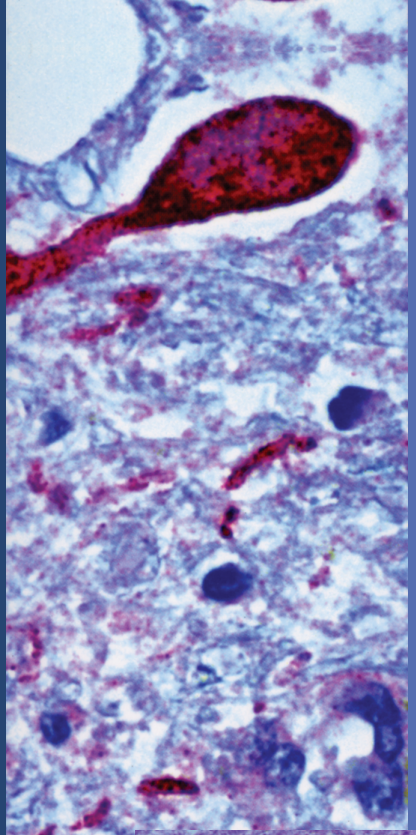

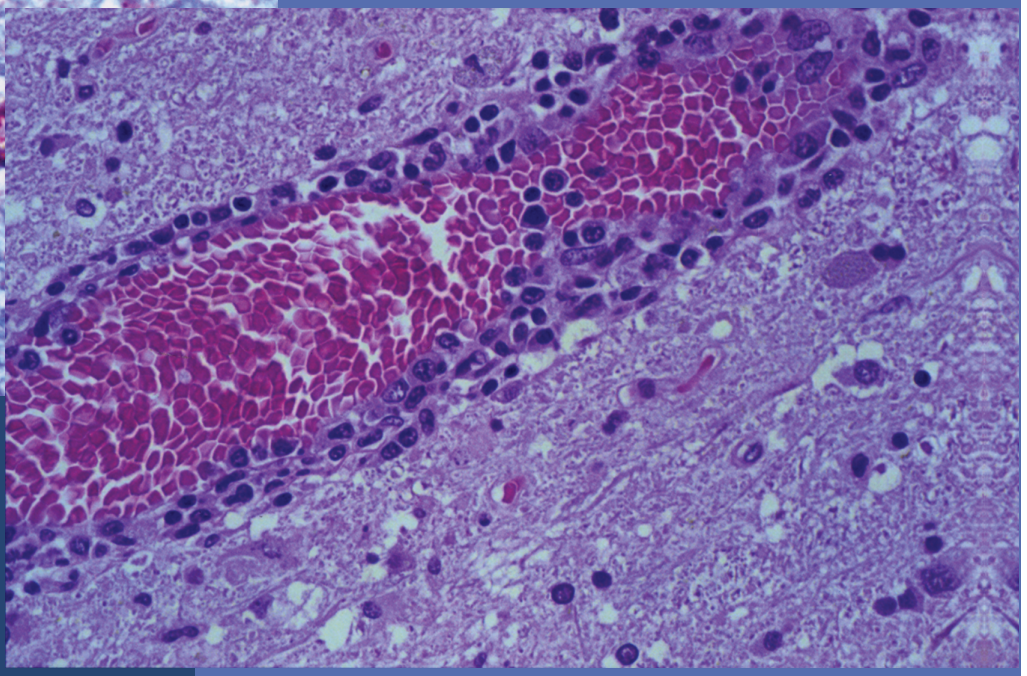

EMERGING INFECTIONS

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Cover photos: West Nile encephalitis. (Upper photo) Viral antigens in neurons and neuronal processes are seen by immunostaining of central nervous system tissue. Naphthol-fast red with hematoxylin counterstain was used. Original magnification, ×158. (Lower photo) Blood vessel in central nervous system showing mononuclear cell perivascular cuffing. Hematoxylin-and-eosin stain was used. Original magnification, ×100. Both photos are courtesy of Wun-Ju Shieh and Sherif R. Zaki, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention.

To our children:

Sarah Walker Scheld,
Bruce Alexander Craig and Lisa Ellen Craig Castiglia, and
Andrew and Mitchell Hughes

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FOREWORD

It is becoming as predictable as New Year's Day: the annual appearance of yet another new human pathogen of public health significance. Sometimes it sneaks up on us with a case here and a case there, as with hantavirus. Sometimes it hits us suddenly and shakes us to the core, insisting on recognition, as with the 1976 outbreak of Legionnaires' disease. And sometimes it does both, as with AIDS. After AIDS was first recognized as a few cases in 1981, we soon found that hundreds of thousands of Americans were already producing billions of viruses a day. But it was not limited to this country. It was quickly realized that the virus was present in all parts of the world, first at relatively low levels and then with an explosion that shook the very structure of countries and global organizations, including the United Nations.

As organisms struggle daily to find an advantage that improves their chances of immortality, the world tinkers in an uncoordinated fashion with relatively small adjustments. The results are new variations of our antibiotics giving only a short-term advantage. Antimalarial drugs give us hope for another decade of protection. Vaccines are for longer-term or even ultimate protection but are limited to a small percentage of the pathogens inflicting humans. But always we worry about the worst-case scenarios such as human engineering of agents to be used in warfare or terrorism or agents with the destructive power of human immunodeficiency virus that are spread as easily as influenza virus.

What is a logical response? It is finally dawning on us that it must be a response that includes every tool the world can assemble, from a coordinated global surveillance system that ties all current surveillance networks with new systems to fill gaps, to rapid analytical capabilities and a coherent global response capacity. This is not something that can continue to be done on an ad hoc basis, muddling through each new threat.

Will Durant once voiced doubts that the world would ever provide an example of coordination short of a threat of an alien invasion. In recent decades we have seen examples of threats that have served as surrogates for an alien invasion, surrogates because they leave many feeling vulnerable, providing partial examples of what the world might be capable of organizing. Work on reducing the threat of nuclear weapons, the smallpox eradication effort, current efforts to eradicate polio, and efforts to avert global warming come to mind. It is time to see emerging infections as true surrogates for an alien invasion. The response must involve every lesson and tool of the infectious disease community. But it will also require political leadership and the support of national governments, global agencies, social scientists, corporations, nongovernmental organizations, and indeed every segment of society. The AIDS pandemic has finally resulted in the mobilization of the global

community in the interest of global health. This new interest must now be used to provide a generic response to all emerging infections. Our response must be so complete that leaders in 100 years will judge our actions to be exactly what was needed.

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PREFACE

As a result of improvements in sanitation and overall living conditions during the early part of the 20th century and the subsequent introduction of many vaccines and antibiotics, tremendous progress has been made in the prevention and control of infectious diseases. Globally, smallpox has been eradicated and target dates have been established for the eradication of poliomyelitis and dracunculiasis. Impressive progress toward eradication of both of these diseases has been made, but major challenges remain. In the United States, the annual incidence of several vaccine-preventable diseases is at an all-time low.

