 times higher than in the former and this high level shows little or no downward trend. In view of the world's population growth the absolute number of cases of tuberculosis is increasing and there are certainly more cases now than at any previous time in history. It is estimated that, every year, between 50 and 100 million people are infected by the tubercle bacillus and, of these, 10–20 million develop overt disease and three million die. Put another way, the bacillus causes more than 5000 deaths every day or one about every 15 seconds.

The lung is the most frequently infected organ but other sites in which the disease often occurs include lymph nodes, bone, kidneys, the reproductive system, intestine, skin and central nervous system. No organ or structure is, however, exempt from infection and occurrence of disease in unusual sites may pose serious diagnostic problems.

Tuberculosis is usually acquired by the inhalation of small moist cough droplets containing a few bacilli. Such particles lodge in a peripheral part of the lung. The events following such infections vary enormously from patient to patient but it is nevertheless possible to discern a general pattern of progression or 'timetable' of disease (Wallgren, 1948). Local multiplication of bacilli at the site of implantation leads to the formation of a small lesion termed the Ghon focus. Bacilli are carried by the lymphatics from this focus to the draining lymph nodes where additional bacillary multiplication occurs. The lesion consisting of the Ghon focus and the enlarged regional nodes is termed the primary complex of Ranke (*Figure 1.1*).

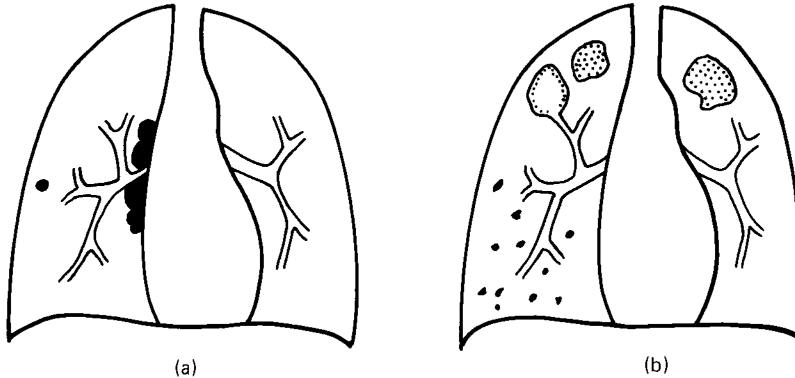

Figure 1.1 Comparison of primary and post-primary tuberculosis of the lung. (a) Primary focus with the small peripheral Ghon focus and enlarged hilar lymph nodes, comprising the primary complex of Ranke. (b) Secondary tuberculosis with apical cavities but no obvious lymph node involvement. One cavity has ruptured into the bronchus, causing spread of lesions to the lower lobes

The protective immune response in tuberculosis is of the 'cell-mediated' type and is due to the activation of macrophages by chemical mediators termed lymphokines released by lymphocytes following their contact with the appropriate antigen. Activated macrophages and lymphocytes form a compact aggregate around the bacilli, thereby forming the histological structure termed the granuloma. Such 'cell-mediated immunity' (CMI) takes about ten days to develop and appears to be distinct from the necrotizing 'delayed hypersensitivity' reaction which appears about six weeks after infection. (For further details of the immunology of tuberculosis and other mycobacterial diseases see Ratledge and Stanford, 1983.)

As a result of an effective CMI reaction the primary complex may heal or, alternatively, the disease may progress in one or more ways. Bacilli may spread from the primary complex to other sites by the blood stream, particularly in young children. This may lead to tuberculous meningitis which often occurs about three months after infection or to more slowly progressive lesions in bones, joints or the kidney, which are usually detected a year or more after infection. Alternatively a more widespread dissemination may lead to miliary tuberculosis with small granulomas resembling millet seeds (hence the name miliary) throughout the body. Vaccination with Bacille Calmette Guérin (BCG) reduces the incidence of such dissemination but it is not clear whether this is due to CMI as described above or to some other action of the vaccine. Frequently, the disseminated bacilli lie dormant for several years or even decades before they reactivate giving rise to post-primary tuberculosis. The usual site for such reactivation is the apical region of the lung. Unlike primary disease, post-primary tuberculosis is usually characterized by tissue necrosis and the formation of cavities which play an important role in the transmission of the bacilli.

Not all patients with tuberculosis are infectious. For transmissibility to occur, there must be a communication between the active lesion and the outside world. Such communication usually results from the ulceration of a tuberculous pulmonary cavity into a bronchus: this enables the bacilli to be expectorated in the sputum. As a rule, only patients whose sputum contains enough bacilli to be seen on a standard but competent microscopical examination are infectious. Such patients are said to have open or smear-positive disease. As a further rule, patients with primary disease, in whom cavity formation is rare, tend not to be infectious.

Leprosy

This disease is considered in Chapter 11.

Other mycobacterioses in man

Other mycobacterial infections of man are, in relation to tuberculosis and leprosy, uncommon. Two of the other mycobacteria cause specific named diseases while the remainder cause less specific infections which often mimic the manifold forms of tuberculosis.

The two named diseases are Buruli Ulcer – caused by *Mycobacterium ulcerans* – and Swimming Pool Granuloma (also called Fish Tank Granuloma) caused by *M. marinum.*

Infection due to *M. ulcerans* was first described in the Bairnsdale region of Australia. Later, '*M. buruli*' was isolated from skin ulcers in the Buruli district of Uganda and was found to be identical with *M. ulcerans*. The disease was subsequently found in several restricted localities within the tropics. Although never cultivated from the environment, it is thought to be a free-living organism that is introduced into the skin of patients by spikes on vegetation. The bacilli multiply in the subcutaneous fat which undergoes necrosis and liquefaction. Eventually the overlying skin breaks down and the necrotic fat is discharged leaving a deeply undermined ulcer. At this stage numerous bacilli are present at the advancing edge of the lesion and, as in lepromatous leprosy, the specific immunological reactivity is suppressed. All patients, however, reach a point in time when, for unknown reasons, immune reactivity appears, bacilli disappear and healing commences. Unfortunately fibrosis and contractures occurring during healing usually leave the patient with a severe disability or disfigurement.

Mycobacterium marinum occurs in swimming pools and fish tanks and enters the skin through abrasions acquired by swimmers or fish fanciers. The infection usually manifests itself as a raised, pink, soft warty lesion and it is common to find one or more secondary and proximal lesions along the route of the draining lymphatics. The disease is usually benign and self-limiting: more serious infections involving tendons and joints have been reported but these are extremely rare.

The other mycobacterial species that infect man cause much less characteristic lesions. The relative incidence of the species involved varies from region to region and is related to the occurrence of the species in the environment. The most frequently encountered slowly-growing species are *M. avium* (including *M. intracellulare*), *M. kansasii* and *M. xenopi.* Less frequent as causes of disease among the slow growers are *M. haemophilum, M. malmoense, M. scrofulaceum, M. simiae* and *M. szulgai.* There are only two rapidly-growing species of importance as pathogens: *M. chelonei* and *M. fortuitum.* Other species are not regularly encountered as pathogens but, especially in this age of iatrogenic immunosuppression, any mycobacterium must be regarded as a potential cause of disease until proved otherwise. Thus there are reports of infections due to *M. gordonae, M. triviale, M. nonchromogenicum* and even *M. smegmatis.*

Four main types of 'atypical' mycobacterial infection occur. First, lymphadenopathy, usually cervical and usually caused by *M. avium-intracellulare*, in young children. In the absence of an immunosuppressive disorder such infections are mostly self-limiting, particularly as such nodes are often excised for diagnostic

purposes. Second, progressive lung disease occurs, usually in the elderly male with underlying lung damage. This type of disease often resolves on standard anti-tuberculous therapy, despite *in vitro* resistance to the drugs, provided that there are no serious complicating factors. Third, widespread disease involving many organs may occur in immunosuppressed individuals, particularly children with congenital defects in cell-mediated immunity, individuals with hairy cell leukaemia and certain other neoplasias, transplant recipients and, more recently, victims of acquired immunodeficiency syndrome (AIDS). Such infections are often fatal despite intensive therapy with a wide range of antibacterial agents. Finally, infections result from the implantation of mycobacteria into tissues as the result of injections, accidental trauma and surgery. A number of cases of 'sterile' post-injection abscess have been shown to be caused by the rapid-growers *M. fortuitum* and *M. chelonei.* Such infections are usually self-limiting or respond to adequate drainage but the same species have caused a number of widespread and life-threatening infections following major surgery, particularly open-heart surgery. Very occasionally, superficial skin infections mimic lupus vulgaris or swimming pool granuloma.

It is clear therefore that the mycobacteria are the cause of a very important group of infectious diseases of man. Despite much research since the time of Koch, attempts to develop an immunological or biochemical diagnostic test for these infections have proved unsuccessful. Accordingly a good bacteriological service is essential to the detection and diagnosis of these diseases as well as to their subsequent management.

For a review of mycobacterial diseases see Grange (1980).

Chapter 2

The role and scope of the tuberculosis laboratory in human and veterinary medicine and epidemiology

> It is astonishing to what extent many doctors in most countries, including tuberculosis specialists, underestimate the importance of *finding the tubercle bacillus* for the diagnosis of tuberculous disease.
>
> H. J. Ustvedt

Despite phenomenal advances in immunological techniques for the diagnosis of disease, none have been found that are usefully applicable to tuberculosis or to other mycobacterial infections. Accordingly bacteriological examinations remain of paramount importance in the diagnosis and management of these diseases.

The roles of the tuberculosis laboratory are various and differ from region to region. These roles may be summarized as follows:

(1) detection of acid-fast bacilli in sputum and other specimens by microscopy;
(2) submitting clinical specimens to culture for mycobacteria;
(3) determining whether an isolate is *M. tuberculosis* or one of the other species;
(4) identifying these other species;
(5) conducting drug sensitivity tests;
(6) consulting with clinicians on the diagnosis and management of disease;
(7) collecting and analysing data for epidemiological purposes;
(8) teaching, including the training of technical staff; and
(9) research.

Naturally not all of these functions will be carried out by every laboratory. In most countries bacteriological services are arranged in a hierarchical fashion as described in Chapter 3.

In regions with a high incidence of tuberculosis but with limited provisions for treating the disease the major role of the medical services is to detect infectious cases and to treat them. There is strong evidence (Rouillon, Perdrizet and Parrot, 1976) that, for practical purposes, only those patients whose sputa contain enough bacilli to be detected by microscopy are infectious. There is little to be gained in detecting additional cases by more expensive cultural techniques unless these patients can also be offered courses of chemotherapy.

The culture of specimens for mycobacteria is much more costly than microscopy. It requires incubators, facilities and materials for preparation of media and the necessary skilled staff to undertake decontamination, inoculation of media and detection of positive cultures. Nevertheless it serves three major purposes. First, as indicated above, it detects patients whose sputa contain insufficient bacilli for them to be detected by microscopy. It has been estimated that, for their microscopic detection, there must be between 5000 and 10 000 acid fast bacilli in every ml of

sputum while cultural techniques will detect as few as ten viable bacilli (Rouillon, Perdrizet and Parrot, 1976). Second, culture enables a definite identification of the bacillus to be made. This is not so important in areas where tuberculosis is very common as the great majority of isolates will be *M. tuberculosis*, but in countries in which there is a very low incidence of this disease a substantial number of isolates will be of other species. Third, culture is an essential prerequisite for sensitivity testing.

In many respects, the role of the veterinary mycobacteriology laboratory is similar to that concerned with human disease although, as infected animals tend to be slaughtered rather than treated, sensitivity testing is not a major activity. Veterinary laboratories are concerned principally with the isolation of the bovine strain of *M. tuberculosis*, bird-pathogenic strains of *M. avium*, and *M. paratuberculosis* – the cause of hypertrophic enteritis or Johne's disease of cattle. From time to time though, there may be a need to investigate outbreaks of mycobacterial infections among other captive or free-living animals.

In addition to the above functions, central reference laboratories are often responsible for the quality of mycobacteriology practised in their country. These laboratories are often responsible for the training of staff who work in the dependent laboratories and they may maintain a check on the quality of work by sending, from time to time, specimens containing known mycobacteria including *M. tuberculosis* of known drug resistance patterns. Reference laboratories are often also the source of both medical and technical advice.

Although in some countries tuberculosis is a notifiable disease, this surveillance technique is not perfect – many cases go unreported. Estimates of the incidence of the disease based on the number of positive cultures reported by laboratories are also unsatisfactory as bacilli are not cultured from all cases of the disease. Taken together, however, a fairly accurate estimation of the incidence of disease can be reached. Examination of the tubercle bacilli by using, for example, the methods for subdivision described in Chapter 8, may yield further important epidemiological information. Thus, for example, the behaviour of bovine bacilli in the community may be studied and differences in the bacterial population between various ethnic groups of patients may be found. The results of sensitivity testing are also of epidemiological importance as they draw attention to breakdowns in good therapeutic practice and delineate populations in which there is a high incidence of drug resistance.

Epidemiological studies on infections caused by other mycobacterial species can be conducted only if laboratory reports are available. Such studies are of importance as they delineate populations at risk from such infections and also point to potentially dangerous sources of infection. The colonization of water-softening resins in renal dialysis machines (Azadian *et al.*, 1981) is one example. In some cases, though, 'epidemics' of such infections may be artefactual due to contamination of the specimens or culture media. Reference specialists, with their knowledge of the ecology of mycobacteria, may be able to detect the origin of such contamination (Collins, Grange and Yates, 1984).

A final important role of tuberculosis laboratories at all levels is research. There are, of course, many areas of mycobacterial research, particularly immunology and the more elaborate exercises in taxonomy, that are outside the scope of units providing a diagnostic service. Nevertheless such units are the usual source of organisms used in such work. There are, on the other hand, important fields of research in which the role of the diagnostic laboratory is paramount. One obvious,

yet seriously neglected, field is the improvement of the techniques for detecting mycobacteria in, and isolating them from, clinical material. In 1954 Dubos complained that mycobacteria were still being isolated from clinical specimens by 'primitive bacteriological techniques worked out decades ago . . .'. He went on to remark that standard decontamination methods must kill many mycobacterial cells as well as the contaminants and that media based on egg or serum offer conditions that are far from optimal for bacillary growth. Despite a further three decades of progress, Dubos' comments remain largely true. David (1978) postulated that many mycobacterial cells die as a result of their failure to adapt from *in vivo* to *in vitro* conditions – a form of microbial 'cultural shock'. Various 'cocktails' of antimicrobial agents incorporated in media have been used as alternatives to chemical decontamination (Mitchison *et al.*, 1972) but these, perhaps because of the increased cost, have not been widely used. Likewise various radiometric methods for the early detection of mycobacterial growth have been developed but these costly techniques still rely on standard media and decontamination procedures. It is paradoxical that the most fundamental aspects of mycobacteriology, i.e. the isolation of the bacilli, should have received so little attention. It is therefore open to any worker in a routine laboratory to make revolutionary advances in this neglected area of research.

Chapter 3

The organization of tuberculosis laboratory services

Before the introduction of effective antituberculous drugs, the bacteriology of tuberculosis was a relatively simple part of the work of the bacteriology departments of general hospitals and was usually confined to the examination of direct smears of sputum. This work was also done at tuberculosis dispensaries and clinics. Cultures, guinea pig inoculations and the identification of tubercle bacilli isolated by either method were the province of laboratories in the specialized tuberculosis hospitals and sanatoria.

This pattern of work changed fairly rapidly after the Second World War, when chemotherapeutic agents, first streptomycin and then PAS and isoniazid, became available. Patients rapidly became non-infectious and it was no longer necessary to isolate them for long periods in sanatoria. People with tuberculosis could be treated in general hospitals and as out-patients. Tuberculosis bacteriology therefore moved away from the specialized laboratories into those of general hospital pathology departments.

Good techniques for the culture of tubercle bacilli were already available, methods for testing the susceptibility of the organisms to the new drugs were developed, and a new interest arose in certain other mycobacteria that seemed to be associated with human disease. Unfortunately some laboratories used suboptimal methods and there was frequently a lack not only of experience but also of interest in this work. It was not unusual for physicians to comment on the variations in the quality of the assistance they received from laboratories. A strong argument developed for tuberculosis bacteriology – or at least the more technically demanding procedures – to become once more the province of specialized laboratories. Moreover, the problems of providing a rational laboratory service to assist in the treatment and control of tuberculosis are not confined to developed countries. The organization of tuberculosis laboratory services, in both developed and developing countries, is therefore considered here under six headings:

(1) The grading of laboratories according to the service they provide.
(2) Design of laboratories.
(3) Supply and maintenance of equipment.
(4) Training and motivation of staff.
(5) Proficiency testing.
(6) The prevention of laboratory-acquired tuberculosis.

Grades of laboratories

In most developed countries each local laboratory determines what service it will offer and whether it will submit material to reference or other laboratories. In one European country, on the other hand, all tests, from simple microscopy of direct smears to sophisticated identification and sensitivity testing are done at one central laboratory. The concept of 'grading' laboratories according to the kind of service they offer arose in England and Wales and in the USA. Its successful application and its adoption in developing countries merits discussion here.

England and Wales

A Central Tuberculosis Laboratory was established at Cardiff (South Wales) in 1932 and this became the Tuberculosis Reference Laboratory of the Public Health Laboratory Service in 1959. In 1962 a National Register of Drug Resistant Tuberculosis was opened. The register was intended to monitor the effectiveness of drug therapy and the incidence of drug resistance. Physicians were asked to register patients who were excreting tubercle bacilli that were resistant to streptomycin, PAS or isoniazid and also patients with sensitive or untested strains that were isolated after six months' chemotherapy. The physicians were also requested to send to the Tuberculosis Reference Laboratory two specimens (usually sputum) from patients proposed 'for registration or who had been notified as tuberculous in or after 1962, or if notified earlier, whose strain was recorded as sensitive to all three drugs or resistant only to PAS' (Marks, 1965a).

In earlier work a close agreement had been noted between the results obtained by the Reference Laboratory and those of the Medical Research Council's Unit for Research on Drug Sensitivity (Marks, 1965a) and therefore it was postulated that valid comparisons could be made between the Reference Laboratory's results and those of the laboratories that had tested strains from candidates for the register. This postulate was investigated and the findings (Marks, 1965a) were disturbing. It was evident that in most parts of the country a diagnosis of resistance in a recent case of tuberculosis was more often wrong than right and that in long-standing cases many resistant strains were reported as sensitive. In addition, some laboratories failed to distinguish between tubercle bacilli and other acid-fast organisms. The amount of disagreement was so large that action was needed and, following Marks' (1965a) recommendations, the Public Health Laboratory Service (1966) established six Regional Centres for Tuberculosis Bacteriology in England. The Reference Laboratory (now the Mycobacterium Reference Unit) acts as Regional Centre for Wales in addition to its other duties.

The Regional Centres offer a service to hospital and other laboratories that culture mycobacteria but do not wish to identify them or perform drug sensitivity tests. There is no compulsion to use this service and those hospital and public health laboratories that do so are not bound to send their material to the nearest centre.

The Regional Centres collaborate with the Reference Unit, use agreed techniques and participate in a performance control scheme. The organization in England and Wales may be summarized thus:

(1) Hospital and public health laboratories examine direct smears and culture specimens. If they wish, they send cultures to a Regional Centre.

(2) The Regional Centres identify tubercle bacilli, may identify other mycobacteria and do sensitivity tests to the commonly used drugs. Problem cultures, resistant strains from new patients and requests for sensitivity tests to rarely used drugs are referred to the Reference Unit.
(3) The Reference Unit identifies mycobacteria using sophisticated as well as standard methods, performs sensitivity tests to all antimycobacterial (and other) drugs, issues performance test cultures, monitors results and undertakes research.

In 1974 the work of the Regional Centres was reviewed by Marks (1975), who was the Director of the Tuberculosis Reference Laboratory in Cardiff. It was apparent that the service was working very well and was much appreciated by physicians. The Reference Laboratory and Regional Centres examined more than 90 per cent of strains of mycobacteria isolated by Public Health Laboratories but it was not possible to estimate the proportion of strains isolated and examined elsewhere. Nevertheless, in a survey conducted by the Medical Research Council for the six months from October 1978 to March 1979 it was found that 1611 (96 per cent) of 1676 cultures for identification and sensitivity tests were examined by the Reference and Regional Laboratories (Medical Research Council, 1980; Jenkins *et al.*, 1985). In 1982 The Reference Unit received approximately 3500 cultures from 200 client laboratories, the Dulwich Regional Centre 2600 from other laboratories and the other Regional Centres rather smaller numbers.

USA

Developments in the USA parallelled those in England and Wales, probably for the same reasons, but two separate organizations each proposed slightly different methods for grading laboratories.

The College of American Pathologists (see Sommers, 1974) proposed four 'Extents of Service', to be self-determined by the participating laboratories:

Extent 1 Do not examine material for mycobacteria.
Extent 2 Smears are examined and culture media inoculated. Positive cultures are sent to another laboratory.
Extent 3 Identify tubercle bacilli, do preliminary identification of other mycobacteria and may do sensitivity tests.
Extent 4 Identify mycobacteria to establish correct diagnosis and may do sensitivity tests.

The other system, which uses three 'Levels of Laboratory Service', was introduced by the American Thoracic Society (1974) and further details were given by Kubica *et al.* (1975). The functions of laboratories at the three levels have recently been redefined (American Thoracic Society, 1983). In principle, these are:

Level I These laboratories collect adequate specimens and forward them to laboratories at a higher level. They may also examine direct smears.
Level II Perform Level I tasks, culture specimens on standard media, identify tubercle bacilli and may do sensitivity tests.
Level III Perform the functions of Level I and Level II laboratories, identify all isolates of mycobacteria, do sensitivity tests, provide training, supervise proficiency tests and undertake research.

Developing countries

The organization of tuberculosis laboratory services in developing countries has necessarily been slow because of the enormous logistical problems involved, but considerable advances have been made with the help of the World Health Organization and the International Union against Tuberculosis. There is general agreement that three kinds of laboratories are needed: peripheral, intermediate and central (Mitchison, 1982).

(1) The peripheral laboratory. Tuberculosis bacteriology is restricted to direct smears (Ziehl–Neelsen method).
(2) The intermediate laboratory. This does direct smears (Ziehl–Neelsen and fluorescence microscopy) and may inoculate culture media which are then transported to the central laboratory.
(3) The central laboratory. This should be capable of doing direct smears, culture, identification and sensitivity tests and be responsible for training, supplies, organization, quality control and research.

These three systems of organizing laboratory services have much in common. At the lowest level the laboratory may be a side room of a tuberculosis or primary health care clinic, or part of the multipurpose laboratory of a small hospital. The middle grade laboratories will probably be in district hospitals while the central or reference laboratories are likely to be in important cities, and either part of large general hospital laboratories or in research institutes.

The arrangements may well change, however. In developed countries, where the incidence of tuberculosis is declining, the numbers of laboratories in the lower and middle grades may be reduced. On the other hand, countries that have centralized all their services may find that the research potential of the work does not merit large establishments and that there may be good economic reasons for redistributing the diagnostic work among smaller laboratories. In developing countries there may be a need for more peripheral laboratories to do direct smears and some intermediate laboratories may extend their service to include culture and sensitivity testing (but see p. 91).

Design of the tuberculosis laboratory

Those who work with tubercle bacilli are at risk from laboratory-acquired infection, mainly by the airborne route and it is well known that sensible laboratory design may contribute much to the prevention of such infections (Collins, 1983 and pp. 24–30 below). According to several codes of practice (e.g. Department of Health and Social Security, 1978; World Health Organization, 1983; Centers for Disease Control and National Institutes of Health, 1984), all work with tuberculous material should be done in containment laboratories, physically separated from other laboratory areas. The object is to reduce the risk, not only to tuberculosis bacteriologists, but also to other individuals in the same building. This is entirely reasonable insofar as work with cultures and suspensions of the organisms is concerned but the requirement poses an organizational problem in clinical laboratories because tubercle bacilli may be present in almost any clinical material. It is almost impossible to process every specimen received in a hospital

bacteriological laboratory under containment conditions (Allen and Darrell, 1981). Nor is the risk that great. A sensible solution is to examine all sputum specimens in containment laboratories because they are more likely than any others to contain tubercle bacilli. Other material may then be examined in the ordinary laboratory unless acid-fast organisms are seen in direct smears, tubercle bacilli have already been found in other specimens from the same patient, or if tuberculosis is suspected on clinical grounds. Allen and Darrell emphasized the need for such information on the request forms.

There is also an ethical problem. Containment laboratories are expensive and while there should be no difficulty in providing them in developed countries, they may well be beyond the budget of tuberculosis programmes in developing countries. The question of a double standard arises, whereby some laboratory workers are protected against laboratory-acquired infection and others are not, largely on financial grounds. Only pragmatic answers are possible. In most developed countries the incidence of tuberculosis is low and is declining: laboratory workers are more likely to be exposed to infection in the laboratory than outside. On the other hand, in developing countries, where tuberculosis is usually a serious public health problem, the risk to laboratory workers of becoming infected is probably greater during the 16 hours or so when they are at large in the community than in the eight hours when they are at work. Ultimately, however, it is the responsibility of those in charge of tuberculosis programmes to determine the priorities.

In this chapter we suggest how both containment and ordinary laboratories may be designed and equipped for tuberculosis bacteriology in the most economical way and at the same time offer the staff a reasonable degree of protection from infection. We must stress, however, that even with the best facilities the prevention of infection depends largely on the skill of the worker and his attitude to his job.

Containment laboratories

Contrary to common belief among health service administrators, architects and engineers containment laboratories do not need sophisticated and expensive air conditioning. Indeed, many of the problems and failures of safety cabinets in new laboratories are directly attributable to such airflow and extraction systems, quite apart from the fact that incorrect pressure gradients may place workers in other rooms at risk from airborne infections. The principle of a containment laboratory is that during working hours air is continually extracted to the outside of the building either through a microbiological safety cabinet or through a simple extract fan in the wall or window. A pressure gradient is thus established so that air always flows from the corridor or other laboratories to the containment laboratory and finally outside the building, thus preventing the dispersal of airborne micro-organisms to other parts of the building. As the exhaust air from safety cabinets is filtered to remove bacteria and the dilution factor at the point of exhaust is considerable there is hardly any risk to people in the vicinity.

Containment laboratories require microbiological safety cabinets. Arrangements for fitting these should be made at the design stage. Afterthoughts usually result in poor performance and unnecessary expenditure.

There are three classes of microbiological safety cabinets but only Class I and Class II cabinets are suitable for the tuberculosis laboratory. They are both shown diagrammatically in *Figure 3.1*. In the Class I cabinet air at a velocity between 0.5

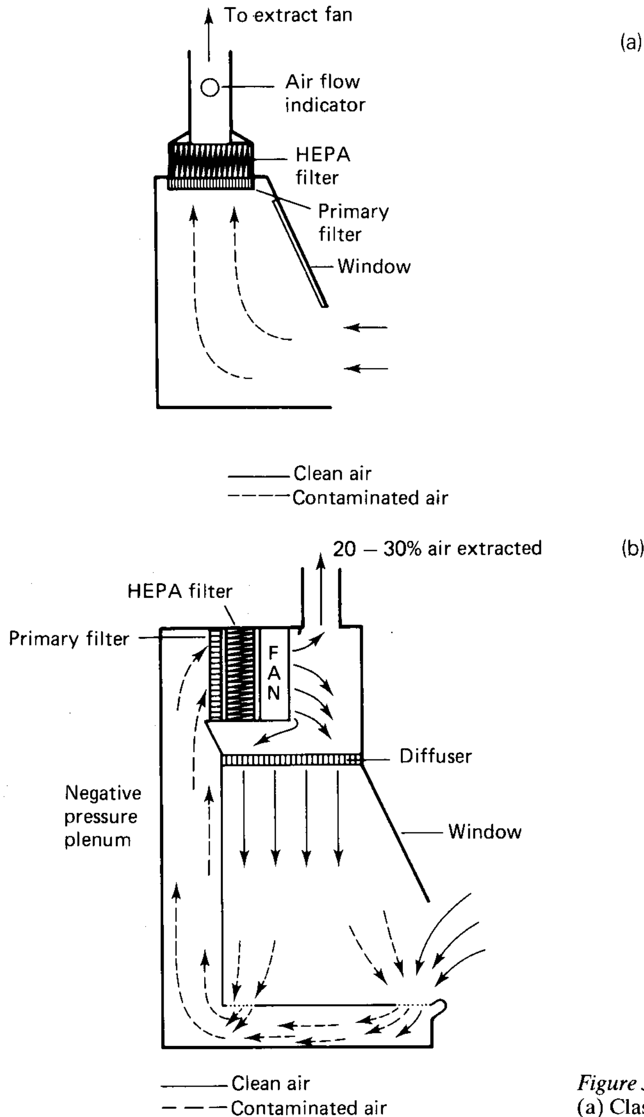

Figure 3.1 Microbiological safety cabinets. (a) Class I. (b) Class II

and 0.75 m/s passes in through the open front, and over the working area where it entrains any airborne bacteria and conveys them into the High Efficiency Particulate Air (HEPA) filter which retains most if not all of them. It is then ducted to the outside of the building.

In the Class II cabinets air is also taken from the room and filtered but some is then recirculated vertically through the cabinet so that a 'curtain' of air descends across the working face and forms a barrier that prevents bacteria leaving or entering the cabinet. The remaining air is exhausted to outside the building.

In the UK there is an official preference for Class I cabinets for work with organisms in Risk Group III, but in the USA and much of the rest of the world Class II cabinets are the norm (Collins, 1983).

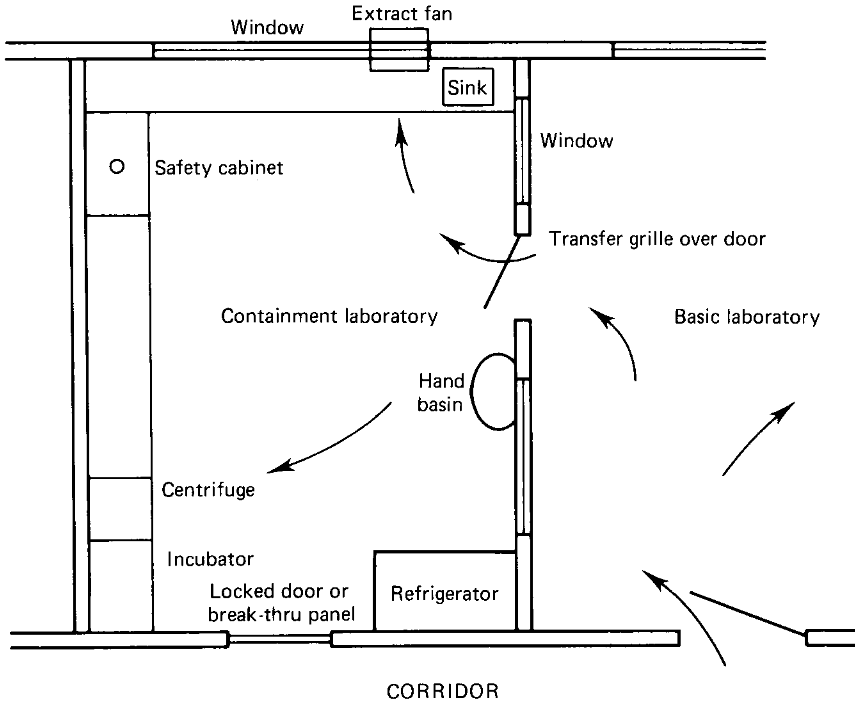

Figure 3.2 Plan for a containment laboratory fitted with a Class I microbiological safety cabinet (arrows indicate direction of airflow)

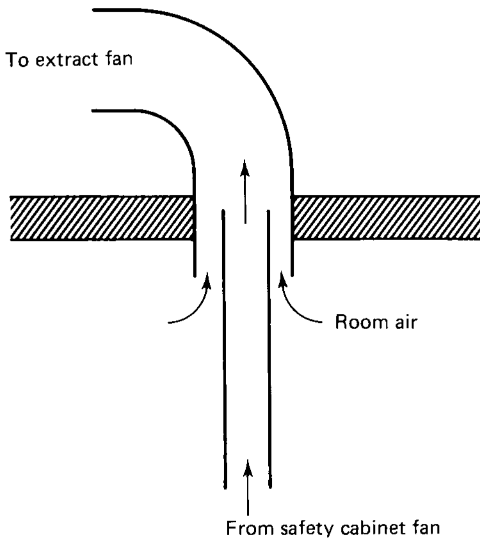

Figure 3.3 'Thimble' unit for a Class II microbiological safety cabinet (arrows indicate direction of airflow)

Figure 3.2 shows a containment laboratory fitted with a Class I microbiological safety cabinet. This example is adequate for laboratories at all levels: rooms incorporating similar features should be provided in hospital and public health laboratories that handle sputum and cultures of tubercle bacilli.