In spite of these successes, infectious diseases remain the leading cause of death worldwide. The World Health Organization (WHO) estimated that approximately 14 million (25%) of the 56 million deaths that occurred worldwide in 1999 were caused by microbial agents. In the United States, infectious diseases are the third leading cause of death.

The Institute of Medicine (IOM) published a report entitled "Emerging Infections: Microbial Threats to Health in the United States" in the fall of 1992. This report, developed under the leadership of Joshua Lederberg and Robert Shope, identified the important factors that contribute to disease emergence and reemergence. These factors include changes in human demographics and behaviors, advances in technology and industry, economic development and changes in land use, increases in travel and commerce, microbial adaptation and change, and deterioration in the public health system at the local, state, national, and global levels.

Recognizing the intense interest and scientific and public health importance of new and emerging infectious diseases, the program committee of the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) and the officers of the Infectious Diseases Society of America (IDSA) organized joint sessions during ICAAC and the IDSA annual meeting beginning in 1995. These joint sessions on new and emerging pathogens were immensely popular, attracting audiences in excess of 4,000, and were planned carefully to span the gamut among new and emerging bacteria, viruses, fungi, and parasites with appropriate discussions on national and international strategies for control.

The chapters in *Emerging Infections 5* were derived primarily from presentations given at the sessions on new and emerging infections at the 2000 ICAAC and are updated and fully referenced for this volume. These chapters focus on a variety of diseases that pose major clinical and public health challenges today; some have been recognized for a century or more, while others have been identified during the past 25 years. Some are important problems in the United States, while others cause disease primarily in other parts of the world. The epidemiology of each has been influenced by one or more of the factors identified in the IOM report. Because

of the nature of the "global village" in which we live, we cannot afford to be ignorant or complacent about any of them.

Experiences with these diseases should alert physicians, microbiologists, researchers, public health officials, policy makers, and the public to the critical importance of ensuring the availability of the capacity to detect, respond to, and control these infections. The ability to address these emerging and reemerging microbial threats requires adequate surveillance and response capacity, ongoing research and training programs, strengthened prevention and control programs, and rebuilding of the public health system at the local, state, national, and international levels. The challenges that these diseases will continue to pose demand a multidisciplinary approach and a supply of trained clinicians, microbiologists, pathologists, biomedical researchers, rodent and vector biologists, ecologists, behavioral scientists, and public health officials. The challenges also require funds to support the people and facilities needed to meet them. This is especially true in the developing world because poverty and malnutrition make populations especially susceptible to emerging and reemerging infections.

Future challenges are difficult to predict but certainly include more problems with antimicrobial-resistant infections, the threat of another influenza pandemic, and the increasingly complex challenges of food-borne disease resulting from the globalization of the food supply. The global human immunodeficiency virus epidemic will continue to put large numbers of people at risk for currently recognized and new opportunistic infections. The roles of hepatitis B and C viruses in chronic liver disease and hepatocellular carcinoma, human papillomavirus in cervical cancer, and *Helicobacter pylori* infection in peptic ulcer disease and gastric cancer are now well established. Additional chronic diseases will certainly be found to have an infectious etiology, providing important new opportunities for disease prevention in the future. Food safety and blood safety will continue to be priorities and to pose challenges. Recent events provide a grim reminder of the threat of bioterrorism, further emphasizing the need to strengthen infectious disease surveillance and response capacity. Two chapters in this volume provide a public health perspective on this issue.

Based on the continued importance of new and emerging infectious diseases as defined by the 1992 IOM report, symposia on these topics are planned for future ICAACs. We plan production of an annual volume on new and emerging infections based on the presentations at each year's ICAAC. This volume, the fifth in the series, should serve as a valuable source of current information for persons responsible for coping with infectious diseases in the new millennium.

W. Michael Scheld
William A. Craig
James M. Hughes

ACKNOWLEDGMENTS

We thank everyone who has helped us in the preparation of this volume. Most importantly, we thank all of the authors for their outstanding contributions. As editors, we are particularly grateful to those members of the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Program Committee and to Barbara Murray, who assisted us in coordinating topic and speaker selection for and/or moderating the joint symposia on emerging infections during the 2000 ICAAC. Numerous other colleagues provided helpful discussion, advice, and criticism. We are also grateful to our secretaries, Natalie Regensburg, Susan Waisner, and Bridget Kelly. We thank Ken April of ASM Press for coordinating production of the book. And, finally, we thank our families for their understanding and support during this undertaking.

Emerging Infections 5
Edited by W. M. Scheld, W. A. Craig, and J. M. Hughes

Chapter 1

Enterovirus 71, an Emerging Virus

Monto Ho

Enterovirus 71 (EV 71) was isolated in 1974 by Schmidt and colleagues (23) from 20 patients in California with central nervous system disease from 1969 to 1974. There was one fatality but no mention of any skin rashes in the patients. A few of the strains produced paralysis in suckling mice consistent with coxsackievirus A. Table 1 provides an overview of the 68 types of enterovirus serotypes including the 3 polioviruses, 29 coxsackieviruses, and 32 echoviruses. Enteroviruses are no longer differentiated into these three different groups, and beginning with enterovirus 68, they were named numerically in sequence.

Since 1974 there have been 13 outbreaks of EV 71 reported throughout the world (Table 2). Hand-foot-and-mouth disease (HFMD) and herpangina (HA) are important clinical hallmarks of enterovirus infection, but ordinarily they are not causes of serious morbidity or fatalities. Aseptic meningitis, along with less specific respiratory, gastrointestinal, or other skin manifestations, may accompany enterovirus infections. EV 71 infection often causes these manifestations. However, in addition, EV 71 causes neurologic complications of greater clinical import. As shown in Table 2, small outbreaks associated with acute flaccid paralysis have occurred (8, 11, 12, 14, 29). In 1975 there was a large outbreak in Bulgaria with 705 cases and 68 deaths (26). The deaths were attributed to bulbar encephalitis initially thought to be due to poliovirus in infants and young children. A similar epidemic occurred in 1978 in Hungary (21).

Unfortunately, there was no good clinical description of the bulbar encephalitis in Bulgaria (26). For example, we do not know what besides flaccid paralysis was observed. It is not known which cranial nerves were involved, and there is no record of tremors or ataxia. What is known is that it afflicted very young children and caused almost all the fatalities. Bulbar encephalitis, like brain stem encephalitis, is a midline encephalitis, and in both cases similar structures may be involved. What is also known is that HFMD, HA, and pulmonary edema, which are now thought to be characteristic of EV 71 infection (16), were not described.