If a Class II microbiological safety cabinet is preferred then the 'thimble system' should be used instead of an extract fan (*Figure 3.3*). The thimble allows air to be extracted continuously from the room and from the safety cabinet when it is in use. Because of the differences in the velocities of air extracted from Class I and Class II cabinets thimble systems are not recommended for the former.

For information about the design, choice, installation and testing of safety cabinets see Clark (1983) and Collins (1983). Firm arrangements should be made for these cabinets and their filters to be tested regularly according to the national standard or the manufacturer's recommendations.

The containment laboratory should have five clearly defined working areas:

(1) A reception bench for specimens.
(2) The cabinet bench with room either side for specimens before and cultures after processing in the cabinet.
(3) A staining area with a sink.
(4) A microscopy bench, preferably in the darker part of the room, especially if fluorescence microscopy is used.
(5) A 'clean' area for record keeping.

Floor spaces should be reserved for a discard bin, a centrifuge and an equipment cupboard.

There should be a hand basin (staff should not be expected to wash their hands in the sink used for staining slides), a cupboard for clean gowns and a linen bag for used gowns. Ideally these should all be in the lobby, but if this is not provided they should be as near as possible to the door.

Other design features should conform to established recommendations, for example, of the World Health Organization (1983), the Centers for Disease Control and National Institutes of Health (1984) and the Advisory Committee on Dangerous Pathogens (1984). For more detailed information on laboratory design see Collins (1983).

The peripheral laboratory

A peripheral laboratory may be in a small hospital or a primary health care clinic. In addition to the examination of sputum smears the work may include other laboratory tests and space must, therefore, be provided for all of these activities. Ideally the tuberculosis work should be done in a separate room, but if this is not possible a separate area should be reserved for it.

Figure 3.4 shows how such a tuberculosis laboratory could be designed. If it is not possible to fit a safety cabinet there should be as much natural ventilation as possible and the tuberculosis bench should be placed so that air from open windows does not blow across the bench and carry infectious particles released during the work into the face of the worker.

A peripheral laboratory may not have an autoclave and an alternative must be provided for the decontamination of discarded specimens, containers and other items. Disinfectants are bulky and costly to transport as well as being unreliable in

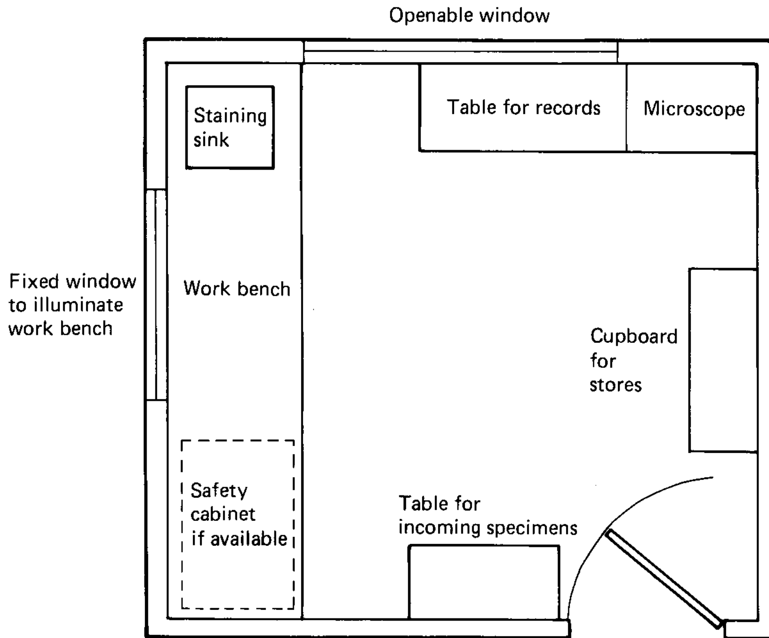

Figure 3.4 Plan for a peripheral laboratory

some hands. A pressure cooker might be one answer. The simplest methods of treating infected material are boiling and burning. A bucket of water over a primus stove is probably adequate for small articles and may be used in the laboratory, but a larger 'boiler', adapted from an oil-drum or petrol-can suspended over a wood fire, should be placed outside. An effective incinerator may be made from an oil-drum.

Equipment

Space does not permit a detailed inventory for every level of laboratory and this section is confined to listing the essential equipment for a containment laboratory. Advice on general equipment for work above the peripheral level is given by Collins and Lyne (1984) and Cheesbrough (1984). Small equipment, suitable for peripheral laboratories is described by the International Union against Tuberculosis (1978). The large or expensive items are microbiological safety cabinets (see above), microscopes, centrifuges, incubators and refrigerators.

Expensive and elaborate microscopes are not necessary for tuberculosis bacteriology, but they should be binocular and comfortable to use as the worker may spend many hours with them every day. Microscopes, especially the fluorescence types, need regular maintenance. This should not be a problem in the larger laboratories, but instruments provided for field work, many miles from the nearest instrument repairer, should be simple and robust, and easily maintained by the staff of a peripheral laboratory. Such a laboratory may be equipped with only one microscope and therefore a spare oil immersion lens and spare eyepieces should be provided. Arrangements should be made for the prompt replacement of damaged optical equipment.

An autoclave is necessary in all but the most remote peripheral laboratory (see above for alternatives). It need not be in the tuberculosis laboratory but should be nearby and in the same building.

Centrifuges are essential in laboratories where tubercle bacilli are cultured (although as indicated on page 46 there are methods that do not involve centrifugation). The machine should be large enough to accept at least six buckets each capable of holding a 28 ml screw-capped bottle ('Universal Container'). Sealed buckets ('safety cups') should be provided. Sealed rotors are not recommended.

Incubators are usually electrically operated, but oil-heated models are still available and have proved to be very reliable in places where there is no electricity supply. In large establishments walk-in incubators ('hot rooms') are more convenient. Problems arise in warm countries with incubation below the ambient temperature, e.g. for the identification of some mycobacteria. Small incubators, or water-baths, may be placed inside refrigerators, with the electricity supply taken in through a hole drilled through one of the walls.

Domestic-type refrigerators are adequate for most kinds of tuberculosis bacteriology but they should have a freezing compartment. Deep-freezers might be considered a luxury.

Smaller equipment should include the following: slide staining and electrically-heated drying racks; slide holders; a Vortex test-tube mixer; a Waring type aerosol-free blender; Griffith or Ten Broeck glass–PTFE tissue grinders; alcohol sand jars (autoclavable 500 ml jars containing 200 g of sand and 250 ml of alcohol for removing material from wire loops); hooded or microbunsens*; pipetting devices; an atomizer for disinfectant; diamond writing styli. Some pieces of small equipment are shown in *Figure 3.5*.

It is an unfortunate fact that in some developing countries laboratories receive gifts of unsuitable equipment. It may even be purchased for prestige purposes. Care should be taken to avoid these embarrassments. One senior and very experienced tuberculosis officer whose work takes him into many developing countries has told us that they do not need electron microscopes and automatic analysers, but pots, slides, bottles and stains.

It must be remembered that equipment and supplies may take a long time to reach peripheral and intermediate laboratories, and even some central laboratories. Supplies officers must therefore think well ahead and maintain considerable stocks of the basic materials, especially slides, containers, stains and chemicals. We have heard of peripheral laboratories and primary health care clinics that have been unable to perform microscopic examinations for months because the supplies of slides and stains failed to arrive.

Training and motivation of staff

Training

Graduate microbiologists are rarely recruited directly into tuberculosis laboratories and usually work for some time in clinical or biomedical laboratories before they consider transferring. Posts are usually limited to reference or central laboratories

* A. R. Horwell, 2 Grangeway, Kilburn High Road, London NW6 2PB, UK; Denley Instruments, Daux Road, Billinghurst, Sussex RH14 9SJ, UK

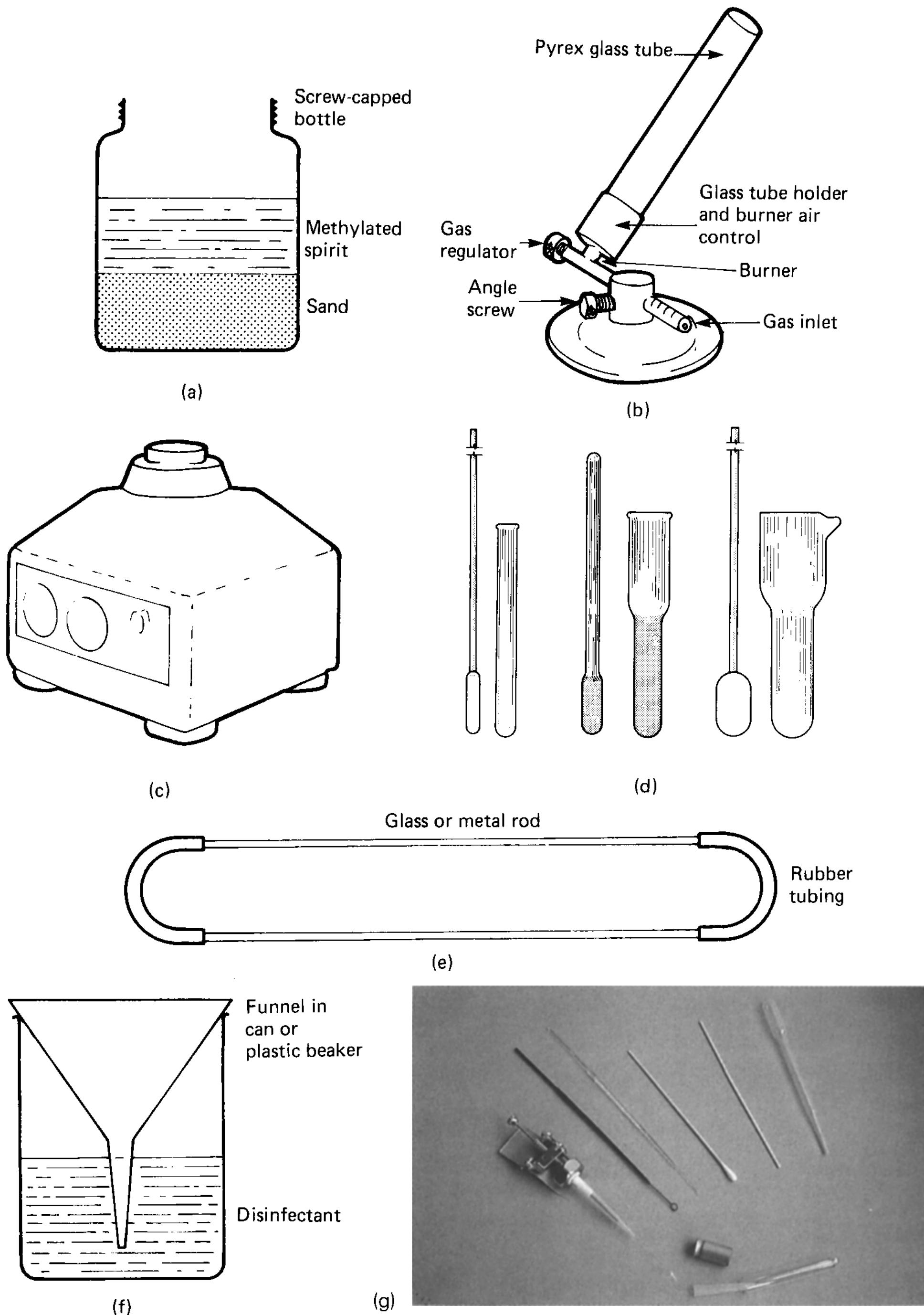

Figure 3.5 Small equipment for a tuberculosis laboratory. (a) Alcohol–sand bottle for cleaning loops before flaming. (b) Hooded (Kampff) microbunsen for safely flaming loops. (c) Vortex mixer. (d) Tissue homogenizers (Camlab). (e) Slide rack for staining. (f) Discard pot for pouring infected fluids into disinfectant. (g) Loops and pipetting aids

(Extent 4 or Level III in the US systems). Serious 'in-service' training of newcomers, irrespective of their qualifications and experience in other fields, should begin at once, by apprenticing them to experienced workers. Before they are allowed to embark on research or sophisticated investigations they should be taught, and obtain considerable experience with, the basic techniques such as making and examining direct smears and the preparation of material for culture. This early training should also include studies on laboratory-acquired tuberculosis and its prevention. It should lead naturally to the more demanding techniques and decision making.

Prospects for promotion in tuberculosis laboratories are not very good, however, and if young microbiologists have any ambitions it is necessary for them to move to other work and obtain experience and possibly higher qualifications. The wastage is therefore high and only a small number of individuals remain permanently dedicated to tuberculosis bacteriology.

Technical staff (technicians or technologists in some countries; medical laboratory scientific officers in the United Kingdom) may be employed at all levels other than in peripheral laboratories. They usually obtain their qualifications by part-time or short-term attendance at colleges while employed as junior staff in clinical laboratories. The college courses, however, are tending to become more and more academic to the detriment of technical training which may then be obtained only if the laboratory in which they work maintains high standards. Unfortunately many senior technical staff are less concerned with good laboratory practices than with management.

Tuberculosis bacteriology is only one of many activities with which technicians are involved and proper training in that field is usually deferred until they have two or three years' experience and have obtained their initial qualifications. Indeed, in the United Kingdom it is unusual for junior medical laboratory scientific officers to be employed in tuberculosis work. When they have obtained their first qualification they are usually 'rotated' through the various laboratory departments or sections to gain experience for the next examinations. This means that they may spend only a month or two in the tuberculosis laboratory and indeed may never learn more than how to make direct smears and prepare cultures. Even after they are fully qualified they may have to continue with the 'rotation' system rather than take a full-time appointment in any one section.

As with graduates, the urge for promotion results in a fairly rapid turnover of staff and the permanent staff of tuberculosis laboratories spend a great deal of their time training people who move on as soon as they become reasonably competent. The American Thoracic Society (1983) recommends that technical staff do not rotate through tuberculosis laboratories but remain committed to that particular kind of work. The success of this scheme depends partly on motivation (see below) but much more on a satisfactory grading and salary structure.

Wastage of trained staff can be a serious problem, as Hiriart (1981) illustrates: In Chile, between 1968 and 1978, 26 qualified technicians were trained in a central laboratory for work in regional or intermediate laboratories. By the end of 1979, 13 no longer worked in that field, nine continued to work but only part-time and only four remained to do the work for which they had been trained.

Laboratory assistants, who work in peripheral laboratories, often have some experience in primary health care and at best will have received some training at an intermediate laboratory. Sometimes, however, training may take place at the peripheral laboratory itself, when it may vary from good to very bad. It should

include the collection of good specimens, making smears of proper material, staining and examining them competently and the prompt shipping of material to the nearest intermediate or central laboratory. The assistant should also be trained to repair and maintain his equipment as the nearest service agent or instrument repairer may be hundreds of miles away. He should also be taught the simple management methods that ensure adequate stocks of materials are maintained (Mitchison, 1982).

Motivation

'Job satisfaction' is a well-known principle in management: a person will do a job well only if he is interested in it and finds it psychologically, if not financially, rewarding. This is summed up in the word 'motivation'. There are no glib answers to the problems of staff motivation – or lack of it – in tuberculosis bacteriology. Our experience has taught us that only a few people really enjoy the work and for that reason alone it is often done badly outside those laboratories where there is enthusiasm and dedication, and above all, good leadership.

One outstanding reason why many people dislike working in a tuberculosis laboratory is that they have to manipulate sputum. Many years ago one of us was in charge of a laboratory where members of the staff often arrived late in the morning. He decreed that the last to arrive each day would do the sputum tests. This did much for punctuality but little for the quality of the tuberculosis work. Oddly enough, no-one ever objected to working with faeces, possibly because they are 'natural' whereas sputum certainly is not.

Another reason stems from the training programmes outlined above. Scientists and technicians who have spent four or five years obtaining their qualifications frequently resent doing what they consider to be menial tasks such as preparing and examining sputum smears. They look for what they imagine to be the glamour of research or prefer the excitement of the clinical laboratory, where they may meet and identify a dozen different organisms each day, often by 'kit' or automated methods that require little initiative or professional skill. Even if graduate and technical staff do adapt to what may appear to be mundane tasks and then progress and become proficient at the more interesting work, such as the identification and sensitivity testing of mycobacteria, they may not become sufficiently motivated to continue with it.

Motivation may be fostered in several ways. One is by associating all members of the laboratory staff with the clinical and epidemiological aspects of tuberculosis therapy and control. This may be achieved by arranging visits to clinics and hospitals, and talks and demonstrations by physicians. The laboratory staff should also be involved in clinical trials and in contact tracing. All this will help them to appreciate the problems of tuberculosis at the 'sharp end' and enable them to identify with a team.

Furthermore, all members of the staff should be involved in some kind of research. This may be as members of a group which is engaged on some major programme, in which case their contribution, however small, should be acknowledged when the results are published, preferably in the title line but at least in the acknowledgements. Some editors may object to the former, but their diffidence can be overcome by ensuring that the persons named were in fact given a real job to do and so made a proper contribution. Alternatively, individuals may be given small but useful technical projects such as comparisons of methods or the

extraction of information from records. Either way, they will be made to feel involved and will gain valuable experience in conducting investigations on their own.

Unfortunately there are two large snags in schemes such as these. One, mentioned above, is the rotation system, where a technician may stay for only one or two months in each department or section, including the tuberculosis laboratory. The American Thoracic Society (1983) recommends that technicians are permanently allocated to tuberculosis work but this arrangement will work only if the staff are happy about it. The other problem relates to promotion. It is unfortunate that the highest paid jobs in the technical grades go not to those who have proved their worth at the laboratory bench but to those who hanker for the manager's chair and the committee room. This is unlikely to be resolved until there is a radical restructuring of career grades so that the best scientist or technician – and the distinction, as already suggested, is rapidly becoming blurred – can command the highest salary and status without engaging in what is essentially the work of clerical and personnel assistants and store keepers.

In developing countries the problems of staff motivation, particularly in the peripheral laboratories, can be even more acute. The best qualified and motivated staff are likely to be concentrated in the central laboratories, with fewer at the intermediate level and perhaps none in the peripherals, which are the front line in the war against tuberculosis. The policy of sending technicians from developing countries to the big city laboratories of the western world results in their determination, whether or not they have the skill, to do only the more sophisticated work and therefore to stay in their own big cities when they return home. Like some graduates and technicians in developed countries, they will claim that they have been trained for better things than sputum smears and cultures. The inevitable result is that the central laboratories become 'ivory towers' while the work at the peripheral, or even at the intermediate laboratories is left to the least skilled who are often the most poorly motivated. This results in wastage and rapid turnover of staff at this level and to a low standard of work. Then, as Hiriart (1981) has pointed out, the intermediate laboratory becomes the most important of the three laboratories.

Motivation of staff in peripheral laboratories needs a different approach as those suggested for higher levels are inappropriate. Health workers in the field cannot be expected to do any kind of research and may be very much 'out on a limb'. Mitchison (1982) suggests increased status and pay linked to an examination system. Those who pass would be awarded a certificate or diploma. Possession of one of these would enhance the personal standing and prestige of the health worker in his own community and motivate him to continue with the work.

Proficiency testing

Proficiency testing or quality control is highly desirable but not easy to achieve. Ideally a central laboratory sends out natural or simulated material. Some samples contain no acid-fast bacilli, others contain varying numbers. The recipients make direct smears and cultures and report their results to the central laboratory which can then assess proficiency. There are several variations on the procedure.

Peripheral laboratories need examine only prepared and stained or unstained slides. At higher levels slides and material, or material only, may be required, and

for laboratories that identify mycobacteria and test their sensitivities the material may contain opportunists and/or resistant organisms. Participation in proficiency testing schemes may be compulsory, as in hierarchical laboratory organizations, where the central laboratory is responsible for those at the lower levels. Or it may be voluntary, as in the UK, where the Quality Control Laboratory (QCL) of the Public Health Laboratory Service provides a variety of material to any interested laboratory. Each laboratory has an identity code known only to itself and the QCL. The QCL sends out collective reports so that each of the participants can see how their proficiency compares with that of others. Furthermore, the Mycobacterium Reference Unit of the Public Health Laboratory Service sends four cultures of freshly isolated, known sensitive, tubercle bacilli each week to Regional Centres, who include them in batches of sensitivity tests to determine the Modal Resistance (see p. 95). This allows each Regional Centre to control the results of its own tests and the unit to monitor the performance of the Regional Centres (Marks, 1975).

The College of American Pathologists has been conducting similar proficiency testing programmes for several years (Sommers, 1974; Kubica *et al.*, 1975; Sommers and MacClatchy, 1983). These are geared to the Extent system (see above) and have 'proved to be very useful in providing a broad assessment of the abilities of diverse laboratories'. Nyboe (1978), Aber *et al.* (1980) and Mitchison *et al.* (1980) have all reported on the usefulness of quality control in tuberculosis bacteriology. The quality control system used in the UK for sensitivity tests was discussed by Marks (1975). (See also Chapter 10.)

In addition to assessing the abilities of laboratories to report correctly, a proficiency testing scheme may detect technical and clerical errors, e.g. where positive cultures are obtained from negative material or reports are inadvertently switched.

Although such schemes are practical in areas where the laboratory services are generally well developed, there may be problems elsewhere. As Mitchison (1982) has pointed out they must take the form of continuous assessment and to be successful require skilled and dedicated staff.

Not the least of the problems is a supply of suitable material, although paradoxically this is easiest in developing countries where the establishment of proficiency testing schemes is most difficult.

The simplest method is for the central or quality control laboratory to send out prepared slides of selected material, e.g. homogenized sputum, diluted to give none, few or large numbers of acid-fast bacilli (Mitchison, 1966). Preparing material for culture is more difficult as larger volumes are required and in some of their investigations Aber *et al.* (1980) used a methyl cellulose–egg solution to create artificial material to which tubercle bacilli could be added.

Prevention of laboratory-acquired tuberculosis

Precautions that should be taken by laboratory workers against infection with the tubercle bacillus were listed in the early 1950s in the USA by Fish and Spendlove (1950) and by Long (1951). It was recognized at the time, however, that the long incubation period of the disease and exposure to the organisms outside the laboratory made it difficult to determine if infections were job-associated.

The incidence of the disease among the staff of clinical laboratories in the UK was first quantified by Reid (1957). He compared it with that of groups of Post

Office staff and found that laboratory workers were from two to five times more likely to contract the disease than the matched controls. He identified 151 cases of tuberculosis among a total laboratory staff of 4824 in 368 laboratories during the years 1949–53 but excluded 51 who might not have acquired the disease in the laboratory.

It is interesting to compare Reid's findings with those obtained some years later by Harrington and Shannon (1976) who confined their investigations to single years, 1971 in England and 1973 in Scotland. When they compared their figures with those for the general population they found that the risk to laboratory workers was five times greater than that to the community at large. There appeared to have been little improvement in the UK in 15 or 16 years in spite of recognition of the problem by the health authorities and the publication by them of excellent recommendations for the safe handling of tuberculous material (Report, 1958; Department of Health and Social Security, 1970). The problem persisted, however, and responsibility for not following the official recommendations must be shared by both employers and employees. Eventually, a Code of Practice for the Prevention of Infection in Clinical Laboratories (Department of Health and Social Security, 1978) was produced and the implementation of its requirements was monitored by a new statutory body, the Health and Safety Executive.

Such was the situation in the UK. Other states have also had problems and some have found ways of dealing with them. For reviews see Collins (1982, 1983.)

Tubercle bacilli and the classification of pathogens on the basis of hazard

Various schemes exist for classifying micro-organisms into groups, classes or categories on the basis of the hazard they offer to those who work with them and to the community. These are shown and compared in *Table 3.1*. The tubercle bacillus is included in Risk Group III in the WHO (1983) classification along with other micro-organisms most likely to infect laboratory workers by the airborne route. It is in comparable classes in the other systems.

Risk Group III micro-organisms include those that are particularly associated with infection by the airborne route. Precautions therefore involve measures to minimize the production and dispersal of aerosols and infected airborne particles and to prevent the worker and other people from inhaling those that might be released, as well as measures intended to prevent infection by accidental ingestion and inoculation.

Specific precautions are mentioned at appropriate places in this book and only a brief review can be given here. (For more detailed information see Collins, 1983.)

Inhalation hazards

It is generally accepted that most of the workers who acquire tuberculosis in the laboratory become infected by inhaling airborne tubercle bacilli released during laboratory manipulations. Many microbiological techniques generate aerosols. These are minute droplets of liquid that are formed when bubbles burst and other thin films are separated. They are also formed when liquids are squirted through small orifices, or impinge on surfaces, even of other liquids. Each droplet may contain one or more micro-organisms. The smaller droplets settle very slowly and dry rapidly, leaving the organisms they contain as 'droplet nuclei' or infective airborne particles. These particles float in the air of a room and are moved about by

TABLE 3.1. Summary of systems for classifying micro-organisms on the basis of risk to laboratory workers and the community

World Health Organization[a]	Risk Group I	Risk Group II	Risk Group III[e]	Risk Group IV
	Low individual, low community	Moderate individual, limited community	High individual, low community	High individual, high community
United States[b]	Biosafety Level 1	Biosafety Level 2	Biosafety Level 3[e]	Biosafety Level 4
	Not known to cause disease in healthy adult humans	Moderate risk, present in the community; associated with human disease of moderate severity	Real potential for infecting by airborne route; serious or lethal consequences	High individual risk of life-threatening disease
United Kingdom[c]	Hazard Group 1	Hazard Group 2	Hazard Group 3[e]	Hazard Group 4
	Most unlikely to cause human disease	Might be a hazard to laboratory workers but unlikely to spread in the community	Serious hazard to laboratory workers; may present risk of spread in community	Serious hazard to laboratory workers; high risk of spread in community
European Federation of Biotechnology[d]	Class 1	Class 2	Class 3[e]	Class 4
	Have never been identified as causing disease in man; no threat to environment	Might offer hazard to laboratory workers; unlikely to spread in environment	Severe threat to laboratory workers; small risk to population at large	Serious hazard to laboratory workers and to population at large

[a] World Health Organization, 1983
[b] Centers for Disease Control and National Institutes of Health
[c] Advisory Committee on Dangerous Pathogens, 1984
[d] European Federation of Biotechnology, 1985
[e] *Mycobacterium tuberculosis* (all variants) is in these categories

very small air currents, even from room to room. If they are inhaled their small size allows them to be taken down into the lungs, where they may initiate an infection. The larger droplets do not dry but settle rapidly and contaminate the hands and laboratory surfaces. If they are inhaled they are removed by the filtering mechanisms in the upper respiratory tract.

A considerable measure of protection against the inhalation of aerosols and airborne infection is given by the controlled airflows in the containment laboratory (p. 14) and by microbiological safety cabinets (p. 15) but neither of these is a substitute for good technique. Nor will they protect the worker against a massive release of aerosol or contact with spilled infectious material.

The following activities generate aerosols.

Work with bacteriological loops

Vigorous spreading of culture media and/or preparation of films from cultures should be avoided. Loops should be used gently. For making films saturated mercuric chloride, not water, should be used. This kills tubercle bacilli rapidly. Loops charged with infectious material should not be placed in the flames of ordinary bunsen burners or the material may spatter before it is burned and contaminate surfaces. Loops should first be cleaned by rotating them in an alcohol sand bath or by being held in the flame of a hooded bunsen (*Figure 3.5b*). Disposable plastic loops are preferable.

Pipetting

Pipettes should be drained, not blown out violently or the last drop will form bubbles which burst and form aerosols. Pasteur pipettes are particularly likely to generate bubbles.

Opening culture bottles and plates

If there is a thin film of fluid between the rim of the bottle and its cap, or between the rim and lid of a Petri dish, aerosols will be formed when they are separated. Bottles should be opened in a safety cabinet.

Centrifugation

Fluid will spill from uncapped centrifuge tubes, be aerosolized by the action of the rotor in the bowl and will issue with considerable velocity from between the lid and the bowl and through the ventilation ports. Even if tubes are capped there is always the risk that one will break, releasing a large amount of aerosol. Sealed centrifuge buckets should always be used in tuberculosis laboratories.

Mechanical homogenizing

Some homogenizers leak at the seals and release aerosols. Unless the special aerosol-free Waring model* is used homogenizers should be covered during use with Perspex boxes, which are afterwards disinfected.

* Christison Ltd, Albany Road, East Gateshead Industrial Estate, Gateshead NE8 3AT, UK.

Pouring into disinfectant

When infectious material is poured into disinfectant it may splash and contaminate the surrounding surfaces and it may produce aerosols. The apparatus shown in *Figure 3.5f* should be used. The end of the funnel should be beneath the surface of the disinfectant in the jar or can and the material to be discarded poured carefully down the side of the funnel.

Dispersion of dried material

Not all infectious airborne particles arise from aerosols. Dried material may be dispersed, e.g. when ampoules of freeze-dried cultures are opened by methods other than those prescribed by the issuing laboratory. Dried pathological material and sputum homogenates, around the screw threads of containers may be released as fine powders when the container is opened.

Ingestion hazards

Tuberculous materials may be ingested by direct aspiration as in mouth pipetting and by putting into the mouth fingers and articles that have been contaminated while on the laboratory bench. This hazard may be overcome by a complete ban on mouth pipetting, licking labels, sucking pencils, smoking and eating and drinking in the laboratory. Fingers may also pick up tubercle bacilli from the outside of sputum containers. Allen and Darrell (1983) found that 65 per cent of 279 sputum containers were contaminated in this way. It may be necessary to disinfect containers as a routine measure.

Inoculation hazards

'Needle stick' accidents are regrettably common, where an individual is accidently stabbed by a hypodermic needle which may be infected. These instruments should not be used as substitutes for pipettes. There are many safer instruments on the market that measure small amounts of fluid. Cuts from infected glassware also occur and glass Pasteur pipettes are the most dangerous objects. The soft plastic varieties are much safer.

Personal precautions

Laboratory workers should maintain high standards of personal hygiene and in particular wash their hands frequently and certainly after removing any protective clothing and before leaving the room.

Personal outdoor clothing, shopping bags and other articles that will be taken home should be placed in lockers outside the containment laboratory.

Protective clothing should be worn at all times. The choice is between gowns of the operating-room type or coats that wrap over the body, fasten at the side and cover the upper chest and neck. Sleeves should be wrist length and gathered to prevent contamination of the sleeves of normal clothing and to prevent particles entering the sleeves when the worker is using a safety cabinet.

Protective clothing should be removed before the wearer leaves the containment laboratory.

Medical supervision

Staff who work with tubercle bacilli should be healthy and well-nourished.

They should be vaccinated with BCG before they are accepted for employment or produce evidence that they have a positive skin test or have had the vaccine.

Wherever possible, and with due regard to medical contraindications, all workers should have an annual chest X-ray.

Disposal of infected material

No infected material should leave the laboratory except when it is properly packed for transport to another laboratory. All pathological material, smears, cultures and the vessels in which they were grown should be sterilized or disinfected before disposal or re-use.

The words sterilization and disinfection do not have the same meaning. Sterilization implies the complete destruction of all organisms; disinfection implies the destruction only of those organisms that are capable of causing disease. In laboratories sterilization is usually accomplished by heat and disinfection by treatment with chemicals.

Methods for the safe disposal of infected material in peripheral laboratories that do not have autoclaves are suggested on p. 18.

Sterilization

Autoclaving is the optimal initial procedure and staff should be carefully instructed in the procedure. Autoclaves should be tested periodically to ensure that they reach a temperature in the chamber that is high enough to kill all bacteria. The minimum should be 121 °C and this should be maintained for 15 minutes. Testing is best done by placing thermocouples in the load and connecting them to an external recorder. If these instruments are not available sterilizer indicator tubes are the best alternative. These are placed in the load and are examined when the autoclave is opened after use. If the process was adequate the indicator will change colour (the range of colours is given by the manufacturers).

After autoclaving waste material may be burned, buried or otherwise dumped. Re-usable articles may be washed.

A domestic pressure cooker is used in the same way as an autoclave, but its load is severely limited.

If neither an autoclave nor a pressure cooker is available re-usable articles containing infectious material should be boiled for at least 60 minutes before washing. Material to be discarded should be incinerated.

Disinfection

The only proprietary disinfectants suitable for laboratory use are those containing phenols, hypochlorites or glutaraldehyde. Sweet-smelling 'antiseptics' should not be used. To have any effect phenolic and hypochlorite disinfectants should be diluted accurately according to the manufacturers' instructions for 'dirty or worst possible situations'. The dilutions should be freshly prepared each day, not stored or their activity will diminish. Glutaraldehyde does not require dilution but an activator (provided separately by the manufacturer) must be added. The activated disinfectant usually remains effective for several days or a week.

Glutaraldehyde may be used for decontaminating bench surfaces, etc. after a spillage or at the end of a day's work.

Articles to be disinfected are usually placed in buckets or bench discard pots. They must come into intimate contact with the fluid. No air bubbles should be seen in submerged objects. The contact period should be at least 18 hours (usually overnight). The fluid should then be poured away and the disinfected articles or materials washed or disposed of. The buckets or pots should be washed in very hot water.

Two other chemicals used as laboratory disinfectants are formaldehyde and alcohol.

Formaldehyde is a gas and is supplied as an approximately 38 per cent solution in water, known as formalin or as a solid polymer, paraformaldehyde. The gas is evolved when either is heated. Formaldehyde is used for disinfecting microbiological safety cabinets. The manufacturer's instructions should be followed as the amounts and methods of heating vary according to the make and size of the cabinet. The gas is also used for disinfecting rooms but public health or hygiene officers trained in the technique should be employed for this purpose as respirators should be worn. For emergency disinfection of surfaces formalin may be sprayed from an atomizer, but the operator should work rapidly and leave the room as quickly as possible.

Alcohol, usually 70 per cent ethanol (methylated spirit) or propanol is used in alcohol–sand baths and for decontaminating benches and surfaces. It should also be used instead of water to balance centrifuge buckets.

All these disinfectants are toxic and exposure may result in respiratory distress, skin rashes or conjunctivitis. Used normally, however, according to the manufacturers' instructions, they are usually safe.

Chapter 4

Collection, preservation and transport of specimens

Tubercle bacilli may be present in almost any pathological material but success in finding them in microscopic preparations and cultures depends initially on the care taken in selecting and collecting the specimens.

Essential prerequisites for the safe collection of satisfactory specimens are the containers which should be robust and leakproof. As far as possible, all containers should be sterile.

Sputum

Containers

These present quite a problem. In spite of the wide variety of glass, plastic and metal jars, pots and bottles no really satisfactory sputum container exists. If the patient is to produce sputum directly into the container it must have a wide mouth, otherwise the outside will certainly become contaminated. Wide-mouthed containers, however, are difficult to stopper. Screw-on caps easily become cross threaded; large press-on lids become distorted and the wads or liners in both kinds frequently fall out. In any case, the contents will leak and may contaminate the hands or person of anyone who handles them. In hot climates and when the specimen has to travel some distance the contents of improperly closed containers are likely to dry up before they can be examined.

When there is a choice, the least unsatisfactory containers are glass or plastic pots 5–6 cm deep with 4–5 cm diameter mouths. Plastic pots suitable for use in developing countries may be obtained from UNICEF. These are easily burned after use.

It is good practice to supply patients with plastic bags in which they can place their specimen containers after use.

Collection

Sputum is the specimen of choice in the investigation of pulmonary disease and should be collected whenever possible, preferably before commencement of chemotherapy.

It is best to collect the first specimen at the clinic. The health worker can explain why the specimen is needed and that sputum, coughed up from deep in the lungs is required, not saliva. The patient should be instructed to place the container against the lower lip and to expectorate carefully so that the outside of the container is not contaminated.

Not all patients can cough up sputum from the lower respiratory tract at their first attempt; supervision and encouragement by the nurse, physiotherapist, technician or laboratory assistant not only ensures that a good first specimen is obtained, but that subsequent specimens, preferably collected early in the morning on each of the next few days, are also satisfactory. A good specimen for laboratory examination should be between 2 and 5 ml.

At least three and possibly as many as six consecutive sputum specimens may be necessary as tubercle bacilli may be released intermittently from lesions in the lungs. A positive smear or culture may be found only after a series that have been negative. Specimens should not be bulked: this could dilute a positive, distribute contaminants and also introduce problems in preparing cultures.

Patients should be asked not to use antiseptic mouth washes just before providing a sputum specimen.

Laryngeal swabs

If sputum cannot be obtained laryngeal swabs are the next best specimens but they are almost useless for direct smear microscopy.

The swabs are made of stiff wire, about 25 cm long and bent at one end to give an angle of about 35 degrees. This end is roughened with a file so that the cotton wool swab, not more than 0.5 cm thick, may be wound on tightly and will not become detached in the patient's larynx. Bicycle spokes, which have a screw thread at one end, make excellent laryngeal swabs. The swabs are placed in glass tubes, with about 5 cm of the straight end protruding; the tubes are plugged with cotton wool and sterilized. Two swabs should be taken from each patient by a physician or trained nurse who is skilled in visualizing the larynx, and who should wear a visor because the patient will cough violently when the swab is inserted into his throat.

Induced sputum; bronchoscopy; bronchial washings; transtracheal aspiration

Descriptions of these clinical procedures for obtaining specimens from the respiratory tract are outside the scope of this book. Our personal experience, however, is that the Lukan traps used in the collection of bronchoscopy specimens and bronchial lavage are highly unsuitable specimen containers. They are particularly liable to leak and frequently arrive at the laboratory in a messy and unsatisfactory condition.

Urines

Containers

The most satisfactory containers for urine specimens are the 28 ml straight-sided screw-capped glass or plastic bottles known as Universal Containers (Collins and Lyne, 1984). One advantage of these is that they fit 50 ml centrifuge buckets, obviating transfer of fluid to centrifuge tubes.

Collection

The best results are obtained from early morning midstream specimens. Women may have difficulties with these containers and should be instructed to pass a midstream specimen into a wide-mouthed vessel which has been boiled and to fill the specimen bottle from that. Three consecutive daily specimens should be provided. Twenty-four hour specimens should be discouraged; they usually become contaminated.

Aspirated fluids and pus

Containers

Again, the 28 ml screw-capped Universal Containers are ideal. Plain bottles and bottles containing a non-bactericidal anticoagulant should be provided for the collection of aspirated fluids. Plain bottles are suitable for pus and exudates.

Collection

Fluids that are likely to form clots should be divided between two bottles, one plain and the other containing citrate or some other non-bactericidal anticoagulant. Samples for cytological and chemical examination may also be required.

As much pus as possible should be collected into a plain bottle. Throat swabs dipped in pus are rarely satisfactory specimens for tuberculosis bacteriology and should be regarded as a last resort and used only when a very small amount of material can be obtained.

Tissues

Containers

Universal Containers, sputum pots or larger glass jars may be used depending on the size of the specimen. The containers used for histological specimens should not be used. The fixative kills tubercle bacilli, and even if this is poured off enough may remain to affect bacteriological examination.

Collection

This is the province of the surgeons, but they should be persuaded to divide the material between the microbiologist and the histopathologist and not expect both to work with the same material.

Milk samples

Screw-capped bottles, at least 100 ml capacity, should be provided. Quarter samples should be collected, about 100 ml from each teat. Foremilk may be included. Samples from bulk milk containers are useless.

Water samples

Water is occasionally examined for opportunist mycobacteria, e.g. in tracing contamination. At least 2 litres should be collected in sterile screw-capped winchester bottles.

Identifying specimens

Labels

The container should be labelled at the time the specimen is collected. If it is labelled earlier it may be used by someone else; if later it may be confused with the specimen of another patient. The patient's name and some other identification such as his clinic or hospital number, should be written clearly on the label. Hospitals and clinics frequently have several patients with the same names.

Request forms

In all but peripheral laboratories and primary health care clinics, where details on the labels are usually copied into the registers, the specimen should be accompanied by a request form completed, or at least signed, by the doctor requesting the examination. The laboratory should provide sensible forms and those who make requests should write legibly and give the information required. The request form should ask for the minimum of information. The more questions asked the less likely they are to be answered.

The information required by the laboratory is:

(1) The name of the person or organization requesting the test and who expects to receive the report.
(2) The address to which the report should be sent.
(3) The surname or family name of the patient, followed by his/her given or local name.
(4) Some other identification of the patient, e.g. hospital or clinic number.
(5) The sex and age or date or birth of the patient.
(6) The nature of the specimen, e.g. sputum, urine, pleural fluid. If the specimen is pus or tissue then the site from which it came must be stated.
(7) The examination required.
(8) Diagnosis or very brief clinical information.

There may be some problems in developing countries in supplying names and ages but in developed countries, where medical records are or should be in good order, there is no excuse. Failure to give the information in this list is discourteous and may indicate a low standard of care, especially when specimens or cultures are sent from one laboratory to another.

Preservation of specimens

In the laboratory specimens may be preserved for several weeks in a refrigerator, preferably at or just below 0 °C. Preservation during transport at ambient

temperatures requires chemical treatment. Three methods give reasonably good results:

(1) The fresh specimen is mixed with an equal volume of 1 per cent cetyl pyridinium chloride in 2 per cent sodium chloride. Tubercle bacilli will survive in this for at least a week.
(2) The fresh specimen is mixed with anhydrous sodium carbonate in the proportion 2 ml of specimen to 50 mg of the reagent.
(3) If the delay before examination is to be less than 24 hours the specimens may be mixed with an equal volume of 23 per cent trisodium phosphate.

Transport

When specimens are sent from a clinic to a laboratory or from one laboratory to another it is important that they arrive in good condition for the tests required and that they do not leak or offer any hazard of infection to those who transport them or handle them while in transit.

Speedy transport is essential for good results. Delays allow unwanted organisms, present in most specimens, to multiply with the result that cultures are contaminated and the organisms sought cannot be found. The fact that there are preservation methods (see above) is no reason for complacency; they are all suboptimal. Managements often seek to 'integrate' transport of pathological material with that of other material. This usually operates to the detriment of the specimens, the result of the examinations and the care of the patients.

Requirements and recommendations for the safe transport of pathological specimens, including cultures, are given in various national and international codes of practice and guidelines; see, for example, those published by the Department of Health and Social Security (1978), the Centers for Disease Control and National Institutes of Health (1984) and the World Health Organization (1983). The postal and transport authorities of most countries, and the International Air Transport Association make regulations about conveying such material. All these should be consulted.

In principle, pathological material intended for postal or air transport should be in approved, robust, leak-proof, 'primary' containers which are packed into 'secondary' containers, made of metal, wood or strong cardboard, with enough absorbent material so that if they are damaged or do leak the fluids will be absorbed. For sending material across international or state boundaries this container may have to be packed in the same way in an outer container and special administrative arrangements with the postal authorities and airlines may be necessary.

Many specimens, however, travel by local transport. Sputum specimens, for example, are sent by peripheral laboratories or clinics to intermediate or central laboratories. Boxes of metal or wood, preferably the former, should be provided. They should be made to hold a number of specimen containers, packed vertically so that they are not likely to leak. These boxes should not be too large or too heavy for one man to carry as he may have to travel long distances. The lid should be securely fastened and in some areas it may be desirable for it to be locked. The boxes should be disinfected and then scrubbed clean at regular intervals.

Chapter 5

Examination of direct smears

Direct microscopical examination of pathological material for acid-fast bacilli may be a valuable or quite useless procedure depending on the nature and quality of the specimen. If this is satisfactory it is either spread directly on a microscope slide or concentrated by centrifugation, if necessary after treatment to lower its specific gravity so that any mycobacteria are more easily sedimented. The smears are then stained by variations of two standard methods.

Preparation of specimens

Sputum

The microscopical examination of direct smears of sputum is probably the most useful test in the diagnosis and control of pulmonary tuberculosis as it permits the rapid detection of infectious patients as well as the monitoring of the progress of the disease and its treatment.

The sputum specimen is examined by eye and its consistency and appearance recorded, e.g. as mucoid, mucopurulent, purulent, blood stained. Local policies should determine whether obvious saliva should be examined.

The next part of the procedure is hazardous to unskilled operators and great care is necessary to avoid contaminating the surrounding areas or releasing airborne droplets (fortunately, sputum is not easily aerosolized).

The smears may be made of sputum in its natural state or of the centrifuged deposit after it has been digested with various reagents.

Sputum is not an easy material to manipulate and the normal tools of bacteriologists, loops and wires, are usually too thin and springy to transfer the most rewarding parts of a specimen to a slide or to a tube or bottle for digestion. Very thick (2 mm diameter) nicrome wire is available but requires special loop holders. The flaming of loops may be hazardous and alcohol–sand bottles and hooded bunsens should be used (*Figure 3.5a,b* and p. 27).

The best tools for handling sputum are undoubtedly the plastic disposable loops now commonly available. The larger, 10 μl, size is the most convenient, but the smaller, 1 μl, loops are useful for separating the chosen part of a specimen from the remainder. It is often necessary to use two loops to do this. Alternatives are throat swabs and the wooden sticks from which they are made. Another advantage of all

these tools is that they may be discarded into disinfectant: they do not have to be flamed.

Although these instruments are satisfactory for making direct smears of sputum they are not optimal for the preparation of centrifuged deposits as larger volumes of the specimens are required: encouraging purulent or very mucoid material from the original containers into those used for processing can be a very messy and hazardous business if it is done with loops or sticks. Ordinary pipettes are unsatisfactory because sputum is too thick to be sucked through the small holes in their tips. The most useful tools are pieces of glass tubing, about 4 mm internal diameter, cut into 200 mm lengths. One end is rounded off in a bunsen flame to accept a rubber teat or pipetting device; the other end is left rough and sharp. The teat is compressed and the sharp end of the tubing placed in the sputum and rotated. It will cut the sputum, allowing it to be sucked into the tube. When the tube is full, rotating it again will free the end of sputum and the contents may be discharged into another container.

New slides should be used whenever possible. It is not good practice to wash and re-use them, especially if they have already been used for material containing acid-fast bacilli. The slides should be numbered with a diamond-pointed stylus. Grease pencil or felt-tipped pen marks are frequently removed during staining.

To make the smears the slides should be placed on glass or ceramic tiles, preferably black, or on pieces of plain paper. The tiles are disinfected or the paper incinerated after each batch of slides has been made. In some laboratories the slides are placed on special racks or slide holders while the smears are made (*Figure 3.5e*). These holders should be placed on the tiles or paper. It is not good practice to spread sputum on slides resting on the bench or the floor of the safety cabinet.

A small portion of purulent or mucopurulent material is selected, separated from the remainder with loops or sticks and transferred to the slide. Care must be taken to avoid placing too much on the slide or it will not spread properly. The smear will then be too thick and may float off the slide during staining. The material is then spread carefully over an area equal to about two-thirds of the slide (*Figure 5.1*) using a loop or stick. This should not be done energetically or sputum will be

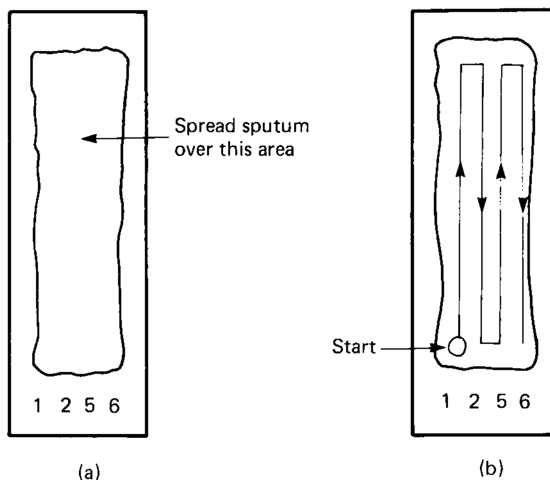

Figure 5.1 Spreading and examining a slide. (a) Sputum, etc. should be spread thinly over the area indicated. (b) Systematic scanning of stained smear, field by field for 100–300 fields

splashed over the surrounding area. It requires some practice to make evenly distributed smears. It is difficult, if not impossible, to see acid-fast bacilli in thick smears but thin smears may contain too little material to find them.

Sputum should on no account be squashed between two slides. The old technique of pressing sputum between slides and drawing them apart, while passing them through a bunsen flame is particularly hazardous. Dried particles become airborne and are easily inhaled. Safety cabinets cannot be relied upon to protect workers against such dangerous practices.

Care must be taken to avoid contamination of the fingers. At this and all subsequent stages slides should be handled with forceps. Slides may be dried in the safety cabinet on racks, or removed and dried by gentle heat over a slide drying rack. As soon as they are dry the slides are fixed by passing them through a bunsen flame. This does not necessarily kill tubercle bacilli (Allen, 1981) and the slides should still be regarded as infectious. They should not be left unstained or uncovered on the bench. If it is necessary to leave them for any period they should be placed in buffered formalin solution (Appendix) for 5 minutes and then dried.

Concentrated sputum

In some laboratories direct smears are made of the centrifuged deposits after digesting and concentrating the sputum. Any of the methods of concentration described for culture (pp. 45 *et seq.*) may be used.

The procedures for making the smears are the same as those for making smears of sputum, but as the deposit is aqueous and much less viscous there are greater hazards from aerosol production. This procedure should *not* be done on the open bench under any circumstances. The smears should be made with great care to avoid splashing and aerosol formation and they should be thin or the material will float off during staining. Concentrated material is less adherent to slides than sputum.

Induced sputum; bronchoscopy material; transtracheal aspirates

Specimens of these may be treated in the same way as ordinary sputum specimens.

Gastric washings

Direct smears should be avoided as the results can be misleading. Acid-fast bacilli are frequently present in food and water and hence in the stomach. There is no way of distinguishing such organisms from tubercle bacilli in microscopical preparations and positive smears must be regarded with suspicion.

Laryngeal swabs

Direct smears are almost useless. A negative result is meaningless. It is best to conserve what little material there is for culture.

Pus and thick aspirates

Direct smears of these specimens should be very thin. Thick smears tend to float off the slide and even if they are retained acid-fast bacilli may be difficult to see after

staining. Smears of concentrates may be prepared as for sputum. Problems may arise with either kinds of smear if a large amount of blood is present in the specimen. Blood sometimes produces acid-fast artefacts.

Pleural fluids

The fluid should be centrifuged and smears prepared from the deposit. Again, these should be thin or they may float off the slide.

Cerebrospinal fluids

Specimens of these should be centrifuged. In tuberculous meningitis there will be very little deposit and care is necessary to conserve it. Two parallel marks, about 10 mm long and 2 mm apart, should be made on a clean glass slide. A loopful of the deposit is then spread between these marks and the smear allowed to dry. Another loopful is then spread over the first. When this is dry the process may be repeated, depending on how much deposit is available. Some should obviously be left for culture. This procedure clearly marks the area to be searched for acid-fast bacilli. It is desirable that the smears are examined by two independent microscopists.

Some workers recommend examining any clot that may be present. This is difficult and we consider that clots should be saved for culture.

Urines

Direct smears of centrifuged urine deposits are very unreliable and should be avoided. Mycobacteria other than tubercle bacilli are sometimes present in urine, either when it is voided or as a result of poor collection techniques. The so-called 'smegma bacillus' (*Mycobacterium smegmatis*) is in fact rarely found in urine. There is no truth in the story that tubercle bacilli and 'smegma bacilli' may be differentiated by their relative acid- and alcohol-fastness (see pp. 43, 84). The commonest mycobacteria in urine specimens are scotochromogens which are often present in hospital water supplies. The presence of acid-fast (or acid- and alcohol-fast!) bacilli in urine should be viewed with suspicion.

Faeces, bone marrow, menstrual blood and biopsy material

Direct smears of faeces are worthless. Acid-fast bacilli are frequently present in the intestinal contents. Smears of bone marrow and menstrual blood are not particularly helpful either; acid-fast artefacts may confuse the diagnosis. It is better to await the result of a culture. Direct smears of biopsy material may be more rewarding. Smears made from 'tubercles' or lymph nodes may reveal acid-fast bacilli, especially if caseous matter is avoided and scrapings made from the inner walls.

Milk

Again, direct smears are of very little value, even in milk collected from individual teats. Other acid-fast bacilli are frequently present and cannot be distinguished microscopically from tubercle bacilli.

Staining

There are two standard methods of staining smears for acid-fast bacilli: the Ziehl–Neelsen (ZN) and the fluorescence microscopy (FM) techniques. The former uses an ordinary light microscope and the latter an instrument with ultraviolet illumination. The choice of method is influenced by the numbers of slides to be examined at any one time. The ZN is the method of choice if this number is not large, e.g. about ten or twelve, but for larger numbers much time may be saved and operator fatigue reduced by using the FM method.

In a comparative study reported by Kubica (1980) it was found that if the criterion for a positive (FM) smear was finding three fluorescent acid-fast bacilli then the method was as reliable as the ZN stain. On the other hand we have noted that in several British laboratories the FM method gave more 'false positives' than the ZN procedure. We believe, therefore, that positive FM smears should be confirmed by overstaining the smear by ZN.

The Ziehl–Neelsen (ZN) method

The interesting history of this stain is recounted by Bishop and Neumann (1970). Robert Koch (1882) used hot alkaline methylene blue as the primary stain and vesuvin (bismarck brown) as decolorizer and counterstain. Ehrlich (1882) used fuchsin as the primary stain, aniline as the mordant and a mineral acid as the decolorizer. Ziehl (1882) used phenol as the mordant. Also in 1882 Rindfleisch (cited by Schill, 1883) heated the slide instead of putting it into hot water. Finally, Neelsen (1883) combined Ehrlich's fuchsin stain with Neelsen's mordant. Thus the Ziehl–Neelsen stain as we know it today should properly be called the Ehrlich–Ziehl–Rindfleisch–Neelsen stain (see also Allen and Hinkes, 1982).

There are several formulae for the carbol fuchsin stain, acid or acid–alcohol decolorizing mixture and the counterstain (see Appendix). Some workers prefer a blue and others a green counterstain. Colour-blind workers are advised to use a picric acid solution which gives a yellow background. Two methods for ZN staining are given here: the 'traditional' technique and that of Tan Thiam Hok (1962).

The traditional ZN method

The smears are fixed by passing the slides, held in forceps, rapidly through a bunsen or spirit lamp flame twice in quick succession. They are then placed on the staining rack over a sink.

Freshly filtered carbol fuchsin stain is poured over the slides so that the smear is completely covered and the slides are then gently heated from below with a bunsen or spirit lamp flame until steam rises. The stain is allowed to act for 3–5 minutes but no longer or it may be precipitated and give an unsatisfactory microscopical appearance. The slide should not be heated more than once, nor should the stain be allowed to boil or the smear may be dislodged. The stain should not be allowed to dry on the slide.

The slides are then washed in running water, either by holding them individually under the tap or with a length of rubber tubing attached to the tap, in which case they may be left on the staining rack. They are then drained.

The decolorizing solution is then poured over the slides and allowed to act for 2–3 minutes, replaced and then rinsed off after another 2 minutes. If the smears are

very thick more decolorizer should be added until no more pink colour is removed. The slides are then washed again with running water and the counterstain poured on. This is left for 1–2 minutes and washed off.

The slides are then placed on edge to drain and allowed to dry. Alternatively each slide may be blotted dry with a fresh piece of filter paper or bacteriological blotting paper (which should not be used again) and dried either by passing them once through a bunsen flame or on a drying rack.

The Tan Thiam Hok (TTH) method

This is a cold staining method, developed for field use when heating slides is difficult. Good results have been obtained by Tan Thiam Hok (1962) and Mokhtairi (1980). The smears are stained with TTH carbol fuchsin for 3 minutes and then washed with tap water for 30 seconds. They are then flooded with Gabbett's solution and left for 1 minute, after which they are washed in water and allowed to dry.

Other cold staining methods

These are essentially the same as the traditional ZN but the carbol fuchsin solution contains dimethylsulphoxide or Tween 80.

The fluorescence microscopy (FM) method

There are several formulae for the reagents and the one we have found satisfactory is given in the Appendix.

The smears are fixed as described earlier and the slides covered with the auramine phenol stain. After 4 minutes this is washed off with running water. They are then decolorized with the acid alcohol for another 4 minutes. The acid alcohol is poured off and the slides covered with the potassium permanganate solution to 'quench' naturally fluorescent material. They are then washed and dried as described earlier.

The ultraviolet light source should be monitored regularly with a Blak–Ray meter.

Bulk staining methods

In bulk staining, i.e. processing a number, and a succession, of slides in the same solution in a single container there is always the possibility that acid-fast bacilli may float from one slide and become attached to others, giving 'false positives'. Many bacteriologists have therefore counselled against this method.

On the other hand it can save a great deal of time and materials. Clancy *et al.* (1976) found no false positives when they used the Shandon–Elliot automatic staining machine for processing large numbers of slides by the FM method. Fodor (1984) rinsed, decolorized and counterstained carbol fuchsin-stained smears in common containers without finding false positives.

Quantitative microscopy

The number of acid-fast bacilli present in a patient's sputum may indicate the severity of his disease and degree of infectivity. A quantitative result is therefore required by some physicians and health workers.

TABLE 5.1. Quantitative microscopy of direct sputum smears

No. of acid fast bacilli seen	*Report*
None in 300 fields[a]	Negative
1–2 in 300 fields	Doubtful: repeat
1–10 in 100 fields	+
1–10 in 10 fields	++
1–10 in 1 field	+++
10 or more in 1 field	++++

[a] High power, oil immersion lens

ZN-stained smears should be scanned systematically as shown in *Figure 5.1* and the number of bacilli counted. At least 100 high power fields (HPF) should be examined before it is concluded that the smear is negative. *Table 5.1* shows a reporting system in common use.

Precautions against false positives

False-positive smears may occur if acid-fast bacilli from one slide are transferred to another. There are several ways in which this may occur. Slides that have been used before may still retain some bacilli. Multiple staining pots (Coplin jars) in which several slides are stained in succession are a possible source: acid-fast bacilli float off one slide and attach themselves to others (but see bulk staining, above). Another source is used blotting paper which is frequently contaminated with portions of stained smears. Scratches on old slides may retain the fuchsin and look like acid-fast bacilli.

False positives sometimes occur as 'outbreaks'. Suspicions should be aroused if the number of positive smears increases suddenly or there is a series of consecutive positives. It is advisable to check the register or day book each week to see if there are departures from the laboratory norm. When we investigated two such incidents we found acid-fast bacilli and fluorescent artefacts in water from a laboratory storage tank in one laboratory and acid-fast bacilli in the demineralized water in another (Collins, Yates and Down, 1981).

Positive smears – negative cultures

It can be embarrassing if a laboratory reports that acid-fast bacilli were seen in a direct smear, but fails to grow mycobacteria from the same specimens. There are several possible explanations.

One is that technical and clerical errors may occur in microscopy and documentation. Another is cross-contamination in staining or from used blotting

paper. Again, the acid-fast bacilli may be artefacts in the rinse water (Collins, Yates and Down, 1981) or psychrophilic mycobacteria that have gained access to the specimens but which do not grow at the incubation temperature used for the culture of clinical material.

There are also non-technical reasons. Dominguez and Vivas (1977) and Taik Chae Kim *et al.* (1984) found that smear positive–culture negative specimens occur mostly in patients with advanced cavitary disease and/or those who have received rifampicin, a drug that rapidly kills tubercle bacilli: the acid-fast organisms seen in the direct smears are dead.

'Acid- and alcohol-fast bacilli'

There are no grounds for the commonly held belief that tubercle bacilli are acid and alcohol fast, but that some other mycobacteria, e.g. the smegma bacillus, are only acid fast. Acid fastness varies with the technique used and the physiological state of the organisms. The alcohol in the decolorizing solution merely gives a cleaner film.

Storage and disposal of slides

Slides of positive smears should be retained for several weeks in case the results are questioned. Immersion oil may be removed with toluene and the slides should be stored in commercially available slide boxes that have tightly fitting lids.

Negative slides and old positives should be discarded into disinfectant, left for 18 hours, and then destroyed.

Chapter 6

Cultural methods

The usual microbiological techniques of plating material on selective or differential media and subculturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. Most pathogenic mycobacteria grow slowly, taking two to six weeks, or even longer, to give visible colonies and special media, not used for other organisms, are necessary. Cultures are usually made in bottles rather than in Petri dishes because mycobacteria, especially tubercle bacilli, are present in very small numbers in most specimens; this necessitates comparatively large inocula which are spread over the surface of the medium without 'looping out'. The bottles are tightly stoppered to prevent drying of the cultures which would certainly happen in Petri dishes.

Most material submitted for culture contains many other organisms which grow in one or two days and, within a week, they would overgrow the entire surface of the medium and probably digest it before the mycobacteria started to grow. There are no antibiotics or dyes, singly or in combinations, that will reliably suppress the growth of some organisms other than mycobacteria (e.g. pseudomonads and fungi) that may be present in pathological material. Antibiotic mixtures are very useful, however, as described below.