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Table 1. Enterovirus serotypes and major diseases

Serotype (no. of types)	Major diseases
Poliovirus types 1 to 3 (3)	Paralytic poliomyelitis, aseptic meningitis
Coxsackieviruses A1 to A22 and A24 (23)	Aseptic meningitis, HA, conjunctivitis (A24), HFMD (A16, A5, A7, A4)
Coxsackieviruses B1 to B6 (6)	Aseptic meningitis, fatal neonatal disease (myocarditis), pleurodynia, HFMD (B2, B5)
Echoviruses 1 to 9, 11 to 27, and 29 to 34 (32)	Aseptic meningitis, rashes, febrile disease
Enteroviruses 68 to 71 (4)	Acute hemorrhagic conjunctivitis (EV 70), HFMD, brain stem encephalitis with deaths, paraparesis, aseptic meningitis (EV 71)

In 1995, Landry et al. (19) described a 3-year-old girl from Connecticut who died with brain stem encephalitis and pulmonary edema. She had fever, rash, truncal ataxia, absent doll's eyes, pulmonary edema, and disseminated intravascular coagulation. EV 71 was isolated from the spinal cord. In retrospect, this was the first description of the hallmark clinical presentation of the Malaysian and Taiwanese epidemics in 1997 and 1998 (15, 30).

Sporadic cases from earlier outbreaks already pointed to brain stem encephalitis. Ishimaru et al. (17) in 1980 reported on 81 patients with neurologic complications from the 1973 and 1978 EV 71 epidemics in Japan. Intention tremor, ataxia, and myoclonus, characteristic of brain stem and cerebellar involvement, were noted. Komatsu et al. (18) in 1999 described 12 patients from Otsu, Japan, who had had these signs in 1979. It was, however, during the Malaysian and Taiwanese epidemics of 1997 and 1998 that this clinical syndrome was clearly recognized and described in large numbers of individuals, along with accompanying pulmonary edema or hemorrhage, which made these two epidemics unique (6, 20).

Very young children were admitted to hospitals with HFMD, fever, tachycardia, and tachypnea. Pulmonary congestion with rapid progression to pulmonary edema, pink frothy sputum, and hemorrhage ensued. Focal neurologic signs may or may not be apparent. Ataxia, myoclonus, absent doll's eyes, and flaccid paresis may be present. Death may occur within 24 to 48 h. The autopsy findings pointed to inflammation and neuronal cell death in areas of the brain stem, cerebellum, medulla oblongata, and spinal cord. These findings supported the hypothesis that the pulmonary edema and deaths were due to disturbances in the vasomotor centers and were neurogenic (6, 20).

Lum et al. (20) pointed out that the neurogenic nature of pulmonary edema was substantiated by an unusually detailed pathologic report in 1957 by Baker (2). Baker collected postmortem data for 150 individuals with bulbar encephalitis due to poliovirus infection during the heyday of polio epidemics in the United States from 1946 to 1951 (2). Among these individuals, 15 (10%) were found to have pulmonary edema or hemorrhage, a forgotten complication of poliomyelitis known in the 1950s. He compared the neuropathologies of the brain stems of 10 affected

Table 2. Outbreaks associated with EV 71[a]

Geographic location	Yr	No. of cases	No. of fatalities	Clinical manifestation(s)[b]	Reference
New York	1972	11	None	Aseptic meningitis (m), encephalitis (s), HFMD (s)	9
Sweden	1973	195	None	Aseptic meningitis (m), HFMD (s)	3
Bulgaria	1975	705	68[c]	Aseptic meningitis (m), paralytic disease (s), encephalitis (s)	26
Hungary	1978	1,550[d]	45[e]	Aseptic meningitis (m), encephalitis (s), flaccid paralysis (s)	21
Japan	1973	3,296	Some	HFMD (m), brain stem	27
Japan	1978			encephalitis (s), paralysis (s)	11, 12
New York State	1977	12	None	Aseptic meningitis (s), HFMD (s), paralysis (s)	8
Australia	1988	114	None	HFMD (m), aseptic meningitis, respiratory disease, encephalitis	10
France	1979	5	None	Acute respiratory disease, CNS[f] complications	29
Philadelphia	1987	5	None	Flaccid paralysis (all)	14
United States	1987	45	None	Aseptic meningitis (s), paralysis (s), encephalitis (s)	1
Malaysia	1997	5,999	31	HFMD, brain stem encephalitis, pulmonary edema	30
Taiwan	1998	129,106[g]	78	HFMD or HA (m), brain stem encephalitis, encephalitis, pulmonary edema or hemorrhage, myocarditis, flaccid paralysis	15

[a]Reprinted from reference 16 with permission from the publisher.
[b]m, most, (i.e., over half); s, minority (i.e., less than half).
[c]Bulbar encephalitis.
[d]Cocirculating tick-borne encephalitis virus chiefly in adults; cases in children were due to EV 71.
[e]Encephalitis.
[f]CNS, central nervous system.
[g]Cocirculating coxsackievirus A16.

individuals with those of 5 controls without pulmonary edema. Individuals with pulmonary edema had lesions in the dorsal nucleus of the vagus and medial reticular cells or cells of the vasomotor nuclei. Apparently, involvement of both sites of the central autonomic nervous system was necessary. Additional evidence that the autonomic system was involved in the Taiwanese outbreak was the occurrence of metabolic disturbances, such as hyperglycemia (7).

THE 1998 TAIWANESE EPIDEMIC

In July 1989 the Taiwan Department of Health instituted a system of clinical surveillance initially to report on influenza, respiratory infections, diarrhea, and

childhood diseases (31). In early 1998, sentinel physicians reported increasing numbers of cases of HFMD and HA. The department asked the sentinel physicians to add HFMD and HA to their list of reportable diseases in March 1998.

Figure 1 shows the weekly trend of all cases of HFMD and HA from Taiwan in 1998. This rose from a handful of cases of HFMD to 15,700 cases during the week of 7 June. The peak was reached a week earlier in the central region and a week and half later in the southern region. During 1998 there were two waves of the epidemic. The first one lasted from the end of March to 25 July and involved all four regions of Taiwan. The second wave was largely limited to the southern region and lasted from 6 September to 12 December. The first wave entailed a total of 98,000 reported cases, and the second one entailed 24,000 cases.

Figure 2 presents the epidemic curve for patients with severe HFMD who required hospitalization. As one can see, the total of 405 cases followed about the same type of curve noted for simple HFMD and HA in Fig. 1. The empty columns represent patients who survived, and the solid bars represent those who succumbed. There were also clearly two waves.

The 78 fatalities in this epidemic were uniformly distributed according to the density of the population in the 22 counties and seven cities of Taiwan. There were greater concentrations of deaths and population in the north, with fewer deaths and fewer people in the east and south. The eastern region of Taiwan is sparsely populated.