Pathological material that is known to contain other organisms must be treated in an attempt to destroy them but to preserve the mycobacteria. The reagents used must also reduce the specific gravity of the specimen so that it can be centrifuged with a reasonable certainty that the majority of the mycobacteria will be deposited.

Unfortunately there are no reagents that will completely fulfil the first requirement and tuberculosis bacteriologists have learned to steer a middle course and to accept that if enough mycobacteria to give a diagnosis are to survive then a proportion of cultures will be contaminated with 'resistant' organisms such as *Pseudomonas aeruginosa*, some enterobacteria and streptococci and a range of yeasts and microfungi. Experience has taught us that a contamination rate of 2–3 per cent is acceptable in laboratories that receive freshly voided specimens, but that if the specimens, especially sputum, take several days to reach the laboratory, or cannot be processed immediately, then losses due to contamination may be as high as 10 per cent. Even refrigeration of specimens does not markedly reduce the contamination rate. It may also be argued that a laboratory which experiences no contaminated cultures is probably using a method that kills many mycobacteria.

Although many chemicals have been investigated, comparatively few have been

reasonably successful. Those which we know to be satisfactory are described below and may be divided into two groups.

(1) 'Soft' reagents that have a minimal effect on both mycobacteria and the other organisms (which are usually termed 'contaminants'). These reagents are suitable for fresh specimens that can be processed immediately, i.e. when the number of contaminants is small and they have not had the opportunity to multiply. Exposure time is not critical.
(2) 'Hard' reagents which will kill tubercle bacilli as well as other organisms unless exposure time is carefully controlled. They are very effective against most contaminants.

After treatment the centrifuged deposit is usually seeded on culture media, but in two of the methods described below the uncentrifuged material may be used. Although these methods are not optimal they avoid the hazards of centrifugation (see p. 27) and reduce the time taken to process large numbers of specimens.

Decontamination of specimens

All operations should be done in a microbiological safety cabinet. Sealed buckets should be used for centrifugation and the centrifuge operated at 3000 rev/min.

Formulae of reagents are given in the Appendix.

It is better to dispense reagents in measured volumes in screw-capped bottles and to sterilize them ready for use than to pipette them from stock bottles during the procedure: this may lead to cross contamination.

Sputum

Bulking of sputum specimens should be avoided. It frequently distributes contaminants. Large amounts, e.g. more than 5 ml, also cause problems. For advice on manipulating sputum see pp. 36–37

Trisodium phosphate method

This 'soft' method may be used for fresh specimens of sputum, gastric fluids, bronchoscopy material, etc. that are not heavily contaminated with other organisms.

About 2 ml of sputum is added to a bottle containing 3 ml of 23 per cent trisodium phosphate ($Na_3PO_4 . 12H_2O$) solution and blended for 2 minutes on a Vortex mixer. The mixture is allowed to stand at room temperature for 12–18 hours.

Distilled water, 16 ml, is added and mixed with the digested sputum. The bottle is then centrifuged. The deposit is cultured.

Trisodium phosphate–Zephiran method

About 2 ml of sputum is added to a bottle containing 3 ml of a mixture of 23 per cent trisodium phosphate solution containing 2 ml per litre of 17 per cent Zephiran*

* Winthrop Laboratories, New York.

(benzalkonium chloride). After blending for 2 minutes on a Vortex mixer the homogenate is allowed to stand at room temperature for up to 2 hours. Distilled water, 16 ml, is added. After centrifugation the deposit is cultured on egg medium. If other media are used the Zephiran must be neutralized with lecithin or a neutralizing buffer (Difco).

Sodium hydroxide method

This is a 'hard' method and is suitable for material that is likely to be heavily contaminated.

About 1 ml of sputum is added to a bottle containing 2 ml of 4 per cent sodium hydroxide and blended on a Vortex mixer. The mixture is allowed to stand for 15 minutes (thin specimens) or not more than 30 minutes (thick specimens) with occasional shaking. Incubation at 37 °C has not been shown to hasten digestion; nor has mechanical shaking (Collins, 1951).

The contents of one bottle (3 ml) of 14 per cent monopotassium phosphate (KH_2PO_4 anyhydrous), coloured with a few drops of phenol red indicator solution, is then added. The colour of the mixture changes from yellow to orange, indicating that the sodium hydroxide has been neutralized.

It is then centrifuged and the deposit cultured.

Sodium hydroxide NALC method

Sodium hydroxide, 2 per cent, is dispensed in 50 ml amounts. Immediately before use 0.5 g of *N*-acetyl L-cysteine (NALC) is added and the mixture redistributed in 2 ml amounts.

About 1 ml of sputum is added to a bottle of the reagent and blended for 2 minutes on a Vortex mixer. It is allowed to stand until digestion is complete, but not for more than 30 minutes.

The contents of one bottle (3 ml) of the monopotassium phosphate solution described above is added, the mixture centrifuged and the deposit cultured.

Oxalic acid method

This is a very 'hard' method but is particularly useful if the specimen is known to contain *Ps. aeruginosa.*

About 1 ml of sputum is added to a bottle containing 3 ml of 3 per cent oxalic acid. After blending for 2 minutes on a Vortex mixer the homogenate is allowed to stand. Distilled water, 16 ml, is added and the mixture centrifuged. The deposit is cultured.

Methods avoiding centrifugation

Kirchner medium may be inoculated with not more than 1 ml of the homogenates of either of the trisodium phosphate or either of the sodium hydroxide methods after dilution or neutralization.

Acid egg medium may be inoculated with exactly 0.2 ml (critical amount) of the 4 per cent sodium hydroxide homogenate without neutralization.

Another method uses 1 per cent cetylpyridinium bromide. Two volumes of this are added to one volume of sputum. After shaking the mixture is allowed to stand

at room temperature for 24 hours. Two slopes of egg media are inoculated and placed in a horizontal position for 1 hour so that the homogenate can soak into the medium.

Laryngeal swabs

The swab, in its original tube, is covered with 3 per cent oxalic acid. This is allowed to act for 15 minutes. The swab is then removed to another tube containing sterile distilled water to dilute the acid. After a few minutes it is lifted, allowed to drain and used to inoculate culture media.

For optimal results the oxalic acid, which might contain tubercle bacilli washed off the swab, is transferred to another tube and centrifuged. The deposit is washed once with distilled water, centrifuged again and cultured.

Urines

About 25 ml of an early morning urine specimen are centrifuged at 3000 rev/min for 20 minutes. The number of other organisms is assessed either by examining a Gram-stained smear of the deposit or by culturing it on blood agar and incubating overnight. The deposit, meanwhile, is refrigerated.

The deposit is suspended in 2 ml of sulphuric acid, 4 per cent. After standing for 15 to 40 minutes, depending on the number of non-acid fast organisms present, it is diluted with 15 ml of sterile distilled water and centrifuged again. The deposit is cultured.

Pleural, cerebrospinal and other fluids

The numbers of non acid-fast bacilli are assessed as for urines. If there are none the centrifuged deposit is cultured without further treatment. Clots should be broken or cut up with sterile scissors.

If other organisms are present the deposit is mixed with 2 ml of 4 per cent sulphuric acid and allowed to stand for 15–40 minutes, depending on the number of organisms present. About 15 ml of sterile distilled water is added and the fluid centrifuged. The deposit is cultured.

Mycobacteria may adhere to glass or plastic surfaces and it is advisable, especially with spinal fluids, to add one of the liquid media containing antibiotics to the original container.

Pus

This may be treated in the same way as aspirated fluids, but if many non-acid fast organisms are present, or the material is very thick, it should be treated in the same way as sputum.

Tissue

Lymph nodes and other tissues should be cut into small pieces, using a sterile scalpel or scissors and then homogenized in a Griffiths tube or a mechanical blender. The numbers of non acid-fast organisms in the homogenate are then assessed and the material treated as described above for fluids or pus.

Milk

At least 100 ml of milk are centrifuged. The cream layer and the deposit are each treated by the NaOH or oxalic acid methods, centrifuged and the deposit cultured.

Water

Two litres of water are passed through a membrane filter. The filter is cut into strips not more than 1 cm wide and each strip placed in 3 per cent oxalic acid in a Petri dish for 15 minutes. They are then removed and placed in distilled water in another dish for 5 minutes. Each strip is placed on culture medium in a screw-capped bottle.

Culture media

Many different culture media have been devised for growing the tubercle bacillus. A wide range of products from dairies, butcheries, grocery shops and pharmacies have been used in different formulations with varying degrees of success. Relatively few of these media are in use today; the survivors may be divided into three groups – egg-based, agar-based and liquid. Formulae are given in the Appendix.

For the culture of sputum the egg-based media should be the first choice. There is increasing evidence, however, that liquid media, especially those containing antibiotic mixtures, may give better results with other specimens (Allen *et al.*, 1983; Mitchison, Allen and Manickavasagar, 1983). Both kinds of media should be used for specimens that are non-repeatable, e.g. biopsy material or cerebrospinal fluid. We also consider that both egg and liquid media should also be used for culturing urine deposits.

Egg-based media

The most popular of these are the various modifications of Löwenstein–Jensen medium. Others include those of the American Trudeau Society (ATS) and the International Union against Tuberculosis (IUTM), Ogawa medium, and that of Stonebrink. We prefer Löwenstein–Jensen medium and in this book the initials LJG are used for medium containing glycerol and LJP for that containing pyruvate.

LJG medium favours the growth of the human variant of *M. tuberculosis* and LJP medium encourages the bovine variant which does not like glycerol. Both should be used in areas where patients may be infected with either organism. Although the human variant grows quite well on LJP medium this should not be used alone as some strains of BCG and some opportunist mycobacteria grow poorly on it.

Agar-based media

The 7H10 and 7H11 media of Middlebrook and Cohn are not often used for primary cultures. They may be useful, however, with the addition of one of the antibiotic mixtures, for non-repeatable material that might be heavily contaminated. They are usually reserved for identification and sensitivity tests and research purposes.

Fluid media

The most useful and least expensive of these for primary culture is that of Kirchner, containing one of the antibiotic mixtures. It has the additional advantage that it can support a large inoculum. Middlebrook 7H9 media is also satisfactory for this purpose, but is more often used in identification tests.

Media with antibiotic mixtures

Penicillin is usually added to egg media used for primary isolation. The addition of mixtures of several antibiotics, notably polymixin, carbenicillin, amphotericin and trimethoprim to fluid media is most useful for recovering mycobacteria from heavily contaminated specimens, and in particular for urines and for biopsy specimens that cannot be repeated (Mitchison *et al.*, 1972).

Several of the egg media may be bought, already tubed, from commercial suppliers, but it is much cheaper to make them. In our opinion, however, it is best to purchase dehydrated 7H9, 7H10 and 7H11 media together with the necessary supplements.

Inoculation of culture media

A common fault in inoculation is the use of too small an inoculum. A plastic Pasteur pipette, rather than a loop, should be used to inoculate each slope with 0.2 to 0.4 ml of the centrifuged deposit. Fluid media can accommodate up to 1 ml.

At least two slopes of egg medium should be used, one LJG and the other LJP. The slopes should be placed in a horizontal position for an hour or two after they have been inoculated to allow the inoculum to be absorbed. Otherwise most of it runs to the bottom of the tube and may never be in contact with the medium.

If possible, some inoculum, especially of non-repeatable material, should be retained and refrigerated at −20 °C.

Incubation

All cultures should be incubated at 35–37 °C. In addition, duplicate cultures of material from superficial lesions should be incubated at 30–33 °C. Cultures on solid media should be incubated in a vertical position so that individual colonies may develop.

Examination of cultures

All cultures should be examined after three days and any that show growth should be removed for further examinations. Colonies of some rapidly-growing mycobacteria and contaminants may appear in this time. Thereafter the tubes should be examined weekly for at least six weeks and if possible for eight or even twelve weeks. Some mycobacteria grow very slowly and extended incubation may result in a small increment of positive results, especially from resected material.

Any tubes showing obvious contamination, e.g. growth of moulds, should be discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of the pH unbinds some of the malachite green from the egg. The colour of the medium changes to dark green. Tubercle bacilli will not grow under these conditions.

Slopes showing confluent growth or isolated colonies of a buff, yellow or orange colour should be removed for microscopical examination. Close inspection, preferably with a hand lens and under a good bench lamp, is necessary to reveal very small colonies or the effuse growth which is characteristic of some opportunist mycobacteria.

In Kirchner medium tubercle bacilli grow as spherical colonies, 1–2 mm in diameter, at the bottom of the tube. The bottle should be shaken gently to observe them. They may be removed with a plastic Pasteur pipette for microscopic examination and subculture on solid medium. It may be difficult to recognize the growth of some other mycobacteria that produce smooth growths. The sediment should be examined microscopically.

Microscopic examination

Stained films of all suspicious growths should be prepared to ascertain if the organisms are acid fast. The ZN stain should be used as the FM method fails to reveal the cellular morphology which is an important diagnostic criterion. It also fails to reveal the presence of non acid-fast organisms.

Three, or with experience, four films may be made on one 3 × 1 inch slide. When large numbers of films are to be made it is worthwhile purchasing the larger, 3 × 2 inch slides, which will accommodate up to eight.

The culture numbers are marked on the slide with a diamond stylus and a small drop (e.g. 10 µl) of a saturated solution of mercuric chloride placed near the number. It is very easy to produce small splashes or aerosols when attempting to emulsify bacterial growth in a drop of liquid and this chemical kills mycobacteria almost instantly. A very small amount of growth is removed from the culture, using a loop (the 1 µl plastic loops are ideal) or a swab stick, and gently rubbed into the mercuric chloride. Violent action may disturb the arrangement of the bacilli (see below). At this point the ease with which the organisms emulsify in the liquid should be noted. Tubercle bacilli, for example, do not form smooth suspensions, unlike some other mycobacteria.

The films are allowed to dry and are then fixed by heat and stained by the ZN method. It is usually unnecessary to heat the carbol fuchsin when smears of mycobacterial cultures (as distinct from sputum, etc.) are being stained.

Figure 6.1. shows the microscopical appearance of several species of mycobacteria. The arrangement of the cells varies from the 'serpentine cords' usually, but not always, characteristic of tubercle bacilli, to a uniform distribution of cells. Cellular morphology is also variable, with individual bacilli ranging in length from 1 to 10 µm and in width from 0.5 to 1.5 µm. Some mycobacteria appear to be almost coccoid and others fusiform. Staining may be regular or show granules or beading. Feebly acid-fast bacilli may also be seen: parts of the organisms stain blue or green or the smear may present a mixed acid-fast and non acid-fast appearance. Care must be taken with these: they may be genuinely pure cultures or a mixture of mycobacteria and other organisms (contaminants).

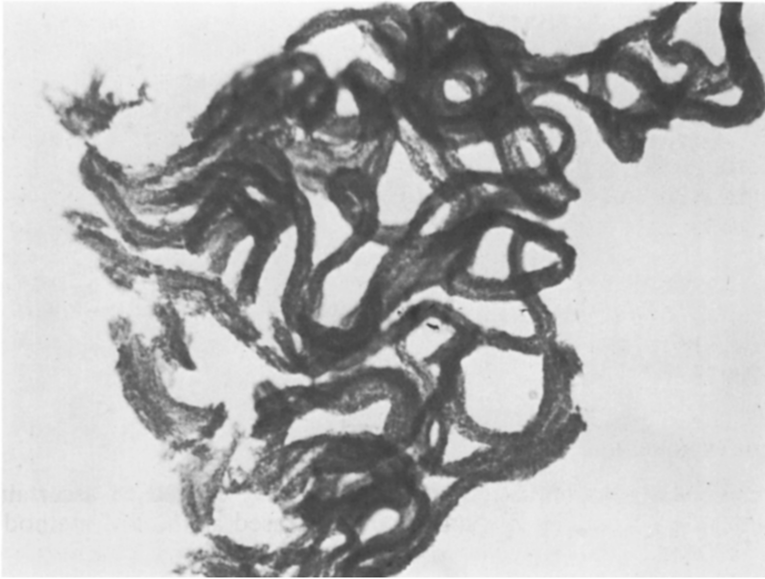

Figure 6.1a Microscopic appearance of mycobacteria. (a) *Mycobacterium tuberculosis,* showing 'serpentine cords'

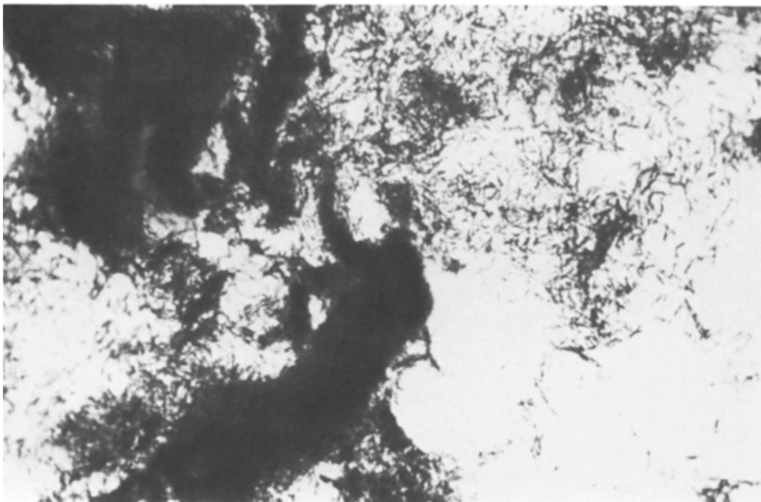

Figure 6.1b Mycobacterium kansasii

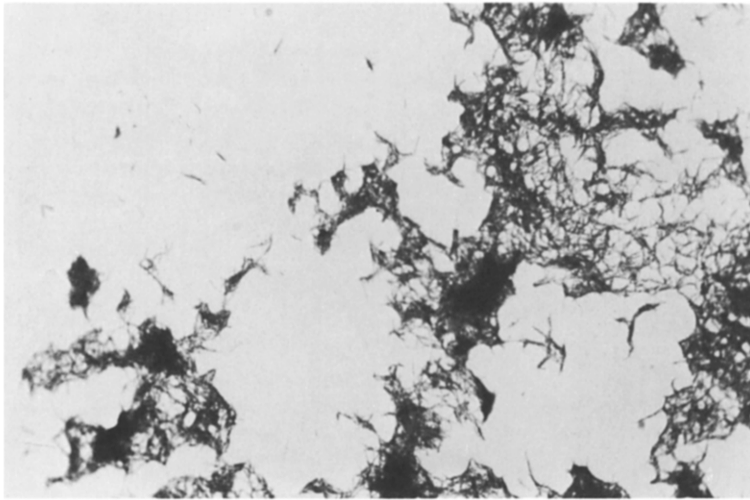

Figure 6.1c Mycobacterium xenopi

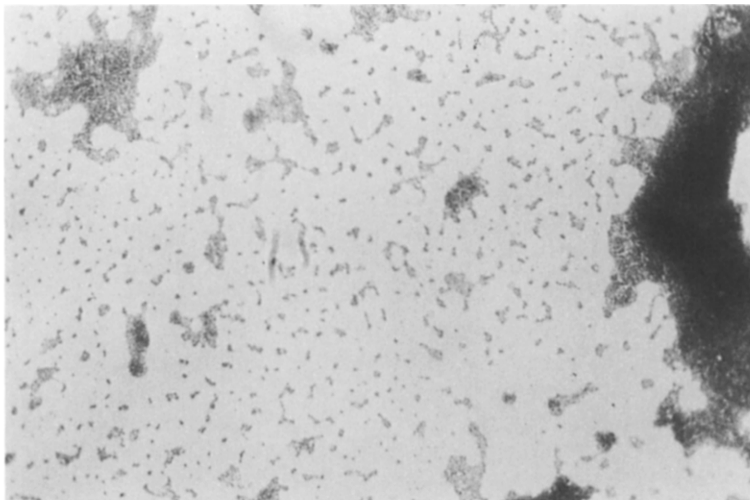

Figure 6.1d Mycobacterium avium-intracellulare-scrofulaceum

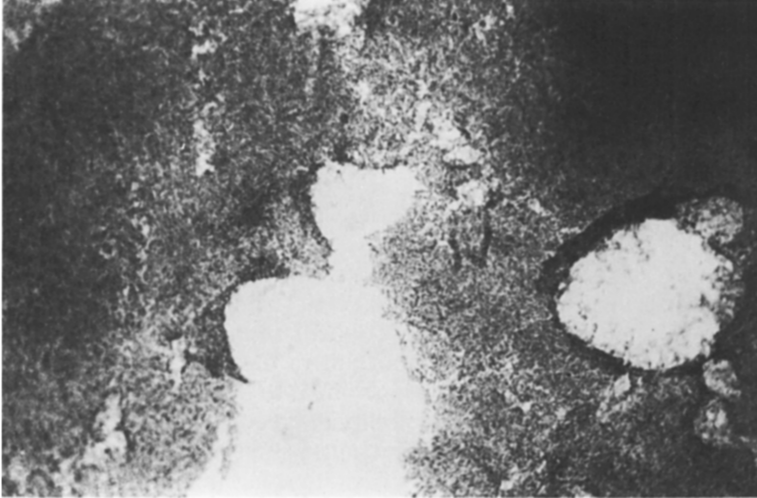

Figure 6.1e Mycobacterium sp. showing absence of any distinctive arrangement or morphology

Preliminary identification

At this stage an experienced worker can usually say whether an acid-fast bacillus is likely to be a tubercle bacillus or another species of mycobacteria.

Tubercle bacilli do not grow in primary culture in less than one week and usually take two or three weeks to give visible growth. The colonies are buff coloured, never yellow; they are rough, having the appearance of breadcrumbs or cauliflowers; they do not emulsify in the fluid used for making smears but give a granular suspension; microscopically they are frequently arranged in serpentine cords of varying length (*Figure 6.1a*), or show distinct linear clumping; individual cells are rarely shorter than 3 µm or longer than 4 µm. Such organisms should be identified by the methods described in Chapter 8.

Other mycobacteria may show one or more of the following characteristics: grow in less than one week on subculture; form yellow or orange coloured colonies; emulsify readily in the mercuric chloride solution (or in water); have cells that are often less than 2 µm or more than 5 µm in length; have a microscopical appearance similar to those in *Figure 6.1(b, c* or *d)*. Such organisms should be identified by the methods described in Chapter 9.

Radiometric method for TB culture

A commercial instrument (BACTEC: Johnston Laboratories) has been developed for detecting the early growth of mycobacteria by a radiometric method.

Sputum or other homogenates, decontaminated if necessary, are added to Middlebrook broth medium containing an antibiotic 'cocktail', to discourage the growth of other organisms, and ^{14}C-labelled palmitic acid. The medium is prepared

commercially (Becton Dickinson) in rubber sealed bottles and is inoculated with a syringe and hypodermic needle. If growth occurs, $^{14}CO_2$ is evolved. The air space above the medium in each bottle is sampled automatically at fixed intervals and the amount of radioactive gas is estimated and recorded. Growth of mycobacteria may be detected in two to twelve days, but positive results require further tests to distinguish between tubercle bacilli and other mycobacteria. In the BACTEC, *p*-nitro-α-acetylamino-β-propiophenone (NAP) (p. 61) is used and this takes another two to five days. Mycobacteria that grow in media containing this substance are subcultured and identified by traditional methods.

Comparative tests (see references below) show that the method is successful and fairly reliable. It is very expensive, however, to purchase and to operate. It could be invaluable for the detection of tubercle bacilli in material such as cerebrospinal fluid, where rapid results are very important in the management of the patient. Two hazards must be considered if the machine is to be used for routine work in the tuberculosis laboratory: one is the possibility of 'needle-stick' accidents (see p. 28); the other is from radioactive waste. This latter is negligible but some regulatory bodies may be deeply concerned. The possibility that infectious aerosols might be released during the sampling procedure was taken into consideration in the design of the apparatus, which is enclosed and air is exhausted through filters.

Further information on this method may be obtained from the manufacturers. Recent references to the use of the BACTEC include Damato *et al.* (1983), Morgan *et al.* (1983) and Roberts *et al.* (1983).

Negative cultures from positive direct smear specimens

This problem is discussed on p. 42.

Chapter 7

Taxonomic principles – the basis of identification

Taxonomy is written by taxonomists for taxonomists; in this form the subject is so dull that few, if any, non-taxonomists are tempted to read it, and presumably even fewer try their hand at it.

S. T. Cowan

Since time immemorial man has struggled to understand the universe in which he lives. As part of this quest for knowledge he has attempted to classify objects and phenomena into various hierarchies. The art of classification has developed into what is, in principle, but alas not always in practice, an exact and logical science called *taxonomy*.

In his book *Species Planetarum* the Swedish botanist Carl von Linne (Carolus Linnaeus, 1707–78) divided living beings into two great kingdoms – animals and plants* – and further divided these into a descending hierarchy of phyla, classes, orders, families, genera and species. He also proposed that individual creatures should be referred to by the generic and specific names only – the so-called binomial system of nomenclature.

To avoid total confusion in nomenclature, the validity of published names is determined by various internationally accepted rules. One of these is the rule of 'chronological priority' according to which the earliest name to appear subsequent to the publication of Linne's *Species Planetarum* on 1st May 1753, was regarded as the valid one. This, however, caused some problems and, in an attempt to make bacterial taxonomy easier (and even duller), history was dismissed 'at a stroke' and it is now only necessary to refer back to the 'Approved Lists of Bacterial Names' published in the *International Journal of Systematic Bacteriology* in January 1980 (Skerman, McGowan and Sneath, 1980). The current list of species, with synonyms or unofficial names is given in *Table 7.1* with appropriate references. Alternative (illegitimate) names are included in inverted commas.

The history of the classification of the mycobacteria is similar to that of many other bacterial genera. Initially the genus and a few species were delineated on the basis of a small number of simple cultural properties. Later, as interest grew, more distinguishing properties were found and many workers described apparently new species, often without comparison with all previously described ones. Consequently, some species were known by a considerable number of different names. Finally order replaced chaos and, as a result of several individual and cooperative studies, the number of 'species' was reduced considerably. Indeed Ratledge and Stanford

* Nowadays a further fundamental division of living creatures is recognized: the prokaryotes and eukaryotes. In the former, which includes bacteria and blue-green algae, the genetic material is not surrounded by a nuclear membrane.

TABLE 7.1. Approved names for mycobacteria[a], synonyms and 'popular' names

Approved name	*Other names*
M. africanum[b]	*M. tuberculosis* African variant
M. asiaticum	
M. aurum	
M. avium[b,c,d]	*'M. avium-intracellulare'* Avian tubercle bacillus, MAIS bacillus
M. bovis[b,c]	*M. tuberculosis* bovine variant Bovine tubercle bacillus
M. chelonei[b,c]	*'M. abscessus'*, *'M. borstelense'* Turtle tubercle bacillus
M. chitae	
M. duvalii	
M. farcinogenes[c]	
M. flavescens	
M. fortuitum[b,c]	*'M. ranae'*, *M. giae'*, *'M. peregrinum'* Frog tubercle bacillus
M. gadium	
M. gastri	
M. gilvum	
M. gordonae	
M. haemophilum[b]	
M. intracellulare[b,c,d]	*'M. avium-intracellulare'* Battey bacillus, MAIS bacillus
M. kansasii[b]	*'M. luciflavum'*
M. komossense	
M. leprae[b]	Leprosy bacillus, Hansen's bacillus
M. lepraemurium[c]	Rat leprosy bacillus
M. malmoense[b]	
M. marinum[b,c]	*'M. balnei'*, *'M. platypoecilus'*
M. microti[c]	*M. tuberculosis* vole variant Vole tubercle bacillus
M. nonchromogenicum	
M. neoaurum	
M. parafortuitum	
M. paratuberculosis[c,d]	*'M. johnei'*, Johne's bacillus
M. phlei	Timothy grass bacillus
M. scrofulaceum[b]	*'M. marianum'*, MAIS bacillus
M. senegalense[c]	
M. simiae[b,c]	*'M. habana'*
M. smegmatis	Smegma bacillus
M. szulgai[b]	
M. terrae	Radish bacillus
M. thermoresistibile	
M. triviale	
M. tuberculosis[b,c]	Human tubercle bacillus
M. ulcerans[b]	*'M. buruli'*
M. vaccae	
M. xenopei[b]	*'M. xenopei'*, *'M. littorale'*

[a] Skerman, McGowan and Sneath, 1980
[b] Well-documented human pathogens
[c] Well documented animal pathogens
[d] Member of MAIS (*M. avium-intracellulare-scrofulaceum*) complex

(1982) noted that of 128 species names listed in the 1966 edition of the *Index Bergeyana*, only 16 are included in the aforementioned 'Approved Lists of Bacterial Names' published in 1980. There were, in fact, so many names that it was virtually impossible to allocate many isolates to any definite species.

When considering the classification of the mycobacteria (or any other bacterial genus) it is necessary to consider the meaning of the word 'species' as applied to bacteria. In the case of animals and, to a lesser extent, plants, it has been possible to work out a fairly comprehensive evolutionary scheme from fossil evidence and also to establish the boundaries of present-day species according to the matings that yield fertile offspring. Such investigative methods are not applicable to the bacteria although a form of evolutionary tree can be adduced from studies of DNA relatedness and antigenic sharings.

As sexual reproduction is absent, or at best rudimentary, among bacteria it has been necessary to seek different criteria for the delineation of species boundaries than those applied to the higher forms of life. Bacterial taxonomists may, like the Scholastic philosophers, be divided into realists and nominalists. The former hold that bacteria are divisible into real evolutionary entities akin to species in higher forms of life. The latter follow the view of the English philosopher John Locke that 'The boundaries of the species, whereby men sort them, are made by men'. They, accordingly, reject the bacterial species as a real entity and instead only refer to a more or less arbitrarily defined taxonomic unit termed the *taxon*. Some have gone even further and have proposed the 'creation' of a *hypothetical mean organism* for each taxon. The fruit of this school of thought is a system for classifying bacteria known as numerical or Adansonian taxonomy. In essence, a large number of characteristics for each bacterial strain are determined and the strains are then clustered according to their degree of similarity. This form of classification has been widely applied to the genus *Mycobacterium*, particularly in a number of international cooperative studies.

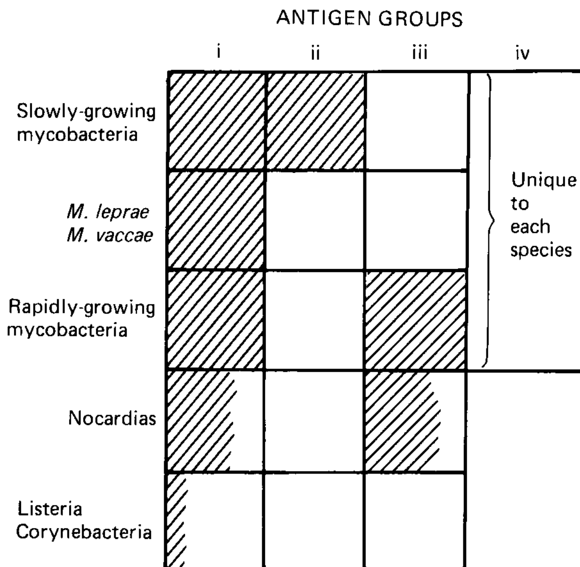

Figure 7.1 The distribution of the four groups of antigens among mycobacteria and related genera, as demonstrated by immunodiffusion techniques.