The four regions of Taiwan and the population at risk, consisting of children 15 years or younger, are listed in the first two columns of Table 3. The numbers of patients who had severe cases and who were hospitalized and the incidences per

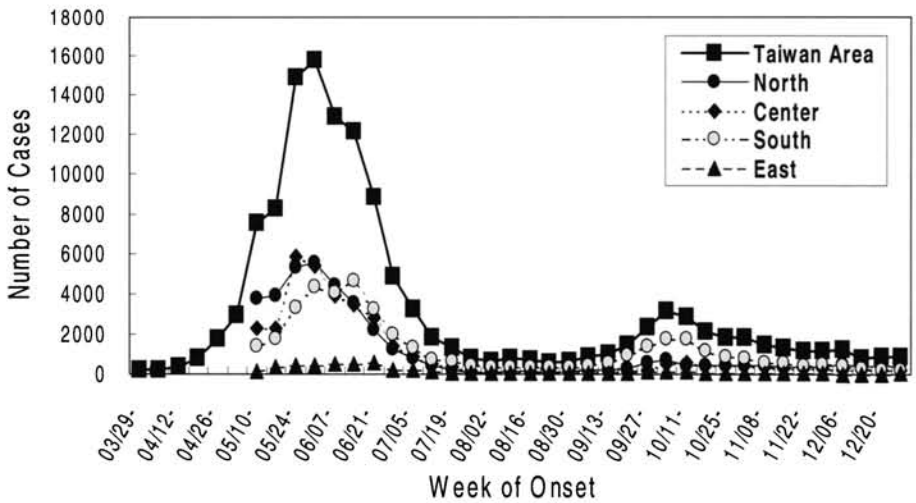

Figure 1. Number of cases of HFMD and HA reported in Taiwan as a whole and in each of its four regions by sentinel physicians from the week of 29 March 1998 through the week of 27 December 1998. The total number of cases was 129,106. Reprinted with permission from the *New England Journal of Medicine* (15). Copyright 1999 Massachusetts Medical Society. All rights reserved.

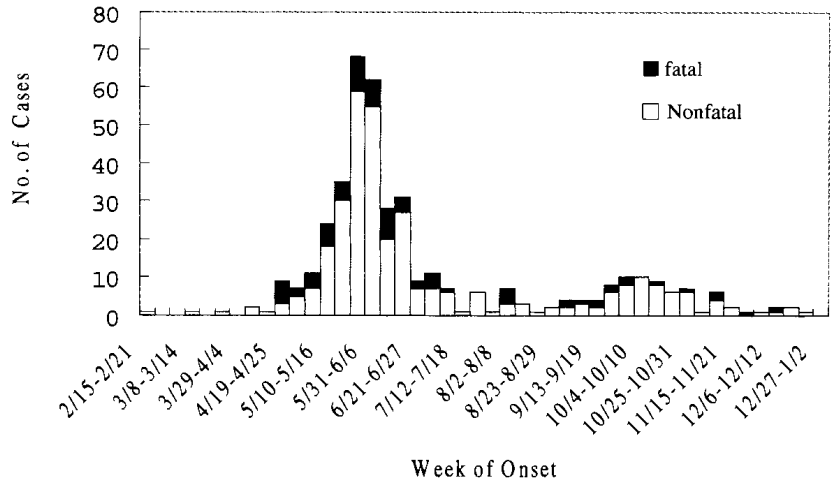

Figure 2. Number of cases and outcomes of severe HFMD and HA in Taiwan from the week of 15 February 1998 through the week of 20 December 1998. A total of 78 patients died, and 327 survived. Reprinted with permission from the *New England Journal of Medicine* (15). Copyright 1999 Massachusetts Medical Society. All rights reserved.

1,000 population are listed in the next two columns. As can be seen, the incidences ranged from 0.055 to 0.093 per 1,000 population and were not significantly different in the four different regions. The number of deaths and the case-fatality ratios are listed in last two columns. The case-fatality ratios varied from 7.7 to 31%. This ratio varied significantly. The central region, for unknown reasons, had a significantly higher case-fatality ratio than the rest of the country.

Table 4 describes the numbers, incidences, and case-fatality ratios for patients who had severe cases and who were hospitalized by age-specific populations at risk. The highest incidence occurred in children less than 1 year old. Most cases

Table 3. Incidence of cases of severe HFMD and HA and case fatality rates, by geographic region of Taiwan[a]

Region of Taiwan	No. at risk[b]	No. of cases	Incidence (no. of cases/1,000)[c]	No. of deaths	Case-fatality ratio (%)[d]
Northern	2,012,802	167	0.083	29	17.4
Central	1,293,462	100	0.077	31	31.0
Southern	1,342,779	125	0.093	17	13.6
Eastern	234,397	13	0.055	1	7.7
Total	4,883,440	405	0.083	78	19.3

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[b]The population at risk consists of children under 15 years of age.
[c]$P = 0.2$ for the differences among the regions.
[d]$P < 0.001$ for the differences among the regions.

Table 4. Incidence and case fatality ratios for HFMD patients by age in Taiwan, January to December 1998

Age (yr)	Population at risk	No. of severe cases	Incidence (no. of cases/1,000)[a]	No. of deaths	Case-fatality ratio (%)[b]
<1	306,666	96	0.313	31	32.3
1–5	1,288,720	218	0.169	40	18.3
6–10	1,622,897	68	0.042	6	8.8
11–15	1,682,943	11	0.007	1	9.1
Total	4,901,226	393	0.080	78	19.8

[a]Chi-square for trend, $P < 0.001$.
[b]Chi-square for trend, $P < 0.001$.

(314 of 393; 80%) were in children 5 years old or younger. Of 78 deaths, 71 (91%) were among children 5 years of age or younger. The case-fatality ratio was significantly higher for children less than 1 year old.

The mortality rate among children 1 year old or younger was assessed in greater detail (data not shown in Table 4). There were 6 deaths among the 38 children age 6 months or younger (15.8%) and 25 deaths among 58 children ages 7 to 12 months (43.1%) ($P < 0.01$). Thus, there were fewer hospitalizations and fewer deaths among those in the youngest age group, presumably because they were protected by passive immunity.

Table 5 gives a picture of the virus isolations from inpatients and outpatients from data collected at two major medical centers in the north and in the south of Taiwan, Chang Gun Medical Center and Cheng Kung University Medical Center, respectively. One of the characteristics of EV 71 outbreaks is that other enteroviruses frequently cocirculate with EV 71 (16). This outbreak was characterized by cocirculation of a number of other enteroviruses, the most prominent one being coxsackievirus A16. A total of 782 isolates were collected. There were 469 isolates of EV 71, of which 62% were from inpatients and 49% were from outpatients. There were 226 coxsackievirus A16 isolates, of which 28% were from inpatients and 37% were from outpatients. Significantly more EV 71 isolates were from inpatients and significantly more coxsackievirus A16 isolates were from outpatients.

Table 5. Enteroviruses isolated from inpatients and outpatients[a]

Virus isolated[b]	Total	No. (%) of isolates from:		Relative risk	*P* value
		Inpatients	Outpatients		
EV 71	469	414 (61.9)	55 (48.7)	1.27	<0.01
Coxsackievirus A16	226	184 (27.5)	42 (37.2)	0.73	<0.04
Other	87	71 (10.6)	16 (14.1)	0.75	<0.90
Total	782	669 (100)	113 (100)		

[a]Reprinted with permission from the *New England Journal of Medicine* (15). Copyright 1999 Massachusetts Medical Society. All rights reserved.
[b]Enteroviruses isolated from patients from Chang Gung Medical Center and Cheng Kung University Medical Center from May to December 1998.

Table 6 depicts the clinical complications for 96 patients who had severe cases and who were hospitalized and the enteroviruses isolated from them. EV 71 was isolated from 78 patients. The most numerous clinical complications were encephalitis, pulmonary edema or hemorrhage and encephalitis, aseptic meningitis, and pulmonary edema or hemorrhage alone. Thirty-nine patients had encephalitis, and 35 patients had pulmonary edema or hemorrhage with or without encephalitis. There were scattered cases of myocarditis and flaccid paralysis.

Table 7 presents the clinical complications of the fatalities and the virus isolates from the patients. Of 78 patients who were fatalities, 51 died of encephalitis and pulmonary edema or hemorrhage and 14 died of pulmonary edema alone. Therefore, adding these two groups together, 83% of all patients had pulmonary edema or hemorrhage. Except for one patient, the only virus isolated from patients with pulmonary edema or hemorrhage was EV 71.

MOLECULAR EPIDEMIOLOGY

Table 8 shows the genotypes of the EV 71 isolates recovered from various outbreaks. These were analyzed by phylogenetic dendrogram analysis after sequencing of the V4-V2 region by Shimizu et al. (25) and the V1 region by Brown et al. (4). The original strain analyzed by Schmidt et al. (23) belonged to genotype A, according to the terminology of Brown et al. (4). The earlier strains from the 1973 epidemic of HFMD in Japan and Bulgaria and Hungary from 1975 to 1978 (data not shown) and a strain from Taiwan recovered in 1980 all belonged to genotype A1 (terminology of Shimizu et al. [25]). There was a shift to genotypes A2 and B with the first description of sporadic cases of brain stem encephalitis in Japan in 1997. The isolates from the outbreaks in Malaysia and Taiwan all belonged to genotypes A2 and B.

By analyzing the V1 region, Brown et al. (4) found that the original U.S. strain and isolates from the United States from 1970 to 1988 all belonged to what they called genotypes A and B. These have since disappeared. In the United States now, all strains belong to genotype C of the terminology of Brown et al. (4) or genotype

Table 6. Clinical complications of 96 severe cases and enteroviruses isolated[a]

Complication(s)[b]	Total no. (%) (n = 96)	No. of EV 71 isolates (n = 78)	% EV 71 isolates	No. of other enteroviruses
Encephalitis	39 (41)	30	77	9
Pulmonary EH and En	25 (26)	25	100	0
Aseptic meningitis	11 (12)	5	45	6
Pulmonary EH	10 (13)	9	90	1
Myocarditis and En	2 (2)	2	100	0
Myocarditis	1 (1)	1	100	0
Paralysis and En	1 (1)	1	100	0
Paralysis	1 (1)	1	100	0
Other	6 (8)	4	67	2

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[b]EH, edema-hemorrhage; En, encephalitis.

Table 7. Clinical complications of fatalities and their virus isolates[a]

Complication(s)	Deaths		Viruses (no. [% of total]) isolated from patients who died			
	No.	% of total (n = 78)	EV 71	Coxsackievirus B5	Other enteroviruses	Total (n = 37)
Encephalitis and pulmonary edema or hemorrhage	51	65	23 (100)	0	0	23
Pulmonary edema or hemorrhage	14	18	9 (90)	1	0	10
Encephalitis	10	13	1 (33)	0	2	3
Other	3	4	1 (100)	0	0	1

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B of the terminology of Shimizu et al. (25). In Japan the original genotype A1 strain, called the Nogaya strain, has also disappeared. The more recent strains from Malaysia and Taiwan are represented by genotypes B and C, respectively, of Brown et al. (4) or by genotypes A2 and B, respectively, of Shimizu et al. (25). Similar conclusions concerning the Taiwan isolates were reached by Shih et al. (24), who analyzed the V1 and 5′ noncoding regions, and Wang et al. (28), who analyzed the 5′ noncoding region.

THE 2000 TAIWAN OUTBREAK

Two years after the 1998 epidemic in 2000, Taiwan experienced another island-wide outbreak of HFMD and HA. This outbreak also lasted most of the year. There were 217 severe cases that required hospitalization and 36 deaths. The number of reported cases of HFMD and the rates of morbidity and mortality were about half of those observed in 1998 (Taiwan Department of Health, unpublished data). The

Table 8. Molecular epidemiology and evolution of EV 71[a]

Yr(s)	Place	Strain	Genotype according to:	
			Brown et al. (4)	Shimizu et al. (25)
1970	California	BrCr-CA-70	**A**	**BrCr**
1970–1988	United States	113 strains	**B**	A2
1985–1998	United States		C	B
1973	Japan	Nagoya		**A1**
1997	Japan	C7/Osaka		A2
1997	Japan	14375, etc 7		B
1997	Sarawak	0756-MAA-97	B	A2
1997	Malaysia	0731-MAA-97	C	**B**
1998	Taiwan	17 strains	C	B
1998	Taiwan	2 strains	B	A2

[a]Based on data from Brown et al. (4), Shimizu et al. (25), Shih et al. (24).
[b]Strains in boldface have disappeared.

predominant enterovirus isolated from patients with severe and fatal cases was EV 71. The 2000 outbreak shows that a serious outbreak of EV 71 can recur only 2 years after a large outbreak, suggesting that only a short interval is needed to build up a reservoir of susceptible young children.

CONCLUSIONS

EV 71 can cause large epidemics, and most recently these have occurred in the Far East. These outbreaks are frequently mixtures of EV 71 and other viruses such as coxsackievirus A16, but serious disease and fatalities are caused only by EV 71. Hallmark clinical manifestations are HFMD, flaccid paralysis, brain stem encephalitis, and pulmonary edema. Typically, severe disease and deaths occur in young children (those less than 3 years old) and are due to the neurovirulence of the virus. Southeast Asia is also the part of the world where other emerging infections have been described, such as dengue hemorrhagic fever (13) and Nipah virus infection (5). EV 71 must be diagnosed by specific virologic procedures. Laboratory surveillance must also be specific. The predominant EV 71 genotypes vary in both time and place. It is a highly mutable RNA virus which evolves at about 1.35×10^{-2} substitutions per nucleotide per year (4). In view of the continued activity of EV 71, despite significant herd immunity, in Taiwan in 1999 and particularly in 2000, the development of a vaccine, preferably of the Salk inactivated type (22), is urgently needed.