There have been a number of more rational and fundamental approaches to the taxonomy of this genus. These include studies on the relatedness of bacterial DNA (Baess, 1979) and the comparison of strains by antigenic analysis of their soluble cytoplasmic antigens. Use of the latter technique has shown that mycobacterial antigens form four main groups (Stanford and Grange, 1974). First, there is a large group of antigens that occur in all mycobacteria and are shared to some extent with related bacteria such as nocardias and corynebacteria. Second, there is a set of antigens peculiar to the slow-growing mycobacteria. Third, there is a set of antigens occurring in rapidly-growing mycobacteria and also nocardias. Finally each species has a set of antigens unique to itself, although there may be minor strain-to-strain variations in the possession of these. The above groups of antigens are referred to as Groups i, ii, iii and iv and are illustrated in *Figure 7.1*. In general, the subdivisions obtained by numerical analysis of the mycobacteria correlate well with those delineated by these more fundamental approaches.

As the result of a large amount of individual and cooperative work (reviewed by Goodfellow and Wayne, 1982) the genus *Mycobacterium* has emerged as one of the best classified of all bacterial genera. It contains about 40 species which are divided into two major groups – the rapid and slow growers. These two groups are quite distinct and could be regarded as two subgenera. The rapid growers are closely related to the genus *Nocardia*. The various mycobacterial species, although often showing a large amount of infra-specific variation, usually have distinct boundaries. In this genus, irrespective of the situation in others, the species do not appear to be merely peaks in a more or less continuous spectrum of bacterial variation.

Although the delineation of the boundaries of species by taxonomists may, at first glance, appear to represent the ultimate in ivory tower or armchair sciences, experience has shown that the development of a rational classification opens the door to many other more practical lines of investigation.

The medical bacteriologist is, however, less concerned with the philosophical basis of taxonomy as with providing his clinical colleagues with an efficient and meaningful service. The physician faced with an ill patient is not concerned with taxonomic niceties: he wants to know whether an isolate is the cause of the patient's disease and, if so, what therapy is indicated. On the other hand, a detailed typing of all strains regarded as pathogens enables us to build up a comprehensive idea of the type of infections that they cause, the likely outcome of such infections and the best way to treat them. We therefore consider it advisable to identify all clinical isolates as well as circumstances and resources permit. There is no universally accepted procedure for identifying mycobacteria in the routine or reference laboratory as the bacteriologist now has a large range of simple cultural and biochemical tests, and an increasing number of more sophisticated analytical tools, to choose from. Most of these techniques are 'spin-offs' from detailed taxonomic studies. Indeed one virtue of the Adansonian approach is that it generates and evaluates a large number of simple tests suitable for routine usage. In the next chapters we give an approach, but certainly not the only one, to routine identification and also an account of some more specialized techniques which are available in central reference laboratories.

Chapter 8

Identification of species and variants of tubercle bacilli

Nomenclature

The term 'tubercle bacillus' is usually synonymous with *Mycobacterium tuberculosis* unless it is qualified with an adjective such as 'avian' or 'cold-blooded'. As outlined in Chapter 1, the species *M. tuberculosis* contains a number of variants, including the human, bovine and African types; the latter two are often, but in our view incorrectly, referred to as *M. bovis* and *M. africanum* respectively. In this chapter we therefore use the term *M. tuberculosis* in this inclusive sense, but in deference to those workers who wish to use the alternative nomenclature we also use the specific names listed by Skerman, McGowan and Sneath (1980).

From a clinical point of view – and we are mainly concerned here with diagnostic mycobacteriology – it is sufficient to determine whether a mycobacterium is *M. tuberculosis* or not. Further subdivision of this species into variants does not provide information that affects the management of the patient but is for epidemiological interest only. For the latter purpose we have found it useful to delineate five variants: the classical and Asian human; the bovine; and two African variants. To these a sixth, the BCG vaccine strain, may be added.

The specific name '*Bacterium tuberculosis*' was proposed by Zopf in 1883 and, in 1896, Lehmann and Neumann assigned the species to the genus *Mycobacterium*. Small but definite differences were observed between organisms isolated from man and cattle; the mammalian bacilli were therefore divided into human and bovine types, known respectively as *M. tuberculosis hominis* and *M. tuberculosis bovis*. The *hominis* strains were those commonly recognized as causing pulmonary disease in man and the *bovis* strains as those primarily responsible for tuberculosis in cattle and which could give rise to non-pulmonary disease in man as a result of drinking the milk from infected cows. Although some bacteriologists referred to the bovine type as *M. bovis* it was not officially given this name until much later (Karlson and Lessel, 1970). For a history of the bovine tubercle bacillus see Collins and Grange (1983).

Another mammalian type, isolated from the vole, *Microtus agrestis*, was described by Wells (1946) as the murine tubercle bacillus and was given the name

M. microti by Reed (1957). This is not pathogenic for man and has been used as a vaccine. The last mammalian type to be identified was named *M. africanum* (Castets, Rist and Boisvert, 1969) and was isolated from patients in west Africa. Later, strains with slightly different properties, isolated from cases of tuberculosis in east Africa were also included in this species. In 1975 Runyon proposed that the term tubercle bacilli should include *M. tuberculosis, M. bovis, M. africanum* and no others. These names have official taxonomic status, having been approved by the International Committee on Systematic Bacteriology (Skerman, McGowan and Sneath, 1980) but we are not alone in disagreeing with the award of specific status to these organisms.

Stanford and Grange (1974), who used antigenic analysis techniques, showed that all these mammalian tubercle bacilli form a single species with some degree of infraspecific variation. Workers using other techniques, such as numerical taxonomy (David *et al.*, 1978; Tsukamura, 1966b, 1976, 1980, 1983), skin test specificity (Magnusson, 1981; Wayne, 1982) and DNA hybridization (Baess, 1979) strongly support this concept. According to the rules of priority, the name of this species would be *M. tuberculosis*.

Furthermore, a variant within those strains formerly designated as *hominis*, and observed in India by Dhayagude and Shah in 1948, was characterized by Grange *et al.* (1977). This is now known as the Asian variant (Collins, Yates and Grange, 1982). Fortunately no-one has attempted to give this variant a specific name (see also Grange, Collins and Yates, 1982; Yates, Collins and Grange, 1982).

Finally, there is BCG – the bacillus of Calmette and Guérin – extensively used as a vaccine. Although this is often referred to as an attenuated strain of the bovine tubercle bacillus there are reasonable doubts about its provenance (see the review by Grange and others, 1983). Certainly it is a laboratory product and Runyon (1975) considered that, although it is a distinct taxon, it should not have a specific name but merely be labelled as BCG. For many years it was not easy to identify, and after observing the growth pattern in various media, testing its pathogenicity to laboratory animals and taking note of the site of the lesion and the case history, all that could be said of strains isolated from man was that they were neither *M. fortuitum* nor *M. chelonei* and that their properties were or were not consistent with those of the vaccine strain. Although it may not be essential to identify BCG recovered from local lesions after vaccination, it is important to identify it when it is suspected of causing disseminated disease in vaccinated neonates or immunosuppressed patients.

Laboratory-acquired BCG infections are not unknown (Collins, 1983), and positive identification may be required for medico-legal reasons.

Fortunately it is now possible to identify BCG with some certainty, however, by two different procedures: the phage typing methods used by Yates, Collins and Grange (1978), who confirmed an earlier observation (Jones, 1975) that it could be characterized by its resistance to lysis by mycobacteriophage 33D; and by the methods of Collins, Yates and Grange (1982) described below.

From the point of view of physicians, therefore, who have to compete with continual changes in nomenclature in many other areas, we consider that all tubercle bacilli should be reported using that name or the more scientific one, *Mycobacterium tuberculosis*, with the proviso that the bovine variant should be specified (because of its natural resistance to pyrazinamide, used in some therapeutic regimens), and that BCG should be reported by that name only (Collins, Yates and Grange, 1984).

Identification

Although preliminary examination of the cultures may suggest that the organisms are tubercle bacilli it is best to do confirmatory tests. Unfortunately there is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Tests that have been used are rate of growth and pigmentation (Timpe and Runyon, 1954); growth at temperatures other than 37 °C (Runyon, 1959; Marks and Trollope, 1960); inhibition by *p*-nitrobenzoic acid (PNB) (Tsukamura and Tsukamura, 1964; Collins and Yates, 1979); hydroxylamine (Tsukamura, 1970; Collins and Yates, 1979) and by *p*-nitro-α-acetylamino-β-hydroxypropiophenone (NAP) (Eidus, Diena and Greenberg, 1960).

We favour the screening method of Marks (1972b, 1976). This has been used successfully by the Reference and Regional Tuberculosis laboratories in England and Wales for a number of years. Some laboratory workers still use the niacin test, however, in spite of its limitations, so that technique is also described.

This screening test is adequate for clinical purposes but does not permit differentiation of the classical human, Asian, bovine and African variants of *M. tuberculosis* and of BCG. Identification of the variants may be useful for epidemiological purposes. It may also be desirable to identify BCG in lesions occurring after vaccination, especially of neonates and when the organism has been used in the treatment of malignancy. Methods for the identification of these variants and of the vaccine strain are also given below.

The screening test

This uses three tubes of media to test for the rate of growth at 37 °C; pigment production, whether or not this is induced by light; growth at 25 °C; and growth on LJG medium containing 500 mg/litre of *p*-nitrobenzoic acid.

Preparation of suspensions

Suspensions of the organisms are made by placing two or three colonies, or the equivalent amount of growth, in 'suspension bottles' which contain phosphate buffer, a few small glass beads and a wire nail (Appendix). The bottles are then placed on a magnetic stirrer, when the rotating nail and the glass beads break up most of the clumps of bacteria. If this is not done the tubes of media will receive unequal inocula and this may give unreliable results. The bottles are allowed to stand for a few minutes so that any remaining large clumps of bacilli may settle.

Inoculation of media

The suspension bottles are carefully placed, at an angle, on modelling clay. Two tubes of LJG medium (and one of LJP if primary growth on this is better than on LJG) and one of PNB medium (LJG containing 500 mg/litre of *p*-nitrobenzoic acid) are each inoculated with approximately 10 μl of the suspension. The loop is withdrawn edgewise from the suspension so that a large drop is not transferred. The inoculum should not be spread over the surface of the medium but streaked down the middle. This facilitates early examination as the border between growth and medium is more easily observed. An alternative method of inoculation, when sensitivity tests are done at the same time as identification, uses the MicroRepette* (*see Figure 3.5*).

* Jencons (Scientific) Ltd, Cherry Court Way Industrial Estate, Leighton Buzzard, Bedfordshire.

Incubation

One LJG tube and its accompanying PNB tube are incubated at 37 °C in an internally illuminated incubator. The other LJG tube is incubated at 25 ± 0.5 °C (room temperature is too variable).

If the 37 °C incubator is of the 'walk-in' kind the light should be left on and the racks of tubes sloped so that the whole surface of the medium is illuminated. If a cabinet incubator is used it may be adapted by fitting a 6 W fluorescent lamp inside. The very small amount of heat given out by this will not affect the thermostat setting. Alternatively the slopes may be incubated in the dark until growth is evident. They are then placed facing daylight, but not direct sunlight, on a windowsill, or facing and about 100 cm away from a tungsten filament bench lamp. After illumination for 1 hour the tubes are returned to the incubator. Loosening the caps to admit oxygen is said to enhance pigment production.

Incubation at 25 °C may also present problems especially in centrally heated laboratories and warm climates. Incubators fitted with refrigerated coils are available and we have found them reliable. Refrigerated water-baths are not suitable for this work, even if the water level is constant: there is always the possibility that a tube may break or leak and that the water will become infected with tubercle bacilli.

Reading

The tubes should be examined after 3, 7, 14 and 21 days' incubation. Tubercle bacilli require about ten days to give visible colonies; they do not produce pigment; they do not grow at 25 °C nor do they grow on the PNB medium. At 21 days classical human and Asian strains of *M. tuberculosis* will show characteristic, dry, breadcrumb or cauliflower growth that is greyish white or buff in colour on the 37 °C LJG or LJP culture only. There will be no growth on the PNB medium or at 25 °C. Growth of the bovine and African variants is usually poor on LJG but quite good on LJP medium. The colonies are flatter and may not appear to be as rough as those of the human variant. Occasional strains of the bovine variant may show very poor growth on PNB and even at 25 °C and these require further identification as described below.

Mycobacteria that grow within three days, produce yellow, orange or pink pigmented colonies, grow at 25 °C or on PNB medium, or show any combination of these characteristics are not tubercle bacilli and should be identified by the methods described in Chapter 9.

The niacin test

This test, which was originated by Konno (1956), depends on the ability of some mycobacteria to synthesize niacin (nicotinic acid) which is detectable in aqueous extracts of the organisms and medium.

Reagents

(1) Aniline, 4 per cent in ethanol (some books prescribe benzidine or *o*-toluidine but these are carcinogenic and best avoided).
(2) Cyanogen bromide. This is a very toxic chemical and should be kept closely stoppered in a fume cupboard. Small amounts of an approximately 10 per cent solution in water should be prepared when required.

Technique

A heavy growth of mycobacteria is required and the organisms should be grown on large LJG or LJP slopes for at least four weeks. Distilled water, 1 ml, is added to each slope and the cell mass is broken up with a loop so that the medium is exposed. Much of the niacin is extracellular and accumulates in the medium. The tubes are placed in a sloping position so that the medium is covered with the water. Extraction is allowed to take place for 15 minutes. Some workers autoclave the tubes to extract the niacin and this certainly makes the procedure less hazardous. Control cultures of known niacin-positive and niacin-negative mycobacteria should also be tested.

Approximately 0.5 ml of the extract is removed to a screw-capped bottle, 0.5 ml each of the aniline and the cyanogen bromide solutions are then added.

A yellow colour, indicating the presence of niacin, is given by most strains of the human variant of *M. tuberculosis* but niacin-negative strains have been reported (Tsukamura, 1974). Positive reactions are also given by some strains of the African variant, *M. africanum*; by *M. simiae*; by some strains of *M. avium*; and by some rapidly growing mycobacteria ('*Mycobacterium borstelense* var. *niacinogenes*'; Bönicke and Ewoldt, 1965).

Other mycobacteria, including the bovine variant of *M. tuberculosis* (*M. bovis*), give negative reactions, but there is always the possibility that negative results, obtained with other mycobacteria, are due to poor growth.

A paper strip method is available commercially (Difco) and a biological test has also been described (Marks, 1965b).

Identification of variants of M. tuberculosis and BCG

Suspensions of organisms identified as tubercle bacilli by the methods described above are used for the following tests. Suspensions are prepared in the way described on p. 92.

Sensitivity to thiophen-2-carboxylic acid hydrazine (TCH)

The medium is LJG containing 5 mg/litre of the chemical and tubed in 2 ml amounts in small screw-capped bottles. Cultures are incubated for two to three weeks at 37 °C. The growth is compared with that on LJG control tubes.

According to Harrington and Karlson (1966) the human tubercle bacillus is resistant and the bovine bacillus is sensitive to this compound at comparable concentrations. Yates and Collins (1979) confirmed this but found that many strains from Asians, otherwise of the human type, were also sensitive. This invalidates the use of the test for identifying bovine strains.

Nitratase test

Middlebrook 7H9 medium bottled in 2 ml amounts is inoculated and incubated for about 18 days at 37 °C. This is then tested by the method of Collins and Lyne (1984). Other methods have given variable results.

When a heavy growth is evident 0.05 ml of 4 per cent aqueous potassium nitrate solution is added and the bottles incubated for 4 hours at 37 °C. The fluid is

acidified with a few drops of N hydrochloric acid and 0.05 ml of 0.2 per cent sulphanilic acid and 0.05 ml of 0.1 per cent 1-naphthyldiethylenediamine dihydrochloride added. These two reagents should be kept in the dark in a refrigerator and freshly prepared each month.

A pink colour indicates nitratase activity. Some organisms reduce nitrite, however, so if no colour is observed a very small amount of zinc dust should be added. If the original nitrate is still present it will be reduced to nitrite which will react with the reagents to produce a pink colour. A pink colour in this part of the test therefore indicates an absence of nitratase activity, while no colour indicates that nitrates have been completely reduced.

Oxygen preference

The method of Marks (1972b) is used. The semisolid Middlebrook or Kirchner medium is inoculated by introducing about 0.2 ml of the suspension about 1 cm below the surface and then carefully mixing to avoid air bubbles and aeration. The bottles are incubated and undisturbed for 18 days.

Aerobic growth occurs at or near the surface of the medium, sometimes extending as much as 10 mm below it. Microaerophilic growth occurs as a band, 10–20 mm below the surface sometimes extending upwards.

Pyrazinamide sensitivity

It is well known that the bovine variant is resistant to this drug. The routine method for assessing the clinical usefulness of the drug is used. It is described on p. 97.

Cycloserine sensitivity

Rist *et al.* (1967) noted that BCG was more resistant than *M. tuberculosis* to cycloserine.

The medium used is LJG containing 20 mg/litre of cycloserine. Inoculated cultures are incubated for 28 days at 37 °C and the growth compared with that on LJG control tubes.

Pyruvate preference

The bovine variants usually grow better on LJP than on LJG.

A tube of each medium is inoculated and growth is compared after 28 days' incubation at 37 °C.

Interpretation

Table 8.1 shows the reactions of the different variants and of BCG. It should be noted, however, that identification of a strain of tubercle bacilli as 'human', 'bovine', 'Asian' or 'African' does not indicate the origin of the strain nor the ethnic group of the patient. These variants are widely distributed.

In practice, time and labour may be saved by including a tube of TCH medium in the tests used to identify *M. tuberculosis* (above). Any strain that grows on this medium may safely be regarded as a classical human variant. The other tests need be done only on strains that are inhibited.

TABLE 8.1. Species and variants of tubercle bacilli

Species[a]	*Variant*[b]	*TCH*	*Nitratase*	*Oxygen preference*	*Pyrazinamide*	*Cycloserine*
M. tuberculosis	Classical human	R	+	A	S	S
	Asian human	S	+	A	S	S
M. africanum	African I	S	−	M	S	S
	African II	S	+	M	S	S
M. bovis	Classical bovine	S	−	M	R	S
	BCG[c]	S	−	A	R	R

TCH, Thiophen-2-carboxylic acid hydrazide; S, Sensitive; R, Resistant; A, Aerobic; M, Microaerophilic

[a] Species according to Skerman, McGowan and Sneath, 1980
[b] Variants of *M. tuberculosis* according to Collins, Yates and Grange, 1982
[c] Not recognized as a species

Variants and species of tubercle bacilli

The classical and Asian variants of Mycobacterium tuberculosis

These two variants belong to the original species *M. tuberculosis* described by Lehmann and Neumann (1896) and redefined by Runyon *et al.* (1967) and Kubica *et al.* (1972).

Colonies on egg media at 18 days are 1–2 mm in diameter, rough, heaped and resemble breadcrumbs or cauliflowers. There is no preference for glycerol or pyruvate media.

The bacilli are 2–4 μm × 0.3–0.5 μm but occasionally shorter or longer cells are seen. In carefully prepared smears from young cultures the bacilli show a 'serpentine cord' pattern (*see Figure 6.1a*).

Growth is restricted to 35–38 °C. These variants are nitratase positive, aerobic, and are sensitive to pyrazinamide and cycloserine. The classical human variant is resistant to thiophen-2-carboxylic acid hydrazide (TCH) but the Asian variant is sensitive. Contrary to some opinions, we have found that this difference is independent of resistance to isoniazid (Yates, Grange and Collins, 1984). Both variants are usually niacin positive. Isoniazid-sensitive strains give a strongly positive catalase reaction but isoniazid-resistant strains usually give weak or negative reactions.

The bovine variant of Mycobacterium tuberculosis (Mycobacterium bovis)

The bovine variant was defined as *M. bovis* by Karlson and Lessel (1970). Growth on pyruvate media is usually much better than that on glycerol media.

Colonies on egg media at 18 days are about 1 mm in diameter, flatter and smoother than those of the human variant and older colonies may be umbonate.

The bacilli are generally shorter and may be plumper than those of the human variant. They are usually arranged in clumps but there is less tendency to produce long serpentine cords.

Growth is restricted to 34–38 °C. This variant is nitratase negative, microaerophilic, sensitive to TCH and cycloserine but resistant to pyrazinamide. It is niacin negative.

The African variants of Mycobacterium tuberculosis (Mycobacterium africanum)

The original African variant was found in west Africa and named *M. africanum* by Castets, Rist and Boisvert (1969) but slightly different strains have also been found in east Africa.

Colonies on egg media at 18 days resemble those of the bovine variant, but may not show preference for pyruvate.

The bacilli are similar in appearance to those of the bovine variant.

Growth is restricted to 34–38 °C.

We recognize two African variants. Both are microaerophilic, sensitive to TCH, pyrazinamide and cycloserine. The African I variant, apparently of west African origin, is nitratase negative, but the African II variant, of east African origin, is nitratase positive. The niacin reaction of both is variable.

BCG

The cultural and morphological features of this are similar to those of the classical human variant of *M. tuberculosis*. There is no preference for pyruvate.

It is nitratase negative, aerobic, sensitive to TCH but resistant to both pyrazinamide and cycloserine. There is often a minor resistance to isoniazid and ethambutol (Resistance Ratio 2) but this is of no clinical significance.

Mycobacterium microti

The 'vole or murine tubercle bacillus' of Wells (1946) and named *M. microti* (after the vole, *Microtus agrostis*) by Reed (1957) has been included in the '*M. tuberculosis* complex' of Tsukamura (1966b) because its cultural characteristics resemble those of tubercle bacilli. Only a few culture collection strains exist and the five that we have examined had the characteristics of the African I variant (Yates, 1984), although *M. microti* is not pathogenic for man.

Other variants

Hein and Tomasovic (1981) isolated 25 strains of *M. bovis* from water buffaloes in Australia. These strains grew well on LJG medium and in the presence of 5 per cent sodium chloride.

Kotian *et al.* (1983) isolated strains that were intermediate in their properties between *M. tuberculosis* and *M. bovis*.

Chapter 9

Identification of 'other mycobacteria'

Mycobacteria that are not obligate parasites may be opportunist pathogens, transient colonizers or contaminants in pathological material. It is important to identify them, especially when they are isolated on more than one occasion from the same patient. In most instances, only those that a preliminary examination suggests are opportunists need be identified to species level. Mycobacteria that have not been incriminated in human or animal disease may usually be recognized as such by simple tests, although on the rare occasions that they are isolated from immunocompromised patients they may require full examination.

Terminology

The study and identification of mycobacteria that were obviously not tubercle bacilli was clouded by the 'tuberculocentric' philosophy of medical bacteriologists, who considered that only one member of the genus was important, and the lack of a general term to describe the others, which, because of indifference, were very poorly characterized. The history of mycobacteriology was changed, however, after the Second World War, when non-medical microbiologists became interested in the genus.

The early bacteriologists recognized that the mycobacteria could be divided into two groups, the 'rapid growers' and the 'slow growers'.

The 'rapid growers'

In 1938 Gordon and Hagan cast some doubts on the validity of the names of some of the 20 or more 'species' of rapidly-growing mycobacteria that had so far been described. The subsequent investigations of Gordon and Smith (1953, 1955) and Gordon and Mihm (1959) resulted in the reduction to synonymity of quite a number of them. Some stock cultures in national collections were found to be incorrectly named and three 'good' species emerged: *M. phlei, M. smegmatis* and *M. fortuitum.* Since then, the examination of strains labelled *M. fortuitum* and some other rapidly-growing mycobacteria identified since the original work of Gordon and her colleagues, has revealed that some of them belong to the distinct species *M. chelonei* (Stanford *et al.*, 1972).

The 'slow growers'

Over the years reports had appeared in medical journals of slowly-growing mycobacteria, obviously not tubercle bacilli or other named species, that had been isolated from clinical material. Little interest in these organisms was aroused until the 1950s when the numbers increased and it was suspected that at least some of them were associated with human disease. They were described as 'atypical mycobacteria' (see below) and it became necessary to have some system for classifying them to assist physicians in deciding which were probably of clinical significance and which could be ignored.

A turning point in mycobacterial classification occurred in 1954, when this need was met by the publication by Timpe and Runyon of a paper entitled 'The relationship of atypical mycobacteria to human disease'. In this paper mycobacteria isolated from clinical material were separated into four groups, later modified (Runyon, 1959), on the basis of their pigmentation and rate of growth. Groups I (photochromogens) and III (non-chromogens) contained strains that seemed to be associated with diseases resembling tuberculosis but which came to be called mycobacterioses. The organisms in Groups II (scotochromogens) and IV (rapid growers) were generally regarded as non-significant. This grouping was not ideal as pigmentary variants occurred and the relationship of the groups to pathogenicity could be disputed. Although it was extremely useful for several years the Runyon classification, as it is known, is now virtually obsolete but Runyon's pioneering work undoubtedly led to the renaissance of interest in the genus *Mycobacterium* and its classification.

Other temporary systems of classifying these organisms followed (Collins, 1962, 1966; Marks and Richards, 1962; Marks, 1972b, 1976). These, like that of Runyon, were eclipsed in turn as species were characterized and named.

'Atypical', 'anonymous' or what?

One problem persisted, however, and that was what to call these organisms before they have been identified to species (or group). This could take two or three weeks, even after the organisms had been recognized as not being tubercle bacilli. As no real agreement has ever been reached it is relevant here to review the controversy which this problem has aroused.

The obvious term '*Mycobacterium* sp.' was avoided by workers in the 1950s, possibly because to physicians the word mycobacterium was always equated with tuberculosis. The name 'atypical' was used in a cooperative study (Anon., 1955) and passed into general use (Youmans, 1958). Objections to this word were made by Hardy *et al.* (1958), who regarded it as temporary and inappropriate, and by Marks (1958) who suggested the word 'indeterminate'.

The unsatisfactory nature of the word 'atypical' was obvious. 'Atypical of what?' was the usual question. There was no doubt that the organisms so labelled were not 'atypical tubercle bacilli' and they were certainly not atypical members of the genus *Mycobacterium.* In 1959 Runyon proposed the name 'anonymous'. This seemed sensible, because although they would subsequently be identified they were still anonymous at the time of isolation.

A leading article in *Tubercle* (Anon., 1959), objected to 'atypical' and suggested 'unidentified' but it seemed likely that a physician might interpret this as meaning unconfirmed *M. tuberculosis.* Later, Marks and Trollope (1960), writing in the same journal, used 'anonymous', rejecting 'atypical' as meaningless. In 1961 and

1965 Runyon referred to these mycobacteria as 'unclassified', which was correct in his context. This resulted in some correspondence in the *American Review of Respiratory Disease.* Youmans (1964) agreed that 'atypical' was unsatisfactory but regarded it as the least objectionable term until someone thought of a better one. Wayne (1964) agreed that 'atypical' should be retained according to custom and usage: everyone knew what it meant. Wayne also mentioned that the current Veterans Administration study was using the term MOTT (mycobacteria other than tubercle) bacilli, but this acronym seems to have been abandoned. The Editors of the *American Review of Respiratory Disease* (Anon., 1964) decided that that Journal would use the word 'atypical'.

In the same correspondence Marks (1964c) asked why one word had to do so much work. He suggested that the genus contained (a) 'customary pathogens' (e.g. *M. tuberculosis*); (b) 'opportunists' (that might be pathogens in the right circumstances); and (c) 'casuals' (that were certainly not pathogens). But at the time when the microbiologist might feel obliged to give a preliminary report to the physician he might not know whether the organism was an 'opportunist' or a 'casual' and as that last word is curiously prone to being mis-spelled or misinterpreted as 'causal' this seems undesirable.

The controversy was continued by Hauduroy (1965) who accepted that 'atypical' was bad, but was so generally accepted that it had become a habit. He thought that 'provisionally unclassified' might be better, but the Editors of the *American Review of Respiratory Disease* (Anon., 1965a,b) decided to retain 'atypical'. Hobby (1967), who used 'atypical' in her review of the organisms, pointed out that the word was used as long ago as 1927 at the Twenty-Second Annual Meeting of the National Tuberculosis Association (Pribram, 1927).

The last shots in this semantic battle were exchanged by Francis and Abrahams (1982), who favoured 'tuberculoid' and by Grange and Collins (1983), who suggested 'nyrocine'. Neither of these terms met with favour and it seems that the Americans have settled down to 'atypical' while we in Britain use 'anonymous' or 'opportunist'.

Specific names

Although many individual contributions have been made in the characterization and classification of the mycobacteria, the largest and most useful have stemmed from the cooperative studies organized by the International Working Groups on Mycobacterial Taxonomy. There is still some dissension, however, about the names and validity of some species and although a list of the 'official' names taken from the Approved List of Bacterial Names of the International Committee on Systematic Bacteriology (Skerman, McGowan and Sneath, 1980) is shown in *Table 7.1* this should not be considered as having received the unqualified approval of all mycobacteriologists. Some alternative and unofficial 'popular' names are also included.

IDENTIFICATION METHODS

Cultural and biochemical tests

The tests described here are those that have been found to be reliable and reproducible. Most of them are used by members of the International Working Group of Mycobacterial Taxonomy and the European Society of Mycobacteriologists. The media and reagents are described in the Appendix.

Macroscopic and microscopic examination

Colony appearance, consistency, rate of growth and ease with which the growth is emulsified for microscopy are noted. ZN-stained smears are examined for size, shape, intensity of staining and distribution of bacilli (see *Figure 6.1*) and for the presence of non acid-fast contaminants.

Preparation of inoculum

Suspensions are made as described on p. 72. Culture media are inoculated with approximately 10 μl, using disposable plastic loops or a MicroRepette (see *Figure 3.5*).

Growth at various temperatures

Five tubes of LJG are inoculated. One each is incubated at these temperatures: 20, 25, 37, 42 and 44 °C. If growth occurred at only 30–33 °C on the primary culture an additional tube, incubated at this temperature, is included.

Incubators with cooling coils are almost essential for the lower temperatures and solid incubation blocks are better (and safer) than water-baths for incubation at 42 and 44 °C. For additional temperature stability these blocks may be placed in the 37 °C incubator room.

The cultures are examined at 3, 7, 14 and 21 days but some mycobacteria require even longer to give observable growth.

Pigment production

Two LJG tubes are inoculated. One is placed in an internally illuminated 37 °C incubator (see p. 62) and the other in a light-proof box and also incubated at 37 °C.

The growths are compared after 18 days' incubation. Production of a yellow or orange pigment on both indicates that the organism is a scotochromogen. Photochromogens produce their pigment only in the growth exposed to light. Photochromogenicity may depend on temperature: there are species (e.g. *M. szulgai*) that are photochromogens at 25 °C but scotochromogens at 37 °C.

Some mycobacteria, e.g. *M. xenopi, 'M. avium-intracellulare-scrofulaceum'* and *M. ulcerans*, may produce only a small amount of yellow pigment, unaffected by light. Although strictly speaking these are scotochromogens, some workers do not use this term.

Sensitivity to thiacetazone (TZ)

The medium is LJG containing 20 mg/litre of thiacetazone. One tube and a control tube of LJG are inoculated and incubated at 37 °C for 18 days. The growths are then compared.