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Chapter 2

West Nile Virus Infection in New York City: the Public Health Perspective

Denis Nash, Neal Cohen, and Marcelle Layton

West Nile virus belongs to the Japanese encephalitis serocomplex of flaviviruses that includes the medically important St. Louis encephalitis, Murray Valley encephalitis, and Kunjin viruses (20). It is enzootic in Africa and Eurasia (Fig. 1); its typical reservoirs and vectors include wild, domestic, and peridomestic species of birds and *Culex* species of mosquito, respectively (10). Most West Nile virus infections among humans are subclinical, with overt disease occurring in approximately 1 of 100 infections (21). The incubation period ranges from 3 to 15 days (26). The illness varies in severity from mild (characterized by fever, headache, lymphadenopathy, and/or petechial rash) to more severe neurologic disease manifesting as meningoencephalitis (20, 25, 29); the likelihood of development of more severe clinical illness increases with age (22). Similarly, the case fatality rate during outbreaks of West Nile virus meningoencephalitis has varied from 4 to 13% and is highest among elderly persons (10, 25, 29).

In 1999, West Nile virus was identified for the first time in the Western Hemisphere when it caused an outbreak of meningoencephalitis in New York City and the surrounding metropolitan area (Fig. 2), resulting in hospitalizations of 59 case patients and 7 deaths (2–4, 25). In this outbreak, illness was characterized by severe neurologic disease that primarily affected older adults, consistent with West Nile virus meningoencephalitis outbreaks in other areas of nonendemicity where the level of population immunity to West Nile virus is low (e.g., Romania and Russia) (10, 16–19, 28).

Before the human outbreak, an increase in the number of avian fatalities that primarily affected crows occurred in New York City. After the human outbreak of

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Figure 1. Geographic distribution of the Japanese encephalitis serocomplex of viruses related to West Nile virus. Reprinted with permission from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colo.

arboviral encephalitis was recognized, a separate investigation by veterinary and wildlife specialists determined that these birds also had pathologic evidence of encephalitis (1, 27). A flavivirus was subsequently identified from the avian tissues and was determined to be West Nile virus (13, 14; T. Briese, X. Y. Jia, C. Huang, L. J. Grady, and W. I. Lipkin, Letter, *Lancet* **354:**1261–1262, 1999).

Genetic analysis indicated that the strain of West Nile virus responsible for the 1999 New York City-area outbreak was similar to strains that have caused encephalitis outbreaks in northern Europe and was nearly identical to a strain of West Nile virus circulating in Israel in 1998 (13). The exact mode of introduction of West Nile virus into the United States is unknown; however, its emergence in the New York City metropolitan area was likely caused by the recent introduction of infected mosquitoes, birds, or humans from an area of endemicity. We describe the detection of and public health response to this outbreak in the New York City area.

MOSQUITO SURVEILLANCE AND CONTROL IN NEW YORK CITY BEFORE THE 1999 OUTBREAK

The most common mosquito species in New York City, and many other northern urban areas, is *Culex pipiens*, a competent vector for a variety of mosquito-borne

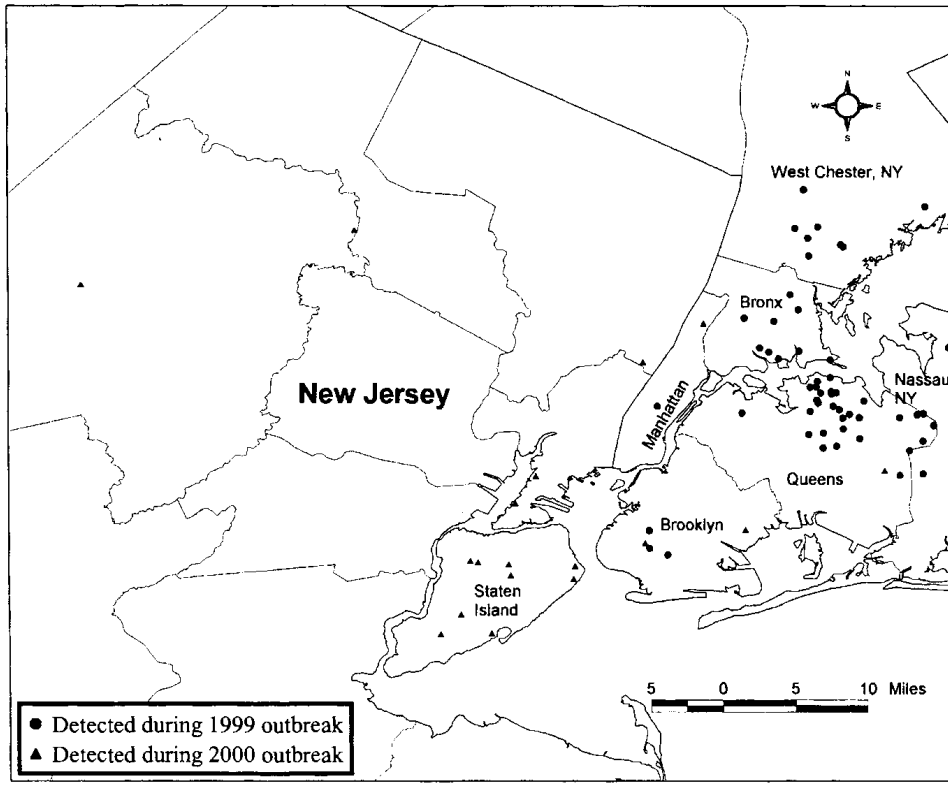

Figure 2. Geographic distribution of human West Nile virus disease detected in the New York City area during outbreaks in 1999 and 2000.

viral encephalitides. Although competent vectors are present in New York City, local transmission of mosquito-borne encephalitis or other arboviral diseases had not occurred for more than a century prior to the West Nile virus outbreak (23). The only mosquito-borne outbreak in recent years was a small, locally acquired cluster of falciparum malaria in Queens in 1993, which was transmitted by an anopheline mosquito species (15).

Since mosquito-borne disease has been rare, mosquito control in New York City has primarily focused on the elimination of the nuisance associated with human-biting mosquitoes. Prior to a sharp reduction in state aid in 1982, this included aerial spraying of pesticides to control the adult mosquito population in coastal areas (23). Since then, the intermittent application of larvicide to kill mosquitoes in the early stages of the life cycle (e.g., eggs, larvae, and pupae) had been the primary strategy used in limited areas of New York City, such as coastal beaches, to prevent the emergence of adult nuisance mosquitoes.

SURVEILLANCE FOR VIRAL MENINGITIS AND ENCEPHALITIS IN NEW YORK CITY

Viral meningitis and encephalitis are 2 of 70 reportable diseases or conditions in New York City and New York State. Unlike many of the other reportable diseases whose diagnosis is based on a single laboratory test (e.g., malaria and tuberculosis), the diagnosis of these conditions is primarily based on clinical criteria, since viral and serologic testing often is incomplete (e.g., convalescent-phase sera are not obtained) or is not done at all. Therefore, the New York City Department of Health relies on physician reporting to monitor these two diseases, and as is true in many other jurisdictions, reporting is far from complete. Among the members of the New York City population of approximately 7.5 million persons, during the decade prior to this outbreak, an average of 9 reported cases of encephalitis occurred per year (1.1 per million population) and an average of 170 reported cases of viral meningitis occurred per year (22.7 per million population). As with population-based surveillance data obtained by other jurisdictions, the specific viral etiology was not determined for most cases.