Nitratase test

Middlebrook 7H9 broth is inoculated and incubated at 37 °C for 18 days. The test is described on p. 63.

Arylsulphatase test

Middlebrook 7H9 broth containing phenolphthalein disulphate is inoculated and incubated at 37 °C until a good growth is obtained (3–18 days). A few drops of ammonia are then added. A pink colour, whose intensity varies with different species, indicates the presence of an arylsulphatase.

Catalase test

A butt of LJG medium in a bottle or tube approximately 12 mm in diameter ('bijou bottle') is inoculated and incubated at 37 °C for 18 days. One ml of a mixture of equal parts of 30 per cent hydrogen peroxide and 0.5 per cent Tween 80 is added and the tubes allowed to stand for a few minutes. The height of the column of bubbles is measured in millimetres. We record as follows: 0–1 mm, negative; 2–10 mm, +; 11– mm, ++; more than 20 mm, +++.

There is a thermostable catalase test in which a suspension of the mycobacteria is heated at 68 °C before testing. We have not found this useful.

Tween hydrolysis

The substrate is a solution of Tween 80 and neutral red in a phosphate buffer. It is heavily inoculated, directly from a well-grown LJG culture, incubated at 37 °C and examined after seven and 14 days. A change of colour, from straw yellow to red indicates that the Tween has been hydrolysed, releasing the dye. Some mycobacteria give negative reactions at seven days but positive reactions at 14 days.

Tellurite reduction

Middlebrook 7H9 broth (2 ml) is inoculated and incubated at 37 °C until there is a heavy growth (three days for rapidly growing mycobacteria, 10–14 days for others). Four drops of sterile 0.2 per cent potassium tellurite are added and the culture returned to the incubator and examined daily. If the organisms reduce tellurite a heavy black deposit will appear within seven days. Grey deposits should be ignored.

Growth in N medium

The medium contains ammonium sulphate as the sole source of nitrogen. A tube is inoculated from the suspension bottle using a straight wire (minimum inoculum) and incubated at 37 °C. Mycobacteria that do not require complex sources of nitrogen grow within three days.

Resistance to antituberculous drugs

Resistance to streptomycin, ethambutol, ethionamide and rifampicin are useful aids in the identification of some mycobacteria. The techniques are described in Chapter 10.

The properties of species that may be encountered in medical and veterinary material are shown in *Table 9.1*.

TABLE 9.1. Usual properties of some opportunist mycobacteria and others that may be encountered in clinical material

Species	*Pigment*	*TZ*	*Nitratase*	*Tween hydrolysis*	*Growth at* (°C) 20	25	33	42	44	*Sulphatase*[a] 3 days	21 days	*Catalase*[b]	*Tellurite*[c] reduced	*Growth on N-medium*	*Rapid growth*
M. kansasii	P	S	+	+	−	+	+	v	−		+	+++		−	−
M. marinum	P	R	−	+ (late)	+	+	+	−	−		++	++		v	+
M. xenopi	−/S	R	−	−	−	−	−	+	+		+++	−	−	−	−
M. avium-intracellulare -scrofulaceum	−/S	v	−	−	v	+	+	+	v		v	−	+	−	−
M. malmoense	−	R	+	+ (late)	−	+	+	−	−		−	−	−	−	−
M. simiae	P	R	−	−	−	+	+	−	−		+	++	−	−	−
M. szulgai	S/P[d]	R	+	+	−	+	+	−	−		+++	+	−	−	−
M. fortuitum	−	R	+	−	+	+	+	v	−	+++	+++	v	+	+	+
M. chelonei	−	R	−	−	+	+	+	−	−	+++	+++	v	+	+	+
M. gordonae	S	R	−	+	+	+	+	−	−		+	++		−	−
M. flavescens	S	R	+	+	+	+	+	−	−		+	+		+	+
M. gastri	−	R	−	+	+	+	+	−	−		+	+	−	−	−
M. terrae	−	R	+	+	v	+	+	−	−		+	+++	−	−	−
M. triviale	−	R	−	+	−	+	+	−	−		+	+	−	−	−
M. nonchromogenicum	−	R	−	+	v	+	+	−	−		+	+++	−	−	−
M. smegmatis	−	R	+	+	+	+	+	+	+	−	+	++	+	+	+
M. phlei	S	R	+	+	+	+	+	+	+	−	+	++	+	+	+
M. ulcerans	−/S	R	−	−	−	−	+	−	−		−	−	−	−	−

TZ, thiacetazone; P, photochromogen; S, scotochromogen; +, usually positive; −, usually negative or none; v, variable

[a] Sulphatase: +++, deep pink; ++, pink; +, pale pink
[b] Catalase: Amount of foam, +++, more than 20 mm; ++, 10–20 mm; +, 5–10 mm; −, less than 5 mm
[c] Test not done on pigmented strains
[d] *M. szulgai:* At 25 °C = photochromogen; at 37 °C = scotochromogen

Lipid chromatography

Marks and Szulga (1965) and Jenkins (1981) showed that mycobacteria could be identified by using thin layer chromatography to determine their lipid patterns. The method is particularly useful for identifying unusual species, e.g. *M. malmoense* and *M. szulgai* and for distinguishing between the various species of rapidly-growing mycobacteria. The method described below is that used at the PHLS Mycobacterium Reference Unit in Cardiff and is given here by permission of the Director, Dr P. A. Jenkins.

Preparation of silica gel plates

Silica gel, 30 g (the exact amount may vary with different batches), and distilled water, 65 ml, are mixed in a stoppered flask to give a slurry that will spread evenly and is free from air bubbles. Glass plates, 20 × 10 cm, cleaned with dichromate solution then washed and dried, are coated with the slurry to give a layer approximately 0.1 mm thick, using Shandon Uniplan apparatus. The plates are left until they have a matt surface and are then dried at 100 °C for 30 minutes in a hot-air oven fitted with a fan. They are washed with acetone, dried at 100 °C and then activated by heating at 120 °C for a further 30 minutes. They may be stored over silica gel for up to seven days.

Lipid extract

Cultures of eugonic organisms are grown on LJ medium. Dysgonic mycobacteria are grown in Kirchner medium. Controls of known species are included with each batch of tests.

The organisms are scraped from the surface of LJ medium, or harvested from Kirchner medium by centrifugation, and transferred to a small glass bottle of known weight. They are dried over phosphorus pentoxide in a vacuum desiccator at 50 cmHg. Their weight is found. At this stage the extract may be stored at 4 °C for several months.

To extract the lipids a freshly prepared mixture of diethyl ether:ethanol:water (17:17:6 v/v) is added in the proportion 16 µl to 1 mg dry weight of mycobacteria. The tubes are stoppered and left at room temperature overnight.

Application

A total volume of 10 µl of the extract is applied to a plate in four separate 2.5 µl amounts, each of which is allowed to dry naturally before the next one is added. For unidimensional chromatograms the extracts are applied 15 mm apart.

Solvents

The following solvents give satisfactory results:

n-propanol:water:ammonia::75:22:3
n-propanol:water:n-butanol:ammonia::57:20:20:3
n-propanol:water:di-isopropyl ether::45:10:45
n-propanol:water:ammonia:di-isopropyl ether::45:9.5:0.5:45

The ammonia is sp.gr. 0.880; di-isopropyl ether is added slowly to the other fluids with continuous shaking.

Location of spots

Chromatograms are run for 10 cm, dried at 100 °C for 30 minutes and then allowed to cool. They are sprayed with a mixture of 2 volumes of sulphuric acid and 1 volume of freshly prepared 1 per cent orcinol in distilled water. The plates are then heated on a hot-plate at about 140 °C when the spots should appear. If too much spray is applied the spots will all be brownish-black in colour.

The chromatogram is read over a grid prepared by ruling 0.5 cm squares with a diamond stylus on the bottom of a glass dish. This is illuminated from below so that the position and shape of the spots may be seen clearly and recorded.

Lipid patterns

A typical chromatogram is shown in *Figure 9.1*. Contributions from the media are easily recognized and the phenol red in the Kirchner medium used for dysgonic organisms is a useful marker as the small amount in the extract gives a narrow band in the centre of unidimensional chromatograms run with the first two solvents.

The patterns of unidentified strains are compared with those of known species.

Additional biochemical tests

In addition to the various biochemical spot tests, a number of 'rows' have been described. These include the carbohydrate row of Gordon and Smith (1953) and the amide row of Bönicke (1962). Although employed in basic taxonomic studies, these methods are infrequently used in routine practice. Furthermore, with few exceptions, their use is confined to the study of rapidly-growing species.

Carbohydrate row

Sugars are incorporated in an ammonium phosphate-based agar medium containing bromocresol purple which turns yellow when acid is produced. Slopes of the media are lightly inoculated with bacterial growth from a Lowenstein–Jensen slope and incubated at 35 °C. They are inspected for colour change weekly for four weeks. The usual sugars are arabinose, dulcitol, erythritol, galactose, glucose, inositol, lactose, mannitol, mannose, raffinose, rhamnose, sorbitol, trehalose and xylose. For diagnostic purposes we have found that three of these sugars are particularly useful (Grange, 1980): these are mannitol, inositol and xylose. Their reactions are shown in *Table 9.2* (Gordon and Smith, 1953). In particular, unreactive strains are likely to be *M. chelonei* or *M. fortuitum* biotype A which is much more frequently incriminated as a pathogen than the other two biotypes.

Amide row

This row is based on the ability of mycobacteria to hydrolyse various amides into free organic acids and free ammonia. A suspension of bacteria harvested from a Lowenstein–Jensen slope is added to the amide solutions which are incubated at 35 °C for 20 hours. The presence of ammonia is indicated by the development of a blue colour on the addition of Russel's reagent. The ten amides usually employed are acetamide, allantoin, benzamide, carbamide (urea), isonicotinamide,

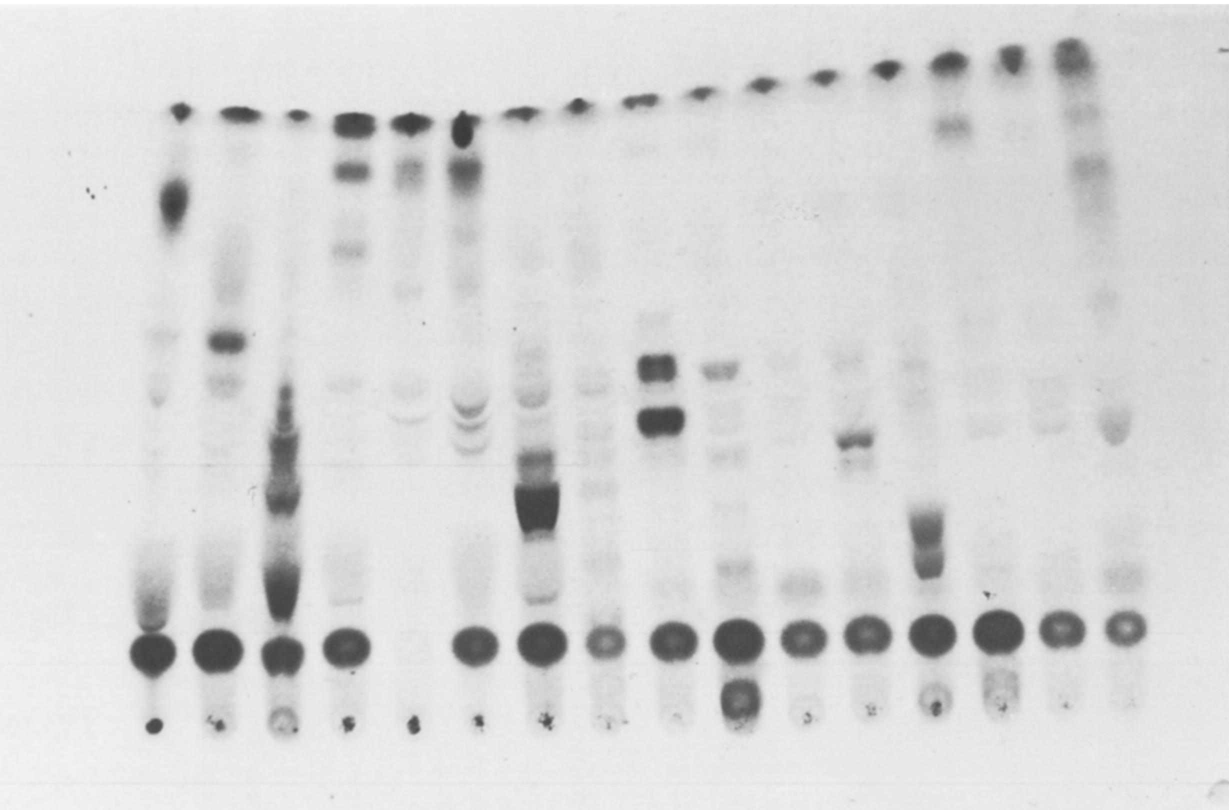

Figure 9.1 Thin layer chromatography of species and strains of mycobacteria. (1) *M. fortuitum* (2) Fortuitum group (3) Fortuitum group (4) *M. peregrinum* (5) *M. chelonei* var *abscessus* (6) *M. chelonei* var *chelonei* (7) *M. marinum* (8) *M. kansasii* (9) *M. gordonae* (10) *M. szulgai* (11) Terrae group (12) *M. malmoense* type II (13) *M. malmoense* type I (14) *M. avium* (15) *M. intracellulare* (16) *M. xenopi* (Reproduced by courtesy of Dr P. A. Jenkins)

TABLE 9.2. Differentiation of some rapidly-growing mycobacteria by allantoinase activity and production of acid from 'sugars'

Species	*Allantoinase*	*Acid from*		
		Mannitol	*Inositol*	*Xylose*
M. chelonei	−	−	−	−
M. fortuitum A	+	−	−	−
M. fortuitum B	+	+	−	−
M. fortuitum C	+	+	+	−
M. smegmatis	−	+	+	+
M. phlei	−	+	−	+
M. gilvum	−	+	+	−
M. duvalii	−	+	−	−
M. flavescens	−	V	−	−
M. vaccae	+	+	+	+

+ = acid produced; − = no acid produced; V = variable reaction

malonamide, nicotinamide, pyrazinamide, salicylamide and succinamide. The only one that we consider useful for diagnostic bacteriology is allantoin which distinguishes *M. fortuitum* from *M. chelonei* (*Table 9.2*) and also between the photochromogenic species *M. marinum* and *M. kansasii*, being hydrolysed by the former species.

Additional reference techniques

There are a number of highly discriminative taxonomic methods which, because of their technical complexity or the cost of the equipment, are confined to a very few major centres. In general, such techniques have been used more for research purposes than for routine identification. They are divisible into two main groups: first, the detection of antigenic differences by immunological methods and, second, the detection of structural differences by sophisticated chemical methods such as gas–liquid chromatography and pyrolysis mass spectroscopy. The general principles of these methods are briefly outlined here.

Immunological methods

Antigenic differences between mycobacterial strains are usually demonstrated by agglutination of whole bacilli by the appropriate antisera or by immunodiffusion analysis of the soluble antigens of disrupted bacilli. Agglutination studies are very simple and require only a small amount of culture. Unfortunately, many mycobacteria, including *M. tuberculosis*, are rough and agglutinate spontaneously. Species in which most strains form smooth suspensions are identifiable, and some are divisible into many serotypes, by agglutination serology. In particular, *M. avium* (including the *intracellulare* strains) and *M. scrofulaceum* have been studied by this technique (Schaefer, 1965). Similar antisera have also been used to identify smooth strains of *M. kansasii, M. marinum, M. chelonei* and *M. fortuitum*, although biochemical tests remain more widely used.

The technique of double diffusion in gel is applicable to both smooth and rough strains and gives much more information on antigenic structure than agglutination

tests. Bacterial cytoplasm, liberated by freeze-pressing or ultrasonication, is placed in one well in an agar plate and an antiserum is placed in an adjacent well. About 14 precipitin lines, depending on the species, may be visually detected between the wells when homologous antiserum is used. Many of these lines represent antigens shared between mycobacteria, as discussed on page 58, but each species has between two and eight precipitating antigens unique to itself. There is also a small degree of antigenic variation within some species. Thus, the technique enables strains to be identified at the species, and sometimes at the subspecific level. For details see Stanford and Grange (1974).

Antigenic differences between species and strains is also demonstrable by comparing the degree of dermal hypersensitivity elicited by bacterial antigens in animals, such as guinea pigs, previously sensitized by challenge with a given mycobacterium. The reagents used for this purpose are usually concentrated filtrates of mycobacterial cultures and are termed tuberculins or sensitins. When such an animal is tested with a set of carefully standardized reagents, the sizes of the dermal reactions reflect the antigenic relatedness between the sensitins and the challenging organism. This technique has been used for taxonomic purposes but may also be used to identify unknown isolates. For reviews and technical details see Magnusson (1981).

Chemical methods

In gas–liquid chromatography volatile bacterial components such as lipids are vapourized by heat and are carried by a stream of nitrogen through a column packed with a non-volatile liquid on a suitable absorbent carrier. The time taken for each component to travel through the column depends on the extent to which each enters the liquid on the packing material. Separated substances emerging from the column are quantitated by ionization detectors and the height of the peaks at different times gives a characteristic profile or 'fingerprint' for the particular strain or species. The equipment is expensive but the analysis requires only a small amount of bacilli and gives rapid results. Technical details are given by Ohashi, Wade and Mandle (1977) and by Tisdall *et al.* (1979). In general, the results of gas–liquid chromatography correlate with those of biochemical tests although a few inexplicable discrepancies occur (Tisdall *et al.*, 1982).

A more discriminative, but even more expensive, technique for obtaining characteristic fingerprints is that of pyrolysis mass spectroscopy in which volatile products are liberated from mycobacteria by thermal degradation in a vacuum and separated according to their masses in an electromagnetic field. For details see Weiten *et al.* (1981). A further sophistication consists of the direct mass spectroscopic analysis of the effluents of a gas chromatograph (Yano *et al.*, 1978).

SPECIES OF MEDICAL AND VETERINARY INTEREST

The cultural and biochemical properties of species of mycobacteria other than tubercle bacilli that are likely to be encountered in tuberculosis laboratories are summarized here in alphabetical order. Most of the names are those in the 'official list' (Skerman, McGowan and Sneath, 1980), also shown in *Table 7.1*, p. 56.

Species of limited interest are listed separately, again with pertinent references, but are not discussed (p. 86).

Mycobacterium avium

Originally called the avian tubercle bacillus, this species (Chester, 1901; Engbaek, Runyon and Karlson, 1971; Grange, 1984b) is included here in '*M. avium-intracellulare-scrofulaceum*'.

'Mycobacterium avium-intracellulare-scrofulaceum' (MAIS)

The three species that make up this complex, *M. avium, M. intracellulare* and *M. scrofulaceum*, are difficult to differentiate except by techniques usually available only in reference laboratories. Stanford and Grange (1974), for example, showed that while *M. avium* and *M. intracellulare* appear to be variants of one species, *M. scrofulaceum* is a distinct species. They are, nevertheless, considered together here. They are often referred to as MAIS bacilli after the initial letter of their generic and specific names.

Colonies on egg media at 14–21 days are about 1 mm in diameter, smooth, white and domed but growth may be effuse. Some strains produce a feeble yellow pigment and those identifiable as *M. scrofulaceum* a deeper yellow pigment, unaffected by light; hence they are sometimes referred to as the scrofula scotochromogens.

The bacilli are small, about 1.0 × 0.5 μm, often appearing almost coccoid (*Figure 6.1*).

All strains grow on egg media at 25 °C, and few grow at 20 °C. Many strains grow at 42 °C and some at 44 °C. These are the avian strains originating from birds. The scrofula scotochromogens are usually sensitive to thiacetazone (20 mg/litre). MAIS bacilli are nitratase negative and catalase negative or weakly positive. They reduce tellurite but do not hydrolyse Tween 80. The sulphatase reaction is variable: *avium* strains are said to be negative, *intracellulare* strains strongly positive. Resistance to all antituberculous drugs is usual but some strains are sensitive to ethionamide.

MAIS bacilli are opportunist pathogens of man, associated with cervical adenitis (scrofula), especially in children, but pulmonary infections do occur. They are also opportunist pathogens of pigs (*intracellulare* strains) and birds (*avium* strains). They are found in water, soil and household dusts and are more common in north America than in Europe.

Mycobacterium chelonei

This is the 'turtle bacillus' of Friedmann (1903) and has an interesting taxonomic history. It was at one time included with *M. fortuitum*, has been known as '*M. abscessus*' (Moore and Frerichs, 1953; Kubica *et al.*, 1972); '*M. runyonii*' (Bojalil, Cerbon and Trujillo, 1962; Kubica *et al.*, 1972) and as '*M. borstelense*' (Bönicke, 1965; Kubica *et al.*, 1972). For details see Grange (1981).

Mycobacterium chelonei grows rapidly (two to three days) when subcultured on nutrient agar and egg medium and produces no pigment. The colonies are white, moist, soft and domed, 2–3 mm in diameter.

The bacilli are about 2–3 × 0.5 μm, and some cells tend to be rather fat. They usually stain solidly although weakly acid-fast cells may be seen.

It grows well at 20 and 37 °C, although occasional strains are psychrophilic and do not grow much above 25 °C. There is no growth at 42 °C. It is nitratase negative, catalase positive, sulphatase strongly positive, fails to hydrolyse Tween in five days, but reduces tellurite rapidly. It grows well in N medium in three days and poorly on MacConkey agar, producing small yellowish colonies. Resistance to all antituberculosis drugs is usual but some strains are sensitive to gentamicin, amikacin, erythromycin or cotrimoxazole.

It is an opportunist pathogen, occurring in superficial wounds, e.g. mechanical trauma and 'injection abscesses' following the use of multi-dose vaccine, etc. bottles. Iatrogenic infections are not uncommon, e.g. from contaminated porcine heart valves used in replacement surgery, following renal transplants and in peritoneal dialysis. Two geographic variants have been recognized: *M. chelonei* var. *abscessus* (USA) which is salt-sensitive and *M. chelonei* var. *borstelense* (Europe), which is salt-resistant (Kubica, 1973; Collins and Yates, 1979). This species would appear to be more common in hospitals than elsewhere in the environment.

Mycobacterium flavescens

This rapidly-growing scotochromogen was described by Bojalil, Cerbon and Trujillo (1962).

Colonies on egg medium at three days are bright yellow, about 2 mm in diameter and may be rough or smooth.

The bacilli show no distinctive morphology, are 2–3 × 0.4 μm and stain well.

Mycobacterium flavescens grows well at 20 and 37 °C but not at 42 °C, is nitratase, sulphatase and catalase positive and hydrolyses Tween 80. It grows in N medium and is resistant to thiacetazone (20 mg/litre). Resistance to streptomycin is variable; most strains are sensitive to ethambutol and ethionamide but resistant to rifampicin. It is only rarely associated with human disease, but is a not infrequent contaminant of pathological material. Its natural habitat is not known but thought to be water and soil.

Mycobacterium fortuitum

This rapidly-growing non-pigmented organism (Da Costa Cruz, 1938; Runyon, 1972; Judicial Commission, ICSB, 1974) has also been known as the 'cold blooded' or frog tubercle bacillus (Küster, 1905), '*M. giae*' (Darzins, 1950), '*M. minetti*' (Penso *et al.*, 1952), '*M. ranae*' (Bergey *et al.*, 1923; Stanford and Gunthorpe, 1969) and as '*M. peregrinum*' (Bojalil, Cerbon and Trujillo, 1962).

Colonies on nutrient agar and egg media at three days are 2–3 mm in diameter, white or buff and either rough or smooth and domed.

The bacilli show no distinctive morphology, being about 2–3 × 0.5 μm, with some fatter forms. They stain solidly.

All strains grow well at 20 °C and most at 25 and 37 °C. Some are psychrophilic and some grow at 42 °C. It is nitratase and catalase positive, sulphatase strongly positive, does not hydrolyse Tween 80 but reduces tellurite rapidly. There is growth in N medium and colonies on MacConkey agar are red. There is a general resistance to antituberculosis drugs but some strains are sensitive to ethionamide, gentamicin, amikacin, erythromycin or cotrimoxazole.

Mycobacterium fortuitum is an opportunist pathogen which may infect superficial wounds. It has been incriminated in injection abscesses, may be a secondary invader of the lungs and has caused serious problems in transplant surgery and peritoneal dialysis. It is normally resident in soil and is a frequent contaminant of cultures of pathological material.

Mycobacterium gastri

This relatively uncommon slowly-growing nonchromogen was described by Wayne (1966) and is important only because it may be confused with the MAIS complex or be mistaken for a non-pigmented strain of *M. kansasii.* Indeed it may be a non-pigmented variant of the latter as the two are antigenically identical (Stanford and Grange, 1974).

Colonies on egg media at 14 days are small, smooth, white and domed.

The bacilli are morphologically undistinguished, being about 2 × 0.3 μm and staining evenly.

Mycobacterium gastri grows at 20 and 37 °C but not at 42 °C. It is nitratase negative, catalase and sulphatase positive, hydrolyses Tween 80 but does not reduce tellurite. It is resistant to isoniazid but sensitive to most other antituberculosis drugs.

Originally isolated from gastric contents this nonchromogen is not known to cause human infections.

Mycobacterium gordonae

This is the official name of the 'tap water scotochromogen' (Bojalil, Cerbon and Trujillo, 1962; Wayne, 1970). It has been known as '*M. aquae*' (Gordon and Smith, 1955; Bönicke, 1962; Wayne, 1970). For its history see Collins, Grange and Yates (1984).

It is a slowly-growing scotochromogen. Colonies at 14 days are 1–2 mm in diameter, smooth and domed. They are yellow when first grown but on continued exposure to light the growth often assumes an orange colour. Crystals of carotene are never seen.

The bacilli are 2–3 × 0.5 μm and undistinguished in their morphology and staining characteristics.

Mycobacterium gordonae grows at 20 and 37 °C but not at 42 °C. It is resistant to thiacetazone (20 mg/litre), is nitratase negative, catalase and sulphatase positive and hydrolyses Tween 80. It is resistant to isoniazid but sensitive to most other antituberculosis drugs.

Although not normally regarded as being clinically significant it has been incriminated in human disease on very rare occasions. It is found in water and is a common contaminant in cultures of pathological material; usually only one or two colonies are seen on only one of the slopes of culture media.

Mycobacterium haemophilum

Identified and named by Sompolinsky *et al.* (1978) this rare organism is important because it does not grow on the culture media normally used in tuberculosis bacteriology. It grows at 30 °C on LJ medium containing 2 per cent ferric

ammonium citrate, on Middlebrook 7H11 medium containing 60 μg/litre of haemin and on blood agar. All the usual tests are negative. Any such acid-fast bacillus should be sent to a reference laboratory.

Mycobacterium haemophilum has been associated with subcutaneous granulomas in immunosuppressed patients.

Mycobacterium intracellulare

This species (Runyon, 1965, 1967) is sometimes called the 'Battey bacillus' after the hospital in the USA where it was first recognized as a pathogen of man. It is regarded by some workers as a serotype of *M. avium* and as it is difficult to distinguish it from that species it is described above under '*Mycobacterium avium-intracellulare-scrofulaceum*'.

Mycobacterium kansasii

This was originally described by Hauduroy (1955) and has been known as '*M. luciflavum*' (Manten, 1957; Runyon, Wayne and Kubica, 1974).

It is characteristically a photochromogen, producing a bright yellow pigment when exposed to light, but occasionally one encounters scotochromogenic or nonchromogenic strains. Continuous incubation in the light of the normal, photochromogenic strains results in the formation of orange coloured crystals of carotene which are embedded in the growth.

Colonies on egg medium at 14 days are 1–2 mm in diameter and either smooth and domed or rough. When cultures are incubated on LJ medium in the dark they may resemble those of *M. tuberculosis*.

The morphology is distinctive; the bacilli are long and thin, 4–5 × 0.3 μm, stain irregularly and often appear beaded or barred (*Figure 6.1*). *Mycobacterium kansasii* grows at 25 but not at 20 °C; occasional strains grow at 42 °C. It is sensitive to thiacetazone (20 mg/litre), is nitratase and catalase positive (more than 20 mm), is sulphatase positive and hydrolyses Tween 80. It is resistant to streptomycin, isoniazid and pyrazinamide but sensitive to other antituberculosis drugs. Some strains are sensitive to erythromycin and cotrimoxazole by the disc test.

It is an opportunist pathogen, associated with pulmonary infections but is occasionally found in other sites, even in skin lesions where it may be mistaken for *M. marinum*. Its natural habitat appears to be piped water supplies and there is circumstantial evidence that infections may arise from the inhalation of aerosols generated by showers and faucets (see the review by Collins, Grange and Yates, 1984).

Mycobacterium malmoense

This is a 'new' species (Schroder and Juhlin, 1977) and is important because bacteriologists have probably failed to isolate or identify it because of its very slow growth and uncertain characteristics.

Colonies on egg medium take as long as ten weeks to develop and are then very small and almost transparent. The organisms grow better after subculture. The bacilli are small and have no particular morphological characteristics.

Mycobacterium malmoense grows poorly at 25 °C and better at 30 and 37 °C but not at 42 °C. It is nitratase and sulphatase negative, is catalase positive and it hydrolyses Tween 80. It is usually resistant to isoniazid and sensitive to other antituberculosis drugs.

It causes pulmonary disease in adults and cervical adenitis in children.

Mycobacterium marinum

First described by Aronson (1926), this species has also been known as '*M. platypoecilus*' (Baker and Hagan, 1938) and '*M. balnei*' (Linell and Norden, 1954).

On primary culture it grows only at 30–33 °C and is therefore missed in laboratories committed to 37 °C. It is a photochromogen and colonies at seven days are 2 mm in diameter, smooth and domed.

The bacilli are 2–3 × 0.3 μm and have no distinctive characteristics.

After subculture *M. marinum* grows at 20 and 37 °C but not at 42 °C. It is resistant to thiacetazone (20 mg/litre), is nitratase negative, catalase and sulphatase positive, hydrolyses Tween 80 and grows poorly in N medium. It is resistant to streptomycin and isoniazid but sensitive to most other antituberculosis drugs.

Mycobacterium marinum is a pathogen of fish and is found in salt and fresh water and in aquaria. It may cause superficial infections in man, such as 'swimming pool granuloma', 'fish tank granuloma' and 'fish fancier's finger' if it enters through trivial skin abrasions. See the review by Collins *et al.* (1985).

Mycobacterium nonchromogenicum

This species (Tsukamura, 1965) is important only because it is sometimes confused with MAIS bacilli.

Colonies on egg medium at 14–18 days are about 1.5–2 mm in diameter, white, smooth and domed.

The bacilli are 2–3 × 0.4 μm and show no distinctive morphology.

Mycobacterium nonchromogenicum grows at 20 °C and may grow poorly at 42 °C. It is nitratase negative, strongly catalase positive, sulphatase positive, hydrolyses Tween 80 but does not reduce tellurite. Strains vary in their susceptibility to antituberculosis drugs.

There are very few reliable reports of human infection, but the organism is occasionally found in cultures of pathological material. Its natural habitat is not known.

Mycobacterium paratuberculosis

This species (M'Fadyean, 1907; Bergey *et al.*, 1923) was called Johne's bacillus after it was first isolated by Johne and Frothingham (1895).