However, although incomplete, historical surveillance data on viral meningitis and encephalitis in New York City still provide useful information on disease trends. Every year, in August and September, there was a peak in cases of viral meningitis; these primarily occurred among young children. For cases in which a laboratory diagnosis was established, the etiology was usually an enterovirus, a member of a family of viruses that are transmitted from person to person through infected stool. Accordingly, when a cluster of viral encephalitis was first detected in the late summer of 1999, an enteroviral cause was initially considered the most likely etiology.

DETECTION OF AN UNUSUAL CLUSTER OF VIRAL ENCEPHALITIS CASES

In recent years the New York City Department of Health has been actively promoting the importance of physician reporting of unusual disease clusters or manifestations because of concerns about both the early detection of new or re-emerging infectious diseases and the threat of bioterrorism. The value of physician reporting in the detection of new or unusual diseases is best exemplified by the New York City West Nile virus outbreak.

At the time that cluster was detected in late August 1999, routine surveillance data did not indicate an increase in the rate of viral meningitis or encephalitis compared with historical trends in New York City (Fig. 3). Rather, a single physician's report of an unusual disease manifestation led to the initial recognition of the human outbreak.

On 23 August 1999, an infectious disease physician in northern Queens contacted the New York City Department of Health regarding two previously healthy older patients whom she thought had viral encephalitis. However, since one of these patients had severe muscle paralysis (an uncommon complication of encephalitis),

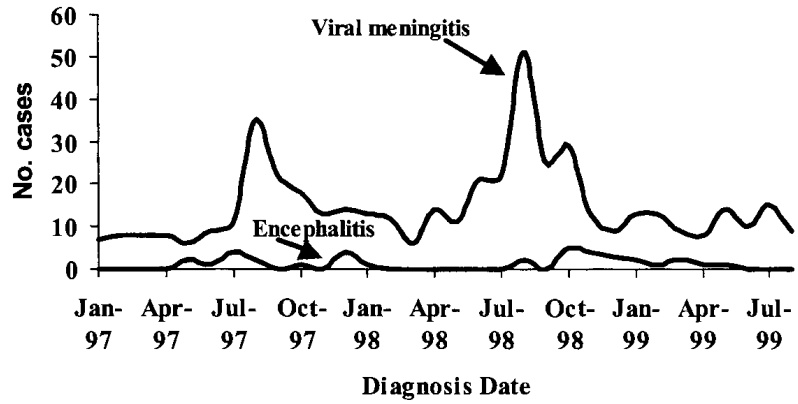

Figure 3. Viral meningitis and encephalitis surveillance, New York City, 1997 to 1999.

she was also concerned about botulism, and the neurologist seeing this patient had preliminarily diagnosed atypical Guillain-Barré syndrome (GBS) with fever.

However, both botulism and GBS were thought to be unlikely, due to the presence of fever and increased cerebrospinal fluid protein levels and pleocytosis, the absence of cranial nerve abnormalities, and a diffuse pattern of weakness (as opposed to the ascending or descending pattern present with GBS or botulism, respectively). Therefore, the New York City Department of Health did not recommend testing for botulism or administration of antitoxin but, instead, encouraged the physician to send cerebrospinal fluid and serum specimens to the New York State Health Department laboratory for viral diagnostic testing.

On 27 August 1999, the infectious disease physician reported a third encephalitis case, and by this time, all three patients had developed severe muscle weakness. On that same day, the neurologist on service at the hospital where the three patients were admitted reported seeing a similar case at a nearby community hospital. Because usually only nine cases of encephalitis are reported citywide per year, the concomitant occurrence of these four cases of an unusual encephalitis associated with muscle paralysis and the clustering of cases in a small area of the city prompted the New York City Department of Health to immediately initiate an outbreak investigation.

By 29 August 1999, active case finding had identified eight suspected cases in nearby Queens hospitals. The case patients were all relatively healthy, older adults (ages, 58 to 87 years) who resided in a 16-square-mile area of northern Queens. Their clinical presentations were nearly identical: a febrile illness associated with mild gastrointestinal symptoms, followed by the onset of altered mental status. Seven of the eight patients had severe, diffuse muscle weakness, with four patients developing paralysis requiring ventilatory support. Hematologic and biochemical tests of cerebrospinal fluid and blood indicated a viral etiology.

The patients' families were interviewed extensively to determine if they had any common epidemiologic associations. The case patients did not know each other

before this hospitalization and had no common exposures, including travel history, social events, restaurants, or commercial food items.

Enteroviruses would have been the expected cause of viral encephalitis in New York City at the time of year when the outbreak occurred (Fig. 2). Enteroviruses are transmitted from person to person and are most commonly found among young children. However, the absence of any secondary illness among the case patients' immediate household contacts and social circles and the lack of reported recent contact with young children made this etiology less likely. The only characteristic that was common among these patients was that they all reportedly spent time outdoors in their backyards or in their neighborhoods, especially in the evening hours, performing activities such as gardening or smoking on a porch or deck. In addition, an environmental investigation revealed the presence of *Culex* species mosquito breeding sites and larvae in most of the patients' yards and neighborhoods (e.g., piles of old tires, large rain barrels, and partially excavated swimming pools).

EXPANDING THE OUTBREAK INVESTIGATION

Consultation with the U.S. Centers for Disease Control and Prevention and neighboring local and state public health agencies did not reveal additional, similar cases in nearby jurisdictions. The immediate priority of the New York City Department of Health was establishment of an etiology, since specific public health or medical recommendations could not be made until a diagnosis was made. Therefore, the New York City Department of Health continued to prioritize retrieval of cerebrospinal fluid and serum specimens and sent the specimens by overnight mail to the New York State Department of Health's laboratory for viral testing.

Although the initial cluster seemed to be localized to one small area in northern Queens, there was concern that additional cases in other parts of the city (i.e., outside the borough of Queens) had occurred but had not yet been reported. Therefore, more active surveillance was begun citywide, and the New York City Department of Health alerted the New York City medical community of an unusual cluster of viral encephalitis cases associated with muscle weakness, using broadcast facsimile and e-mail. In those alerts, physicians were asked to immediately report to the New York City Department of Health any patients with suspected cases of viral encephalitis admitted to their hospitals. To address the concern that physician reporting is often incomplete, active surveillance was also implemented by calling infectious disease and neurology specialists at all 70 acute-care hospitals and querying whether these physicians were aware of similar cases at their institutions. By 3 September 1999, approximately 30 suspected cases had been reported to the New York City Department of Health from hospitals throughout the city.