It can be cultured only on media containing extracts of mycobacteria (mycobactin) and isolation (from lesions in animals and animal faeces) is therefore rarely attempted.

It causes a contagious and enzootic disease of ruminants, usually diagnosed clinically and by the examination of direct smears of faeces.

Mycobacterium phlei

Called the Timothy grass bacillus because it was originally isolated from that plant (*Phleum pratense*), this was described by Lehmann and Neumann (1899) and by Gordon and Smith (1953).

It is a rapidly-growing mycobacterium which produces a yellow or orange pigment unaffected by light. Colonies on nutrient agar or egg media at two days are 2–3 mm, rough and wrinkled or smooth.

The bacilli are 2–3 × 0.4 μm and stain evenly.

Mycobacterium phlei grows at 20 °C (and below), and at 55 °C. It is nitratase and catalase positive, gives a weak positive sulphatase reaction, hydrolyses Tween 80, reduces tellurite and grows in N medium. Strains vary in their susceptibility to antituberculosis drugs.

Although at one time it appeared to be common in farmyard soils and on plants, this organism is rarely encountered in the clinical laboratory. It is of historic interest.

Mycobacterium scrofulaceum

This species (Prissick and Masson, 1956; Wayne, 1975) is identical with '*M. marianum*' (Suzanne and Penso, 1953) but because of confusion with the distinct species *M. marinum* (Wayne, 1968) the name *M. scrofulaceum* was legalized and '*M. marianum*' disappeared into synonymity (Judicial Commission, ICSB, 1978). It is the scrofula scotochromogen and because it is not easy to distinguish it from *M. avium* and *M. intracellulare* it is described above under '*M. avium-intracellulare-scrofulaceum*'.

Mycobacterium simiae

A relatively 'new' species (Karasseva, Weiszfeiler and Krasznay, 1965), also known as '*Mycobacterium habana*' (Valdivia-Alvarez, Suarez-Mendes and Echemendia-Font, 1971; Meissner and Schröder, 1975) this is important because it is niacin positive and may have been identified as *M. tuberculosis* by those who rely on that test alone and who do not incubate cultures in the light or test their ability to grow at temperatures other than 37 °C.

It is a photochromogen; colonies on egg medium at 14–18 days are 1 mm diameter or less and smooth.

The bacilli are 2–3 × 0.3 μm and have no distinctive morphology.

Mycobacterium simiae grows at 25 °C and some strains grow at 42 °C. It is resistant to thiacetazone (20 mg/litre), is nitratase negative, catalase and sulphatase positive, does not hydrolyse Tween 80 nor reduce tellurite. Strains vary in their susceptibility to antituberculosis drugs.

It is associated with pulmonary disease and it may be transmitted from monkeys to man. There is also some evidence that infection may arise from contaminated water (Wolinsky, 1979).

Mycobacterium smegmatis

Another species of historic interest, this was described by Lehmann and Neumann (1899) and characterized by Gordon and Smith (1953).

It is a rapidly-growing mycobacterium. Colonies on nutrient agar and egg media at two to three days are 2–3 mm in diameter, smooth and white or buff. Growth later becomes wrinkled.

The bacilli are 2–3 × 0.4 μm and stain evenly.

Mycobacterium smegmatis grows at 203°C and below and at 44 °C, is nitratase and catalase positive but sulphatase negative or weakly positive, hydrolyses Tween 80, reduces tellurite and grows in N medium. It is sensitive to most antituberculosis drugs, except rifampicin.

It is alleged that this organism was originally isolated from the smegma of a dog and generations of medical bacteriologists have been taught that it occurs in human urine and may be distinguished from the tubercle bacillus because it is acid but not alcohol fast. Both beliefs are unfounded. In recent environmental studies it has appeared with great rarity and is commoner in medical textbooks than in pathological material. We have encountered it less than a dozen times among some 5000 non-tuberculous mycobacteria examined in the last ten years.

Mycobacterium szulgai

A recently identified pathogen (Marks, Jenkins and Tsukamura, 1972), this is important because it may be confused with the common tapwater scotochromogen.

It is a slowly-growing scotochromogen at 37 °C but is a photochromogen at 25 °C. Colonies on egg media at 12–14 days are 1–2 mm in diameter, yellow and smooth.

The bacilli are 2–3 × 0.4 μm and of undistinguished morphology.

Mycobacterium szulgai fails to grow at 20 °C in 21 days, grows at 25 but not at 42 °C. It is resistant to thiacetazone (20 mg/litre), nitratase positive, gives a weak positive catalase but a strongly positive sulphatase reaction and hydrolyses Tween 80. It is usually resistant to streptomycin, always resistant to isoniazid but sensitive to other antituberculosis drugs.

It is primarily a pulmonary pathogen but infections in other sites have been recorded.

Mycobacterium terrae

This species, described by Wayne (1966), is not common but could be confused with MAIS organisms.

It grows relatively slowly. Colonies on egg media at 14 days are 1–2 mm in diameter, white and smooth.

The bacilli are 2–3 × 0.4 μm and stain evenly.

Mycobacterium terrae grows poorly at 20 °C, well at 25 °C and poorly at 42 °C. It is nitratase positive, gives a strong catalase but weak sulphatase reaction, hydrolyses Tween 80 but does not reduce tellurite. It is resistant to isoniazid. Most strains are resistant to streptomycin and rifampicin, but sensitive to other antituberculosis drugs.

It is usually of no significance when isolated from clinical material but has been known to cause superficial lesions (e.g. 'sausage finger').

Mycobacterium triviale

Described by Kubica *et al.* (1970) this is another species that might be confused with MAIS bacilli.

It grows relatively slowly on egg media. Colonies at 14 days are 1–2 mm in diameter, white and smooth.

The bacilli are 2–3 × 0.3 µm and undistinguished morphologically.

Mycobacterium triviale grows at 25 °C but not at 20 or 42 °C, is nitratase negative, catalase positive (thermolabile), gives a weakly positive sulphatase reaction, hydrolyses Tween 80 but does not reduce tellurite. Apart from resistance to isoniazid, strains vary in their resistance to other antituberculosis drugs.

It is usually of no clinical significance, but infections (e.g. septic arthritis) have been reported.

Mycobacterium ulcerans

This species was described by MacCallum *et al.* (1948). It grows slowly, and only at 32 °C and does not adapt to other temperatures in subcultures. Colonies at four to five weeks are about 1 mm in diameter, are colourless or faintly yellow, and smooth.

The bacilli are about 2 × 0.3 µm and stain evenly.

The outstanding characteristic of this organism is its inactivity in all the usual tests and identification is usually based on the clinical diagnosis and the failure to grow at temperatures other than 32 °C.

Although this species was originally identified in Australia, it is the causative organism of Buruli ulcer in east Africa and is found in other tropical regions. Its natural habitat is believed to be vegetation and the organism enters the human skin when it is pierced by the spines on certain plants.

Mycobacterium xenopi

This species was described by Schwabacher (1959) who isolated it from a lesion on a toad (*Xenopus laevis*) and named it '*M. xenopei*'. It was also known as '*M. littorale*' (Marks, 1964b; Runyon *et al.*, 1974) because isolation was mostly made in estuarine and coastal areas.

It grows slowly on primary cultures on egg medium. Colonies on egg media at 21 days are about 1 mm in diameter and smooth but an effuse growth is quite common. Continued incubation results in the formation of a pale, yellow pigment, which is more intense in cultures incubated at 44 °C. In these circumstances it is a scotochromogen.

The morphology is characteristic; the bacilli are long and thin, 4–6 × 0.3 µm, often filamentous and fusiform. They stain poorly and irregularly, sometimes giving a 'lacy' appearance. Some cells or parts of cells do not retain the fuchsin (*Figure 6.1*).

Mycobacterium xenopi is a thermophile, failing to grow at 25 °C but growing well at 44 °C. It is nitratase and catalase negative, gives a strongly positive sulphatase reaction but does not hydrolyse Tween 80 or reduce tellurite. It is resistant to thiacetazone (20 mg/litre). It is more sensitive to isoniazid than most other

mycobacteria (resistance ratio of 4) and its sensitivity to other antituberculosis drugs varies. Some strains are sensitive to amikacin and erythromycin by the disc method.

It is an opportunist pathogen in human lung disease but is rarely significant when found in other sites. It is a common contaminant of pathological material, especially in south-east England, where it occurs in piped water supplies; its thermophilic nature enables it to colonize hot water systems. Human infection may possibly arise from the inhalation of aerosols of tap water (see Collins, Grange and Yates, 1984). *Mycobacterium xenopi* is more common in Europe than in North America.

Species of limited interest in human and veterinary medicine

This list is not complete. The names are given here with pertinent references only.

Mycobacterium asiaticum Weiszfeiler, Karasseva and Kraznay, 1971.
Mycobacterium aurum Tsukamura, 1966b.
Mycobacterium chitae Tsukamura, 1966c.
Mycobacterium duvalii Stanford and Gunthorpe, 1971.
Mycobacterium farcinogenes Chamoiseau, 1979.
Mycobacterium gadium Casals and Calero, 1974.
Mycobacterium gilvum Stanford and Gunthorpe, 1971.
Mycobacterium lepraemurium Marchaux and Sorel, 1912.
Mycobacterium neoaurum Tsukamura, 1972.
Mycobacterium novum Tsukamura, 1967.
Mycobacterium parafortuitum Tsukamura, 1966a.
Mycobacterium rhodesiae Tsukamura, 1971.
Mycobacterium senegalense Chamoiseau, 1979.
Mycobacterium shimoidei Tsukamura, 1982.
Mycobacterium thermoresistibile Tsukamura, 1966b.
Mycobacterium vaccae Bönicke and Juhasz, 1964.

Chapter 10

Drug sensitivity tests

The theory of drug resistance and treatment regimens

For centuries man has sought a cure for tuberculosis. Many bizarre yet ineffectual remedies were introduced and few physicians were willing to admit that they were powerless to modify the progress of this dread disease. In 1782, one hundred years before Koch's discovery of the tubercle bacillus, Sir William Buchan wrote 'Though I do not remember to have seen one instance of a genuine consumption of the lungs cured by medicine, yet I have known a West Indian voyage work wonders in that dreadful disorder'. It is salutary to recall that until only a few decades ago there was still no remedy for the disease and its victims were often submitted to mutilating surgical procedures such as thoracoplasty, in which large sections of the chest wall were excised, in order to close tuberculous cavities.

The therapeutic breakthrough came in 1944 when Selman Waksman and his colleagues isolated the aminoglycoside antibiotic streptomycin from a strain of *Streptomyces griseus.* Two other antituberculous drugs, para-amino salicylic acid (PAS) and isonicotinic acid hydrazide (isoniazid, INH) were discovered in 1946 and 1952 respectively and these, together with streptomycin, formed the mainstay of the treatment of tuberculosis for the next two decades. Although these drugs revolutionized the treatment of the disease, they were not curative in the strict sense of the word. They merely reduced the bacterial load to a level that could be contained by the host's defence mechanism. It was not uncommon, therefore, for patients to relapse into overt disease after completion of their therapy or, on occasions, asymptomatically to excrete viable tubercle bacilli in their sputum.

As time went on other antituberculous drugs were found, namely, ethambutol, ethionamide, thiacetazone, kanamycin, viomycin, capreomycin, cycloserine, pyrazinamide and rifampicin. Of these, rifampicin (a semi-synthetic derivative of rifamycin, an antibiotic from *Streptomyces mediterranei*) is the most important. The inclusion of this drug in the antituberculosis armamentarium in 1969 brought about a second revolution in the treatment of this disease as it at last became possible to achieve true cures. Mitchison and Dickinson (1978) drew an important distinction between antituberculous drugs that are bactericidal *in vitro* and those that actually sterilize lesions *in vivo.* The former include isoniazid and streptomycin while the latter include rifampicin and pyrazinamide. The other drugs listed above are bacteriostatic and they are included in regimens to prevent the emergence of drug-resistant strains (see below).

Principles of treatment regimens

When considering the treatment of tuberculosis it is convenient to regard the bacilli as belonging to certain functional groups (Mitchison, 1980). First, there is a large group (about 10^7 to 10^9 cells) of metabolically active extracellular bacilli, mostly in liquefied caseous material and in the walls of cavities. Second, there is a smaller population (about 10^4 to 10^5 cells) of moderately active intracellular bacilli and, third, there is a small population of bacilli (also about 10^4 to 10^5 cells) that only show occasional bursts of activity. Some of these are intracellular and others are in the midst of solid caseous material. Streptomycin is ineffective at the low pH within macrophages and therefore acts only against the first group. Pyrazinamide, by contrast, is only active on the intracellular bacillary population at this low pH. Isoniazid is effective both inside and outside cells but, as it acts by inhibiting cell wall synthesis, it is only effective against bacilli that are actively growing. These three drugs can therefore cope with all tubercle bacilli save the minority that are virtually dormant. It is against this group that the action of rifampicin is particularly valuable as it can kill bacilli that undergo only short bursts of metabolic activity.

When the above drugs are given there is a very rapid killing of the bacilli in the first of the three bacterial populations. As this group is responsible for infectivity an important advantage of modern therapy is that the patient becomes, for practical purposes, non-infectious within a few days.

The other antituberculous agents contribute virtually nothing to the bactericidal activity of drug regimens. Nevertheless, by inducing bacteriostasis, they prevent the emergence of drug-resistant strains, particularly from the large metabolically active extracellular population. By analogy with infectious endocarditis, it may seem illogical to combine bacteriocidal and bacteriostatic drugs but, in practice, the presence of the latter does not appear to reduce the efficacy of the former in the treatment of tuberculosis.

Development of modern treatment regimens

In the three decades since its introduction, antituberculous chemotherapy has undergone enormous changes. It was originally believed that it was necessary to maintain the serum drug levels continuously above the minimal inhibitory concentration (MIC). The drugs were accordingly given four times a day which, over the usual two-year course of therapy, amounted to about 3000 doses. When it was realized that it was possible, and even preferable, not to maintain drug levels in excess of the MIC all of the time, doses were given daily but this still amounted to 750 doses over two years. The introduction of rifampicin heralded the era of short-course therapy. Thus, for example, the British Thoracic and Tuberculosis Association (1976) advocated a nine-month regimen in which isoniazid and rifampicin were given daily, with the addition of ethambutol for the first two months. This regimen, unlike those containing streptomycin, is an oral one and is virtually always curative. An even shorter regimen, of six months duration, is made possible by the inclusion of pyrazinamide, in addition to ethambutol, during the first two months. Furthermore, a number of intermittent short-course regimens have been evaluated, mainly for use in developing countries, and it is now possible

to achieve a very high cure rate with 64 doses given over a five-month period (for a review see Fox, 1981). These regimens are usually based on a short intensive phase with rifampicin, isoniazid, pyrazinamide and streptomycin, followed by a phase in which the first two drugs only are given intermittently. Apart from being much more desirable for the patient, short-course regimens have two special advantages. First, full supervision of the therapy is much easier and cheaper and, second, many patients are cured during the initial intensive phase of the therapy so that relapses do not occur in all those who abscond before completion of their treatment.

In general, most treatment regimens have been developed for pulmonary tuberculosis. The more limited studies on extrapulmonary disease indicate that standard regimens are equally applicable to tuberculosis of the urinary tract, bones and joints. The therapy of tuberculosis meningitis requires further evaluation and at present it would be unwise to use a short-course regimen. Tuberculous lymphadenitis appears to be more refractory to therapy than other forms of the disease and regimens should thus be continued for longer.

Problems faced in the therapy of tuberculosis

Two great problems have dogged the successful drug therapy of tuberculosis since its inception. The first of these has been the organizational and financial difficulties in ensuring that all patients receive a complete course of the most effective drugs currently available. The second problem has been that of the emergence of bacilli resistant to the drugs. Two forms of drug resistance are recognized: primary resistance, in which the patient is infected with a strain already resistant to one or more drugs, and acquired (or secondary) resistance which emerges during therapy. The identification of primary resistance may be unreliable owing to the failure of the patient or his records to mention previous antituberculous therapy. It is preferable therefore to refer to initial drug resistance when the first isolate from the patient is found to be resistant. Prior to the introduction of an antituberculous drug, strains of *M. tuberculosis* show a remarkably constant degree of sensitivity to it. Indeed few other bacteria, including other mycobacterial species, are as constant in their drug susceptibility as the tubercle bacillus. Unfortunately in every bacterial population mutation to drug resistance occurs at a low but constant rate. The natural incidence of mutation varies from drug to drug and, for example, occurs once in every 10^7 cell divisions for ethambutol, 10^8–10^9 divisions for isoniazid and once in every 10^{10} divisions for rifampicin (David, 1970; see also Gangadharam, 1984). Normally the number of mutants in the population remains very low as they are at no advantage over their sensitive counterparts. The addition of a drug to such a population does not increase the rate of mutation but, by destroying the sensitive population, it permits the small population of mutants to become dominant.

As a result of the natural occurrence of such mutants, the use of a single antituberculous drug is almost guaranteed to lead to the emergence of resistance to it. The chance, however, of two mutations occurring in the same bacterial cell is negligibly low; thus two drugs to which the strain is sensitive can be used in therapy. As an additional precaution a third drug, usually a bacteriostatic one, is often used but this may be safely dropped as soon as the results of reliable sensitivity testing are available.

The treatment of other mycobacterial infections

The therapy of infections caused by mycobacteria other than *M. tuberculosis* still causes much difficulty and the relative rarity of such infections has not permitted any large scale clinical trials. The evaluation of the efficacy of therapy is compromised by the fact that such infections vary considerably in their outcome according to the state of the host's relevant immune responses. The causative agents are often resistant to many antituberculous agents *in vitro* but experience has shown that the results of sensitivity testing cannot be used to predict the effect of the drugs *in vivo*. Uncomplicated pulmonary infections due to *M. kansasii, M. xenopi* and *M. avium-intracellulare* often resolve on an extended standard regimen, i.e. rifampicin, isoniazid and ethambutol, despite any *in vitro* resistance. Some workers however use regimens containing rifampicin with four or five other drugs – a regimen that is often poorly tolerated by the patient.

Recently antibiotics not normally used for treating tuberculosis have been found useful for treating many mycobacterial infections. These include cotrimoxazole, erythromycin, doxycycline and amikacin (for a brief review see Grange, 1984).

The role of sensitivity testing

Methods for sensitivity testing are not designed merely to detect drug resistant mutants as, for reasons discussed above, these will almost certainly be found. Instead, sensitivity testing is designed to show that the great majority of cells in a culture are as susceptible to a given drug as one or more known 'sensitive' strains. The object of sensitivity testing is therefore to determine whether an isolate is as likely to respond to standard therapy as one or more known 'sensitive' strains and the techniques used to attain this goal are described in the following section.

Sensitivity testing is of undoubted value in the evaluation of therapeutic regimens and the planning of widescale treatment. It is also of value in the surveillance of tuberculosis in order to detect breakdowns in good prescribing and management practice with its consequent emergence of drug resistance. There has, however, been considerable debate as to whether a sensitivity test is essential for the management of individual patients. In some countries, a fear of litigation leads to a regular use of the test and in other countries a high incidence of multiple resistance due to uncontrolled 'over the counter' sales of drugs renders 'blind' therapy very chancy indeed. On the other hand, in regions with a low level of resistance, usually restricted to a single agent, the need for routine testing is much more questionable. Studies in Hong Kong (Hong Kong Tuberculosis Treatment Services, 1974) showed that sensitivity reports were of little help to clinicians when the old regimen of streptomycin, isoniazid and PAS was used. They are, accordingly, likely to be of even less use with modern short-course therapy.

If sensitivity testing is undertaken, it is important that it be conducted to a high degree of accuracy. Much harm can be done by prescribing a more toxic yet less effective drug regimen on the basis of a false report of resistance or by trusting a false report of sensitivity rather than the patient's failure to respond to a regimen.

Methods for sensitivity tests

The high level of accuracy referred to above can be achieved only by experienced workers who appreciate the factors that affect sensitivity test results. These include

(1) Variations in the activity of different batches of the same drug, and how this activity is affected when the drug is incorporated into culture media, which may then be heated and stored.
(2) The range of concentrations of the drugs used and the size of inoculum.
(3) The relation between the *in vitro* and *in vivo* activity of the drug.
(4) The importance of using adequate controls.

For a detailed discussion on these problems see Gangadharam (1984).

In spite of attempts to standardize the potency of drugs there is often some variation between batches and between different manufacturers' products. It is best not to change supplier, and to do preliminary titrations on each new batch of drug and on each new working solution. Some drugs are bound by egg protein and the concentrations necessary in Löwenstein–Jensen medium may be much higher than those for Middlebrook media. Heating culture media containing drugs may reduce the potency of the latter; and storing the medium for any length of time, even in a refrigerator, may also reduce the activity of antibacterial agents.

The amount of available drug remaining in the medium also affects the range of concentrations used in the test. We have noted that the ranges offered by some commercial laboratories are too wide, even missing the MIC altogether. If an absolute concentration method is used (see below) with incorrect concentrations the MIC level reported may be unattainable in the patient's serum.

Adequate controls are perhaps the most important factor from a purely technical standpoint. They must include several known sensitive strains and sufficient numbers of test strains so that deviations from normal findings may be detected. For these reasons alone the tests are not normally done in clinical laboratories that isolate small numbers of mycobacteria, but in tuberculosis laboratories at the intermediate and central laboratory levels (ACP Extents 3 and 4; ATS Levels II and III. See Chapter 3).

Methods in current use

There are at least five of these:

(1) The Absolute Concentration method, which is popular in some parts of Europe, and which lends itself to automation (Meissner, 1964).
(2) The Proportion method, also used in Europe and popular in the USA (Canetti, Rist and Grosset, 1963; Canetti *et al.*, 1969; Vestal, 1975).
(3) The Resistance Ratio method, used in the UK and those countries that are influenced by British bacteriologists (Marks, 1961; Leat and Marks, 1970; Collins and Lyne, 1984).
(4) The Disc Diffusion method, which is a modification of that used in clinical laboratories (Wayne and Krasnow, 1966).
(5) The Radiometric method which measures the metabolic activity of mycobacteria in the presence of antituberculosis drugs (Vincke *et al.*, 1982).

We prefer the Resistance Radio method because it uses media in screw-capped tubes whereas Petri dishes are usually employed in the Proportion method and are used by some workers for the Absolute Concentration method. This seems to offer an unnecessary risk of dispersing infectious aerosols (see p. 27). There is no reason, however, why, these tests should not be done in tubes.

It is of interest that in a recent (unpublished) investigation carried out by members of the European Society of Mycobacteriologists, there were only minor discrepancies between the results obtained by different workers, using the first three of these methods.

All five methods may be used for direct sensitivity tests on smear positive sputum and indirect sensitivity tests on cultures. The preparation of stock solutions of antituberculous drugs, dilutions of positive sputum homogenates and suspensions of bacilli from cultures, and the choice of control strains are the same for each method.

For a full discussion on the relative merits and usefulness of these methods in differing circumstances, see Gangadharam (1984).

Stock drug solutions

These are conveniently prepared as 1 per cent solutions in deionized water, except for rifampicin, which must be dissolved in formdimethylamide. These solutions are stable for several months at −4 °C but the aqueous ones should not be thawed and refrozen too often. It is best to distribute the stocks in smaller amounts for use as required.

Smear positive sputum homogenates for direct tests

These are prepared as described on pp. 45–47 and are diluted in sterile water. It is best to use two dilutions, 100-fold apart, according to the number of bacilli seen per high power field in the direct smear:

Acid-fast bacilli per field in concentrate	*Dilution of concentrate to use*
<1	undil. and 10^{-2}
1–10	10^{-1} and 10^{-3}
>10	10^{-2} and 10^{-4}

Bacterial suspensions for indirect tests

There are two methods:

(1) The organisms are grown in Middlebrook Tween 80 broth for ten days when there will be about 10^8 cfu/ml. This is diluted 10^{-3} and 10^{-5} to give about 10^5 and 10^3 cfu/ml. The highest dilution is used for the Absolute Concentration and Resistance Ratio methods and both for the Proportion method.

(2) Two or three colonies, each about 1 mm diameter, or an equivalent amount of growth, are taken from the primary culture and emulsified in 1 ml of phosphate buffer in 'suspension bottles' (Appendix) on a magnetic stirrer. After standing for 5 minutes to allow larger clumps to settle, the supernatant fluid contains about 10^5 cfu/ml and is satisfactory for the Resistance Ratio method.

Control strains

At least three freshly isolated and known sensitive strains should be included in each batch of tests. The stock H37Rv strain may be used as well, but should not be used alone, especially for the Resistance Ratio method (Marks, 1961) as its susceptibility to some of the drugs used does not parallel that of 'wild' strains.

The Absolute Concentration method

This is essentially the determination of the minimum inhibitory concentration (MIC) of each drug for the test organisms.

Media

The concentrations of drug in the medium must be exact, and therefore the media must not be heated after the drugs are added. This excludes egg media and the medium normally used is Middlebrook's 7H10. It is best to use solid rather than broth media because cleaner end-points are obtained. A very small number of resistant mutants would give only one or two colonies on agar, but cause turbidity in broth.

The concentrations used are shown in *Table 10.1* and the appropriate amount of drug is added to the melted medium at 50 °C before it is distributed into Petri dishes or tubes for sloping.

TABLE 10.1. Final concentrations of drugs (mg/litre) in 7H10 agar for the Absolute Concentration and Proportion methods of sensitivity testing

	Absolute Concentration method				*1% Proportion method*		
Isoniazid	0.2	0.4	0.8	1.6	0.2	1.0	5.0
Rifampicin	1.0	2.0	4.0	8.0	1.0	5.0	10.0
Ethambutol	2.5	5.0	10.0	20.0	2.5	7.5	15.0
Streptomycin	2.0	4.0	8.0	16.0	2.0	5.0	10.0

Inoculation and incubation

Each concentration of each drug is inoculated with 10 μl of the diluted homogenate or bacterial suspension using a standard loop or automatic pipette. Each strain should be tested at least in duplicate. Four strains may be placed on one Petri dish (Felsen quadrant dishes are convenient). The inoculum is allowed to dry or be absorbed by the agar by leaving the slopes or Petri dishes in a horizontal position. Tubes may then be incubated vertically. Petri dishes should be sealed and placed in plastic bags for incubation at 37 °C for 18–21 days.

Reading and reporting

MICs are read as the lowest concentration that completely inhibits growth or permits only two or three colonies to grow. Petri dishes should not be opened but examined through their bases. The results should always be compared with those

obtained with known sensitive controls. A strain may be reported as sensitive if it does not grow at the lowest concentration of the drug shown in *Table 10.1*.

The Proportion method

In principle the number of colonies growing on drug-free medium is compared with the number on drug-containing medium and the proportion of resistant organisms is calculated.

Media

Middlebrook 7H10 agar is used, containing the concentrations of drugs shown in *Table 10.1*. It is poured into Petri dishes or tubed. Felsen (quadrant) plates may be used: one quadrant contains drug-free medium; the other three each contain a different drug-containing medium.

Inoculation and incubation

Each quadrant or tube is inoculated with three discrete drops of 0.15 ml of the diluted homogenate or suspension. The cultures are kept level until the inoculum has dried or been absorbed. They are then incubated at 37 °C with Petri dish cultures sealed and in plastic bags, for 18–21 days.

Reading and reporting

The cultures are examined under strong light, with a low-power lens if necessary, and the colonies on each quadrant or tube counted. Petri dish cultures should be examined unopened by viewing through their bases. The criterion for resistance is that the number of colonies on the drug-containing medium is 1 per cent or more of the number developing on the drug-free medium. In practice, with the concentrations given in *Table 10.1* there will be either no growth (sensitive) or good growth (resistant) on the drug-containing medium.

The Resistance Ratio method

In this method the MIC of a test strain is compared with the modal average results of several control strains using the same batch of medium. Within reason, therefore, the exact amount of drug in the medium is not important and egg medium, inspissated after the addition of the drugs, may be used.

Media

Löwenstein–Jensen (LJG) medium is prepared as described in the Appendix and the drugs are added in the concentrations shown in *Table 10.2*. Each laboratory should experiment with concentrations around these levels and select six doubling concentrations. The lowest two of these should give growth and the other four no growth on tests with a batch of about 12 known sensitive strains. With experience only the three highest concentrations may be used for the tests strains, but all five are necessary for the controls.

The medium, including a drug-free batch, is tubed in 2.5 ml amounts in screw-capped bottles. Each batch is placed in an inspissator fitted with a fan and which has already been preheated to 80–87 °C. After exactly 50 minutes the hot

TABLE 10.2. Final concentrations of drugs (mg/litre)* in Lowenstein–Jensen medium for the Resistance Ratio method

Isoniazid	0.007	0.015	0.03	0.06	0.125	0.25	0.5
Rifampicin	0.78	1.56	3.12	6.25	12.5	25.0	50.0
Ethambutol	0.07	0.015	0.31	0.62	1.25	2.5	5.0
Streptomycin	0.78	1.56	3.12	6.25	12.5	25.0	50.0

* Choose six that give near confluent growth on the first two and no growth on the other four.

tubes are removed and allowed to cool. We have found that such media may be stored in a refrigerator for up to 21 days without deterioration, but we do not recommend keeping it any longer.

Inoculation and incubation

One tube of drug-free medium and one set of each drug-containing medium are inoculated with 10 µl of the suspension prepared as described above. It is convenient to use automatic pipettes (MicroRepettes) with plastic disposable tips (*Figure 3.5*). A new tip is used for each strain; used tips are discarded into glutaraldehyde and may be autoclaved, washed and re-used.

The cultures are incubated at 37 °C for 18–21 days.

Reading and reporting

The control strains are read first. Growth is recorded as CG (confluent), IC (innumerable discrete colonies), + (20–100 colonies) and 0 (0–20). *Table 10.3* shows how the Modal MIC is calculated.

TABLE 10.3. Calculating the Modal Resistance of five control strains for the Resistance Ratio method

	Drug concentration (tube number)					
	1	*2*	*3*	*4*	*5*	*6*
Strain A	CG	CG	0	0	0	0
B	CG	IC	0	0	0	0
C	CG	CG	+	0	0	0
D	IC	IC	0	0	0	0
E	CG	CG	+	0	0	0
Modal resistance	CG	CG	0	0	0	0

CG, confluent growth; IC, innumerable discreet colonies; + 20–100 colonies; 0, fewer than 20 colonies

The test strains are then read in the same way. The Resistance Ratio of each test strain to each drug is calculated by dividing the MIC of the test by the Modal MIC, as shown in *Table 10.4.* As doubling dilutions are used the Resistance Ratio is 1 (or less), 2, 4 or 8. Strains giving a Resistance Ratio of 1 or 2 are reported as Sensitive; those giving a Resistance Ratio of 4 as Resistant; and those giving a Resistance Ratio of 8 as Highly resistant.

'Mixed Sensitive and Resistant' strains are sometimes encountered, as are 'Borderline' strains. These should be interpreted with caution and the tests are best repeated. We confirm all Resistant and Highly resistant findings on new patients by repeating the tests.

TABLE 10.4. Interpretation of the Resistance Ratio method for drug sensitivity tests

	Drug concentration (tube number)							
	1	*2*	*3*	*4*	*5*	*6*	*Resistance ratio*	*Interpretation*
Modal resistance	CG	CG	0	0	0	0		
Strain no. 1	CG	0	0	0	0	0	0.5	Sensitive
2	CG	CG	0	0	0	0	1	Sensitive
3	CG	CG	+	0	0	0	1	Sensitive
4	CG	CG	CG	0	0	0	2	Sensitive
5	CG	CG	CG	+	0	0	3	Borderline
6	CG	CG	CG	IC	0	0	4	Resistant
7	CG	CG	CG	CG	CG	0	8	Highly resistant
8	CG	CG	CG	CG	CG	CG	8+	Highly resistant
9	CG	CG	CG	+	+	+	–	Mixed sensitive and resistant

CG, confluent growth; IC, innumerable discreet colonies; +, 20–100 colonies; 0, less than 20 colonies
For practical purposes CG and IC may be regarded as equal

The Disc method

This method was introduced by Collins (1955, 1956) for screening strains of tubercle bacilli that were resistant to streptomycin and isoniazid. Paper discs containing the drugs were placed on LJG slopes that had been inoculated with test and control strains of the organisms. Some discordant results were obtained due to diffusion, with consequent dilution of the drugs from the discs before the organisms began to grow. Wayne and Krasnow (1966) overcame this problem by making the drug content of the disc five times greater than that finally required in the medium. One disc is placed in each quadrant of a Felsen plate and 5 ml of 7H11 medium poured over it. The quadrants are inoculated after the medium has dried. Recommended concentrations of drugs in the discs are shown in *Table 10.5*.

The usefulness of this method for field work was confirmed by Griffith *et al.* (1971).

The Radiometric Method

The BACTEC instrument (p. 53) may be used to obtain rapid results (Laszlo *et al.*, 1983).

TABLE 10.5. Disc contents and final concentrations for the Disc method[a]

	Amount per disc (μg)	*Final concentration[b]*
Isoniazid	1 and 5	0.2 and 1.0
Rifampicin	5	1.0
Ethambutol	25 and 50	5 and 10
Streptomycin	10 and 50	2 and 10

The discs may be obtained from the BBL and the quadrant plates from the Bioquest (Falcon) Divisions of Becton Dickinson and Company, Cockeysville, MD 21030, USA

[a] After Wayne and Krasnow, 1966 and Kubica *et al.*, 1975
[b] mg/litre in 5 ml of medium in quadrants of Felsen plates

Appropriate amounts of antituberculosis drugs, followed by the inoculum, are added to the media vials with a hypodermic syringe and needle (at the risk of 'needle-stick' accidents).

The method gives reasonably reliable results within one week, but the equipment and materials are expensive, even for developed countries. Such rapid results are rarely required by physicians in the UK, who commence treatment on clinical, rather than bacteriological, evidence. The method does offer a distinct advantage, however, in those developed countries where physicians await the bacteriological findings before prescribing, and where the cost is borne by the patients.

Pyrazinamide sensitivity tests

Pyrazinamide acts upon tubercle bacilli in the macrophages which have an acid pH. For reliable sensitivity tests, therefore, the medium should also be acid and a pH of 5.2 will support growth of tubercle bacilli and allow the drug to act. Even so, tubercle bacilli grow poorly on egg medium at pH 5.2 and although Middlebrook media may be used instead of egg medium the results are sometimes difficult to interpret.

Marks (1964a) introduced a 'stepped pH' method. This uses three pH levels in an enriched egg medium and one pH level in a semisolid medium overlaid on egg medium. Only one concentration of pyrazinamide is used and all tubes receive the same inoculum. Growth is compared with that in control tubes containing no pyrazinamide.

We have had good and consistent results with a modification of this method (Yates, 1984) which uses LJG medium overlaid with Kirchner medium, only one pH level, 5.2, but two concentrations of inoculum.

Medium

The method for preparing this is described in the Appendix. Four tubes are used for each test: two are drug-free and the other two contain pyrazinamide.

Inoculation and incubation

One tube of each is inoculated with 20 μl of the suspension used for the Resistance Ratio method. The other pair receive the same amount of a 1:10 dilution of this suspension.

Reading and reporting

Colonies of tubercle bacilli should be distributed evenly throughout the semisolid medium in one or both of the control (drug-free) tubes.

Pyrazinamide sensitive strains give growth in the drug-free control tubes only. Resistant strains give growth in all four tubes but if there is growth in the pyrazinamide tube that received the large inoculum, but not in that which received the small inoculum, the strain is reported as sensitive.

NB The 'classical human' and 'Asian' variants of *M. tuberculosis*, and the 'African' variants (*M. africanum*) isolated from untreated patients are almost always sensitive. The 'bovine' variant (*M. bovis*), BCG and all other mycobacteria found in clinical material are naturally resistant.

Chapter 11

Animal inoculation and assay tests

Although these two procedures are rarely required in tuberculosis laboratories it is advisable for the laboratory worker to be aware of the problems involved and to have some knowledge of the techniques. He is then able to give advice on their value and cost effectiveness.

Animal inoculation tests

In many countries there are regulations governing the use of experimental animals. In the UK a scientist who wishes to use animals for diagnostic or experimental purposes must obtain a licence from the Home Office. The Advisory Committee on Dangerous Pathogens (1984) has made recommendations on the levels of containment necessary for animal experiments with organisms such as tubercle bacilli.

For diagnosis

The animal inoculation test is '. . . no longer a current method in the diagnosis of tuberculosis, thanks to progress in culture methods and due to the difficulty and cost of the maintenance of an animal house and, lastly, to the risk encountered with isoniazid-resistant bacilli which may lose their virulence for the guinea pig'. (Grosset and Truffot-Pernot, 1982.)

As long ago as 1928 Corper and Uyei advocated a change from guinea pig inoculation to culture. The superiority of the culture method was further demonstrated by Norton, Thomas and Broom (1932). Nevertheless, it was not until 1972, when Marks reported that 2000 animal tests had added only one useful positive result to those obtained by culture that bacteriologists were in a position to decline to do such tests with sputum and to relieve themselves of the financial burden of animal houses.

Guinea pig inoculation is justified, however, on very rare occasions and for certain specimens, such as non-repeatable material contaminated with *Pseudomonas aeruginosa* (an organism notoriously difficult to kill by the usual methods of decontamination), and small amounts of spinal fluid and uterine curettings, in which the bacilli may be very scanty. The technique is therefore described briefly

here. See, however, our comments on the radiometric method for detecting tubercle bacilli (p. 54).

Material known to contain other organisms is treated by one of the methods that yields a centrifuged deposit with a neutral pH (pp. 45–46). Uncontaminated specimens, such as spinal fluid or tissues ground to a fine paste in a Ten Broeck homogenizer are used without treatment.

If possible, two guinea pigs are used. Approximately 0.5 ml of the material is inoculated intramuscularly into the thigh of each. The inoculated areas are palpated weekly. If local lesions or enlarged inguinal nodes are detected the animals are killed and autopsied in a microbiological safety cabinet (see p. 14). The local lesions, enlarged lymph nodes and any tubercles in the viscera are removed, examined microscopically and cultured for tubercle bacilli.

If no local abscesses develop after six to eight weeks the animals are killed and autopsied.

For 'typing' tubercle bacilli

Both guinea pigs and rabbits were inoculated to determine if the organism was of the human or the bovine type. Progressive disease in both indicated the latter: progressive disease in only the guinea pig indicated the former. Easier and more reliable cultural methods are now available (Chapter 8).

Similarly, chickens were used to identify the 'avian tubercle bacillus', but this procedure is now rarely used.

Assay of antimicrobial agents in serum and their detection in urine

It is only very rarely necessary to determine serum levels of antituberculous agents. Most of the important agents – isoniazid, rifampicin, pyrazinamide and ethionamide – are destroyed by metabolism or eliminated by biliary excretion. These agents may, therefore, be used safely in patients with renal failure. Streptomycin and ethambutol, on the other hand, are almost entirely eliminated by the kidneys. There are several rapid assays for streptomycin: these include high pressure liquid chromatography, radioimmunoassay and enzyme immunoassays. Standard microbiological assays are, however, usually adequate although it is important to ensure that the test organism is resistant to other antibacterial agents that the patient is receiving. For details of these assays see Collins and Lyne (1984). An accumulation of ethambutol can lead to the serious complication of optic neuritis. Unfortunately the assay of this agent is a complicated one requiring gas–liquid chromatography and is not readily available. Ethambutol is therefore best avoided in cases of renal impairment.

The rate of elimination of isoniazid from the body depends on the rapidity of its acetylation in the liver. This metabolic activity is genetically controlled and individuals are divisible into slow and rapid acetylators (Ellard, 1984). This, however, does not affect the therapeutic efficacy of isoniazid if used daily, thrice weekly or twice weekly. The half-life is only increased by up to 30 per cent in almost complete renal failure, even in slow acetylators. There is, therefore, no need to determine either the acetylator status of the patients or their serum isoniazid levels.

'Spot tests' for antituberculous or antileprotic drugs or their metabolites in the urine have been used to check on patient compliance. Such tests are, however, no substitute for good supervision of the administration of the drugs.

Urine test for isoniazid (Ellard and Greenfield, 1977)

This technique gives a positive result 24 hours or more after the ingestion of 100 mg of isoniazid.

Urine, 0.5 ml (preserved, if necessary, by the addition of a crystal of thymol) is acidified by the addition of 0.2 ml of 4 M sodium acetate/acetic acid buffer pH 5.

The following freshly prepared solutions are added at 15 second intervals:

0.1 ml of 10 per cent aqueous potassium cyanide
0.1 ml of 10 per cent aqueous chloramine T
0.5 ml of 1 per cent barbituric acid in acetone/water 1:1.

A positive reaction is indicated by the development of a blue colour within 30 minutes. Test strips may be obtained from Difco.

Urine test for rifampicin (Burkhardt and Nel, 1980)

This test is based on the red colour of rifampicin and its metabolite desacetylrifampicin. N-butanol, 2 ml, is added to 10 ml of urine and mixed gently by inverting the tube twice. The tube is placed upright to allow the butanol to form a separate upper layer and the presence of the drug is indicated by a pink or red coloration of this layer. An excess of bile in the urine can cause a similar coloration.

Urine test for dapsone (Jopling, 1978)

This method is sensitive to 0.1 mg of dapsone in 100 ml of urine. (The urine samples may be preserved, if necessary, by the addition of 1 part of N hydrochloric acid to 50 parts of urine.)

N sodium hydroxide, 0.5 ml, and 2 ml of ethyl acetate are added to 10 ml of urine and gently mixed for 1 minute. After standing, the upper ethyl acetate layer is removed and 0.2 ml of Ehrlich's reagent (1 g *p*-dimethylaminobenzaldehyde; 20 ml N hydrochloric acid; 80 ml ethanol) is added. A positive result is indicated by the immediate development of a yellow colour or precipitate.

Chapter 12

Leprosy

Mycobacterium leprae, unlike other mycobacteria that cause disease in man, is not isolated or identified in the tuberculosis laboratory as it has never been convincingly cultured *in vitro*. Nevertheless, it is possible to obtain a limited growth of the bacillus in mouse footpads. By incorporating various antileprosy drugs in the diet of such mice, sensitivity testing may be performed. Such investigations, however, lie outside the province of all but a few research or reference centres. Routine bacteriological studies on leprosy are, of necessity, confined to a microscopical detection of acid-fast bacilli in skin, nasal mucosa and nasal discharges. Owing to technical similarities, such investigations are well suited to the tuberculosis laboratory. Accordingly, this disease and the technical methods for the demonstration of the causative organism are discussed here.

Leprosy is, after tuberculosis, the most prevalent mycobacterial infection. It is estimated by the World Health Organization that there are, at present, about 11 million sufferers of the disease throughout the world, though many regard this as being a conservative estimate. As the causative organism, *M. leprae* has so far stubbornly resisted all attempts to cultivate it *in vitro*, laboratory examinations in this disease are usually confined to the examination of smears of tissue, fluid or nasal scrapings for the presence of acid-fast bacilli. Some laboratories also perform histological examinations of tissue biopsies in order to classify patients according to the 'spectrum' of immune responses, as described below.

The nine-banded armadillo is one of the very few animals, apart from man, in which *M. leprae* multiplies well. Although far too expensive to use for diagnostic purposes, this animal has been used to supply large numbers of bacilli for the development of skin-testing reagents and for use in various research projects.

Leprosy is principally a disease of skin and superficial nerves but it affects the nose, from which numerous bacilli are shed, and it is probably these bacilli that are the source of new infections. Leprosy occurs in several forms which have been classified according to a so-called 'spectrum' of immune responses (Ridley and Jopling, 1966). At one end of the spectrum – tuberculoid leprosy – there is a high degree of immunological activity with the formation of tuberculosis-like granulomas containing very few bacilli and the response to therapy is good. At the other extreme – lepromatous leprosy – the relevant immune reactions are suppressed, the lesions are teeming with bacilli and the response to therapy is poor. Between these two polar forms there is a wide range of intermediate forms that, for

convenience, are divided into borderline tuberculoid, mid-borderline and borderline lepromatous. As one approaches the lepromatous end of the spectrum the number of bacilli seen in the lesions, and the infectivity of the patients, increase.

Leprosy is complicated by the occurrence of the two types of tissue-damaging reactions known as Jopling types I and II. The first of these – *erythema nodosum leprosum* (ENL) – occurs in patients at or near to the lepromatous pole of the spectrum and is almost certainly due to complexes formed between antibody and antigens liberated by the bacilli. It often, therefore, occurs shortly after the commencement of chemotherapy. The second reaction is a delayed hypersensitivity type and is said to indicate that the type of disease is shifting towards the tuberculoid pole of the spectrum. This reaction is accordingly termed a *reversal* or *upgrading* reaction and it often leads to rapid and severe nerve damage with crippling sequelae.

Technical procedures

As outlined above, there is a spectrum of leprosy ranging from the immunologically active tuberculoid form with very few bacilli through various intermediate or borderline stages to the anergic lepromatous form with numerous bacilli in the skin and other tissues. It is therefore only possible to detect bacilli in the skin by microscopic examination in cases that are towards the lepromatous end of the spectrum. In such cases, numerous bacilli are also found in the nasal mucosa and in nasal discharges. It is likely that the latter are responsible for transmission of the disease, thus for practical purposes patients with solid-staining bacilli in their nasal discharge must be regarded as being infectious. In new cases it is unusual to find bacilli in the nose if they are not also seen in the skin but they may reappear in the nose before the skin in cases of relapse (Browne, 1966).

The skin

Patients with suspected leprosy are examined by the 'slit skin smear' technique, in which cells and tissue fluid from the dermis are obtained from small superficial cuts. The sites examined usually include both earlobes, at least two lesions and, in the case of suspected lepromatous leprosy, two areas of apparently unaffected skin.

The skin site is cleaned with surgical spirit or other volatile antiseptic and allowed to dry. A fold of skin is then pinched up between thumb and forefinger and a small incision is made into the dermis with a sharp scalpel blade. The incision should be about 5 mm long and 2 mm deep. This procedure takes some practice as the cut must be deep enough to reach the dermis, where the bacilli lie, but not deep enough to draw blood. The tip of the scalpel is then turned through 90 degrees and used to scrape a small amount of fluid and cells from the edges of the cut. This fluid is then smeared fairly thickly on a glass slide, allowed to dry and fixed by passing the slide through a flame.

The slide is stained for acid-fast bacilli by the Ziehl–Neelsen method or by a fluorescent technique if the appropriate microscope is available. The smear is then examined with a 2 mm oil-immersion objective (×100) and the number of bacilli is

recorded. This number is expressed as the Bacillary Index (BI) which has six points, each corresponding to a tenfold increase in the number of bacilli, i.e.:

1+ 1–10 bacilli in 100 microscopic fields.
2+ 1–10 bacilli in ten fields.
3+ 1–10 bacilli in one field.
4+ 10–100 bacilli in one field.
5+ 100–1000 bacilli in one field.
6+ >1000 bacilli in clumps one field.

It is a widely accepted belief that viable leprosy bacilli stain strongly and evenly while dead bacilli stain weakly and irregularly. The percentage of bacilli of the former type in the smear gives the Morphological Index (MI) which is used as a clinical guide to the viability of the bacilli and of the efficacy of therapy. In lepromatous leprosy the MI should drop from between 25 and 80 to zero within five months of commencing treatment with dapsone but within five weeks of therapy with rifampicin. Not all authorities accept that the correlation between bacillary viability and staining is a close one. More accurate tests of viability based on the detection of bacterial enzyme activity do not correlate with the MI (Kvach *et al.*, 1984). Nevertheless there is, at present, no evidence that such tests provide more useful information than the MI in routine clinical practice.

The slit skin smear examination is repeated at intervals during treatment and gives an indication of the efficacy of chemotherapy. An increase in the Morphological Index may give early warning of non-compliance or of the emergence of drug resistance.

The nose

Nasal discharges are examined for bacilli simply by staining and examining smears as though they were sputa. The mucosa is examined by scraping the nasal septum or the lower turbinate with a small curette (Downs Surgical Limited: see Jopling, 1978). Alternatively a simple disposable scraper can be made by straightening a paperclip and hammering one end flat. The scraping is spread on a slide for staining.

Biopsies

When no bacilli are detectable and when the diagnosis remains in doubt a biopsy of a representative lesion is indicated; for which purpose an ellipse of skin, of about 12 × 3 mm and including the full thickness of the dermis, is removed under local anaesthetic. Punch biopsies are less satisfactory. The biopsy is then placed in a fixative, preferably one containing mercuric chloride such as Ridley's solution (see Appendix) for 15 to 24 hours after which it is transferred, without rinsing, to 70 per cent alcohol.

The examination of skin biopsies requires considerable experience, judgement and skill and lies outside the scope of this book. For full details see Ridley (1977).

Appendix

Stains, culture media and other materials

Stains

Ziehl–Neelsen (ZN) stain

(1)	Basic fuchsin	5.0 g
	Phenol crystals	25.0 g
	95% ethanol	50.0 ml
	Distilled water	500.0 ml

Dissolve the fuchsin and the phenol in the alcohol over a warm water-bath, then add the water. Filter before use.

(2)	85% ethanol	970.0 ml
	Conc. hydrochloric acid	30.0 ml
(3)	Methylene blue	2.5 g or
	Malachite green	2.5 g or
	Picric acid	3.5 g
	Distilled water	500.0 ml

Tan Thiam Hok stain

Kinyoun's cold stain and Gabbett's solution are used.

(1)	Basic fuchsin	20.0 g
	Phenol crystals	40.0 g
	95% ethanol	100.0 ml
	Distilled water	500.0 ml

Dissolve as for the ZN stain

(2)	Methylene blue	5.0 g
	95% ethanol	150.0 ml
	Distilled water	250.0 ml
	96% sulphuric acid	100.0 ml

Dissolve the methylene blue in the alcohol, add the water and then the sulphuric acid, carefully and slowly.

Fluorescence stain

(1)	Auramine	1.5 g
	Phenol crystals	15.0 g
	Distilled water	500.0 ml
(2)	Sodium chloride	3.0 g
	Conc. hydrochloric acid	3.0 ml
	95% ethanol	450.0 ml
(3)	Potassium permanganate	0.5 g
	Distilled water	500.0 ml

Culture media

Media marked with an asterisk (*) are available commercially. Not all manufacturers supply all media and their lists and catalogues should be consulted.

Löwenstein–Jensen medium*

Potato starch and asparagine are omitted from this formula: they are unnecessary. LJG contains glycerol; LJP contains pyruvate.

Monopotassium phosphate, anhydrous	4.0 g
Magnesium sulphate.7H_2O	0.4 g
Magnesium citrate	1.0 g
Glycerol (for LJG only)	20.0 ml
Sodium pyruvate (for LJP only)	12.0 g
Distilled water	to 1000.0 ml

Dissolve in this order in a 2000 ml bottle and steam for 2 hours. This is the mineral salt solution.

Clean fresh eggs with soap and water, rinse them in 70 per cent alcohol (methylated spirit) and break them into a graduated cylinder until 1600 ml are obtained. Transfer to a large screw-capped jar containing a few glass beads (about 10 mm diameter). Shake to break yolk and to homogenize fluid. Filter through sterile cotton gauze to remove any shell fragments and add to 1000 ml of mineral salt solution. Add 50 ml of 1 per cent aqueous malachite green and 200 000 units of penicillin and mix well. Tube in 7 ml amounts in 25 ml screw-capped bottles for primary culture, or in 2.5 ml amounts in 7 ml bottles for identification tests, etc. Slope and inspissate at 80 °C for 50 minutes.

IUT medium

(International Union against Tuberculosis, 1955)

Asparagine	3.60 g
Potassium dihydrogen phosphate	2.40 g
Magnesium citrate	0.60 g
Magnesium sulphate	0.24 g
Malachite green	0.40 g
Distilled water	600.00 ml

Dissolve and add 1 litre of homogenized egg fluid prepared as for Lowenstein–Jensen medium, tube and inspissate.

ATS medium

(American Thoracic Society, 1974)

Potatoes	140 g
Glycerol, 2% aqueous	335 ml
Malachite green, 2% aqueous	10 ml

Clean and chop the potatoes, autoclave them with the glycerol solution and when cool add the malachite green. Break three whole eggs, add to them egg yolk to give a total volume of 400 ml. Homogenize as for Lowenstein–Jensen medium and add to the potato–glycerol mixture. Tube and inspissate.

Ogawa medium

(Ogawa and Sanami, 1949)

Dipotassium hydrogen phosphate	2 g
Sodium glutamate	2 g
Glycerol	6 ml
Distilled water	400 ml

Dissolve and add 800 ml of whole egg emulsion prepared as for Lowenstein–Jensen medium. Tube and inspissate.

Kirchner medium

Disodium *o*-phosphate.$12H_2O$	19.0 g
Monopotassium phosphate, anhydrous	2.5 g
Magnesium sulphate. $7H_2O$	0.6 g
Trisodium citrate	2.5 g
Asparagine	5.0 g
Glycerol	20.0 ml
Phenol red, 0.4% aqueous	3.0 ml
Distilled water	to 1000.0 ml

Steam to dissolve. The pH should be 7.4–7.6. Dispense in 9 ml amounts in 25 ml screw-capped bottles and autoclave at 115 °C for 10 minutes. When cool, add 1 ml of horse serum to each bottle For the Mitchison *et al.* (1983) 'cocktail' add (per litre) polymixin B, 200 000 units; carbenicillin, 50 mg; trimethoprim, 10 mg; ampicillin, 10 mg. Incubate to test for sterility.

Acid egg medium*

(Zaher and Marks, 1977)

For highly alkaline uncentrifuged inocula

Monopotassium phosphate	6.3 g
Magnesium sulphate. $7H_2O$	0.3 g
Glycerol	12.0 ml
or Sodium pyruvate	7.0 g
Distilled water	600.0 ml

Dissolve and autoclave at 115 °C for 10 minutes.

Add:

Egg homogenate, prepared as for LJ medium	1100.0 ml
N Hydrochloric acid	32.0 ml
Malachite green, 2% aqueous	11.0 ml
Penicillin, sodium salt	100 000.0 units

Tube and inspissate at 80 °C for 50 minutes.

Middlebrook 7H9 broth*

Base

Ammonium sulphate	0.5 g
L-glutamic acid (sodium salt)	0.5 g
Trisodium citrate.$2H_2O$	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 sulphate.$7H_2O$	0.05 g
Calcium chloride	0.0005 g
Zinc sulphate.$7H_2O$	0.001 g
Copper sulphate.$5H_2O$	0.001 g
Tween 80	0.5 ml
Distilled water	to 900.0 ml

Steam to dissolve. Distribute in 180 ml amounts and autoclave at 115 °C for 15 minutes.

For use add 20 ml of Middlebrook's OADC enrichment (see below) and tube.

Middlebrook 7H10 agar*

Five separate solutions and OADC supplement (p. 108) are required.

(1) Monopotassium phosphate	15.0 g
Disodium phosphate	15.0 g
Distilled water	to 250.0 ml

Store at room temperature

(2) Ammonium sulphate	5.0 g
Monosodium glutamate	5.0 g
Trisodium citrate.$2H_2O$	4.0 g
Ferric ammonium citrate, green scales	0.4 g
Magnesium sulphate.$7H_2O$	0.5 g
Biotin (dissolved in 2 ml of 10% ammonia)	0.0005 g
Distilled water	to 250.0 ml

Store at 4 °C.

(3) Calcium chloride.$2H_2O$	0.05 g
Zinc sulphate.$7H_2O$	0.1 g
Copper sulphate.$5H_2O$	0.1 g
Pyridoxine HCl	0.1 g
Distilled water	to 250.0 ml

Store at 4 °C.

(4) Glycerol.

(5) Malachite green 0.01% aqueous solution.

Preparation

Distilled water	975.0 ml
Solution 1	25.0 ml
Solution 2	25.0 ml
Solution 3	1.0 ml
Solution 4	5.0 ml
Adjust to pH 6.6 with 6 N.HCl (approx. 0.5 ml) and add	
Solution 5	2.5 ml
Agar	15.0 g

Steam to dissolve the agar, bottle in 180 ml amounts and autoclave at 115 °C for 15 minutes. Store in the dark. For use, melt 180 ml, add 20 ml Middlebrook's OADC enrichment* and tube or pour plates.

Store tubes or plates at 4 °C in the dark and use within 14 days.

Middlebrook OADC enrichment*

(Oleic acid-albumen-dextrose-catalase)

Bovine albumin, fraction V	50.00 g
Sodium chloride	8.08 g
Glucose	20.00 g
Citric acid, 10% aqueous	0.4 ml
Sodium hydroxide 0.1 N	25.00 ml
Oleic acid	0.6 ml
Catalase (crude)	0.02 ml
Distilled water	975.00 ml

Dissolve and sterilize by membrane filtration. Bottle in 20 ml amounts. Incubate to test for sterility. Store at 4 °C.

Oxygen preference medium

Add 0.1 per cent pure agar to Middlebrook 7H9 broth or Kirchner medium. Autoclave at 115 °C for 10 minutes and distribute in 12 ml amounts in narrow screw-capped bottles. Before use add 0.5 ml OADC supplement to each bottle.

LJG-*p*-nitrobenzoic acid medium (PNB)

Dissolve 0.5 g of *p*-nitrobenzoic acid in 10 ml of N sodium hydroxide. Add 70 ml of distilled water and a few drops of phenolphthalein. Add N hydrochloric acid dropwise, shaking continuously until neutral. If a white precipitate appears too much acid has been added. Add more alkali, dropwise, to dissolve. Make the solution up to 100 ml with distilled water. Autoclave at 120 °C for 10 minutes. Store at 4 °C.

To prepare PNB medium containing 500 mg/litre, add 50 ml of this solution to 450 ml liquid LJG, mix well, tube and inspissate.

LJG–thiacetazone medium (TZ)

Dissolve 0.1 g of thiacetazone in 10 ml of formdimethylamide. Use glass pipettes and tubes: the solvent attacks some plastics. Store at 4 °C.

To prepare TZ medium (10 mg/litre) add 1 ml of stock to 10 ml of distilled water. Mix 2 ml of this with 200 ml of liquid LJG, tube and inspissate.

LJG–thiophen-2-carboxylic acid hydrazide medium (TCH)

Dissolve 1 g thiophen-2-carboxylic acid hydrazide in 100 ml distilled water. Store at 4 °C.

To prepare TCH medium containing 5 mg/litre, add 1 ml of stock to 9 ml of distilled water and add 2.5 ml of this solution to 500 ml of liquid LJG. Mix well, tube and inspissate. For TCH, 1 mg/litre, add 0.5 ml of diluted solution to 500 ml liquid LJG.

LJG–cycloserine medium (CY)

Dissolve 0.2 g of cycloserine in 100 ml distilled water. Store at 4 °C.

To prepare CY containing 20 mg/litre, add 1.25 ml of this to 100 ml liquid LJG, mix, tube and inspissate.

Tween 80 substrate (T80)

This is not a 'medium' as growth does not occur.

(A) Disodium phosphate.12H_2O	22.88 g
Distilled water	to 1000.00 ml
(B) Monopotassium phosphate anhydrous	9.07 g
Distilled water	to 1000.00 ml

Mix 61.1 ml of A, 38.9 ml B, 0.5 ml Tween 80 and 2.0 ml 0.1% neutral red, bottle and autoclave at 115 °C for 10 minutes.

Sulphatase medium

Dissolve 0.64 g of phenolphthalein disulphate, potassium salt (PDSK) in 100 ml of distilled water. Sterilize by filtration. Mix 180 ml of 7H9 broth, 20 ml of OADC enrichment and 20 ml of PDSK solution. Distribute aseptically in 2 ml amounts, incubate for sterility and store at 4 °C.

N medium

(Collins, 1962)

Sodium chloride	1.0 g
Magnesium sulphate.7H_2O	0.2 g
Monopotassium phosphate anhydrous	0.5 g
Disodium phosphate.12H_2O	3.0 g
Ammonium sulphate	10.0 g
Glucose	10.0 g
Distilled water	to 1000.0 ml

Dissolve by heat, adjust pH to 6.8–7.0, tube and autoclave at 115 °C for 10 minutes.

LJ–Kirchner medium for pyrazinamide sensitivity tests

(Yates, 1984)

(1) Solid phase

Make LJG medium as described above omit penicillin. Adjust to pH 5.2 electrometrically with N hydrochloric acid.

Add 9 ml of filter sterilized 0.22 per cent pyrazinamide solution in water (store frozen) to 300 ml of the medium (test) and 9 ml of sterile distilled water to another 300 ml (control). Tube separately in 1 ml amounts and inspissate vertically to form butts at 80 °C for 60 minutes.

(2) Semisolid phase

Add 1 g of pure agar and 3 g of sodium pyruvate to 1000 ml of Kirchner medium. Adjust to pH 5.2 electrometrically with 5 N hydrochloric acid. Add 15 ml of the pyrazinamide solution (above) to 500 ml (test) and 15 ml of sterile distilled water to the other 500 ml. Steam to dissolve. Cool to 40 °C and add 30 ml of OADC supplement to each bottle.

(3) Final medium

Layer 2 ml of test semisolid medium on the LJ butts of the test solid medium. Do the same with the control media. Store at 4 °C and use within three weeks.

Suspension bottles

Buffer, pH 7.4

Disodium hydrogen phosphate anhydrous	13.3 g
Potassium dihydrogen phosphate	3.5 g
Distilled water	2.0 litres

Wash small iron nails, about 10 mm in length in ether to remove grease. Wash well with water. Treat small glass beads, 3 mm in diameter, in the same way. Place one nail, about six beads and 1 ml of buffer in small screw-capped bottles and autoclave them.

Buffered formalin

This is used to make smears safe if they cannot be stained immediately.

Formalin, commercial (about 37% formaldehyde)	500.00 ml
Sodium dihydrogen phosphate (monohydrate)	22.75 g
Disodium hydrogen phosphate	32.5 g
Distilled water	4.5 litres

Ridley's solution

Mercuric chloride	2.00 g
Formaldehyde 40%	10.00 ml
Glacial acetic acid	3.00 ml
Water	to 100.00 ml

Dissolve the mercuric chloride in water with heating, allow to cool and add the other ingredients.

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