On 2 September 1999, the New York State Department of Health reported that preliminary serologic testing of all eight case patient specimens was most consistent with the presence of St. Louis encephalitis virus. On 3 September 1999, the Centers for Disease Control and Prevention reported that serologic test results for the same specimens were positive for St. Louis encephalitis virus by a nonspecific enzyme immunoassay. The serologic results, in conjunction with the clinical and epidemiologic findings, were suggestive of St. Louis encephalitis virus.

PUBLIC HEALTH RESPONSE

Mosquito Control

The initial laboratory finding of St. Louis encephalitis virus led to the rapid institution of mosquito control measures (2–4, 25), including widespread aerial spraying of adulticide to reduce the adult population of potentially infected mosquitoes. This public health emergency prompted the activation of the New York City Emergency Operations Center. The Emergency Operations Center is housed in the mayor's Office of Emergency Management and facilitates centralized coordination for the management of citywide emergencies that require input and response from more than one city agency or department.

When this outbreak was detected, New York City did not have an existing mosquito surveillance and control program; therefore, emergency contracts were required. Emergency control measures included aerial and ground spraying of adulticides (including malathion, resmethrin, and sumithrin), elimination of potential breeding sites, and application of a biological larvicide (*Bacillus sphaericus*) to standing water that could not be eliminated, incuding sewage treatment plants and catch basins. Emergency control efforts were coordinated with other city agencies, including the New York City Department of Sanitation, to remove potential breeding sites (e.g., to clean up tire piles), the New York City Department of Environmental Protection to apply larvicide to sewage treatment plants, and the New York City Police Department to map the substantial number of backyard swimming pools in the epicenter of northern Queens, many of which had not been well maintained, possibly because of the drought conditions that occurred in the summer of 1999. The Police Department identified unmaintained swimming pools by helicopter, and the New York City Department of Health then sent sanitarians door to door to help clean the pools and provide larvicide. Finally, to avoid shortages at city pharmacies, the city purchased 400,000 cans of mosquito repellent and distributed them to the public free of charge through various city agencies (e.g., firehouses and police precincts).

Public Education

The greatest challenge in response to this outbreak was the need to ensure effective communication to the public to address concerns about both the virus and the city's ongoing mosquito control measures. During the initial investigation, when the outbreak was thought to be localized to only a small area in the borough of Queens, the New York City Department of Health sent hundreds of staff door to door with fact sheets, translated into eight languages, that provided information on St. Louis encephalitis virus, personal precautions to be taken to avoid mosquito bites, and the pesticides being used in aerial spraying. Approximately 1 week later, with the recognition that the outbreak was affecting the entire city, the New York City Department of Health established a public telephone hot line that was staffed 24 h a day for 7 weeks. At its peak, 75 persons worked each shift, including physicians, epidemiologists, and toxicologists who provided consultation to the operator staff. With over 150,000 calls received during this time, it was the busiest

hot line ever established by New York City. Information was also made available through the department's website, which provided downloadable fact sheets that were translated into 18 languages.

Media

Daily communication and coordination with the media were conducted to address the public's concern about the outbreak, pesticide spraying schedules, and the health effects of pesticide spraying. This was accomplished through daily press conferences that were conducted by the mayor and the commissioners of health and emergency management, public service announcements, and press releases from the New York City Department of Health Office of Public Affairs. The media was likely the most effective conduit for informing the public about the disease and the city's public health response.

Communication with the Medical Community

In order to maximize completeness and rapidity of case finding, direct communication between the New York City Department of Health and the medical community was required to provide information on the clinical presentation, laboratory diagnosis, and treatment of West Nile virus infections and preventive measures for avoidance of West Nile virus infection. A broadcast facsimile system, capable of rapidly sending faxes to all 70 New York City acute-care hospitals, was in place at the New York City Department of Health prior to the outbreak. During the outbreak this system was used to send weekly broadcast facsimile alerts to four departments (infectious disease, emergency medicine, laboratory medicine, and infection control) with requests to share this information with all clinical staff. In addition, a separate telephone hot line for providers was established and staffed by New York City Department of Health physicians.

Avian Epizootic and Recognition of West Nile Virus

What was already unexpected (the preliminary identification of St. Louis encephalitis virus in New York City, where it had never occurred before) became even more remarkably unprecedented with the recognition of West Nile virus (an arbovirus never before seen in the Western Hemisphere). The identification of West Nile virus was the isolation of the infectious virus from birds collected during a simultaneous bird die-off. An epizootic associated with substantial numbers of bird deaths (mostly crows) had been observed throughout the New York City area in the weeks before and concurrent with the outbreak among humans. This epizootic was initially assumed to be unrelated to the human outbreak, because St. Louis encephalitis virus normally does not kill its avian reservoir hosts. However, a concurrent investigation of these avian fatalities by veterinarians and wildlife specialists demonstrated encephalitis on pathologic examination, along with viral inflammation of other organs (e.g., heart, kidney, and spleen), prompting viral testing (27). On 22 September, 3 weeks after the recognition of a mosquito-borne encephalitis outbreak among humans, West Nile virus was identified in avian tissue specimens

(27) and was subsequently determined to be the common etiologic agent in the encephalitis outbreaks among birds, horses, and humans (13; Briese et al., letter). On 30 September, West Nile virus was isolated from *C. pipiens* mosquitoes that had been collected in the area of the epidemic almost 3 weeks earlier.

There are a number of reasons for the delayed recognition of West Nile virus. First, serology does not provide a definitive diagnosis for the Japanese encephalitis complex of arboviruses, which includes both West Nile and St. Louis encephalitis viruses, because there is extensive cross-reactivity among these viruses. Second, viral culture tests of all human cerebrospinal fluid and autopsy brain specimens were negative, so the first viral isolate from the outbreak that was identified and able to undergo genetic sequencing was an isolate from one of the dead birds. Finally, it was initially thought that the bird deaths were unrelated to the human outbreak, because neither St. Louis encephalitis virus nor any other member of the Japanese encephalitis virus serocomplex has ever caused simultaneous avian and human encephalitis outbreaks. The valuable information that necropsies of the dead birds found in New York City revealed pathologic evidence of viral encephalitis was not shared with the local public health officials investigating the human outbreak until 2 days before West Nile virus was identified. Mosquito surveillance and viral testing were implemented as part of the epidemic investigation; West Nile virus was isolated on 30 September 1999 from mosquitoes collected in New York City on 11 and 12 September.

SURVEILLANCE FOR HUMAN ENCEPHALITIS AND MENINGITIS DURING THE 1999 OUTBREAK

The ongoing public health response to the West Nile virus outbreak included active surveillance to fully define the geographic extent of the outbreak and to evaluate the need for additional mosquito control measures. The New York City Department of Health implemented a multifaceted surveillance program for human cases that included both passive and active surveillance systems.

Case Definition

Clinical criteria for reporting emphasized the syndrome of the initial Queens cluster (i.e., encephalitis with severe muscle weakness), but also included encephalitis without muscle weakness, fever with paralysis, or the milder syndrome of viral meningitis. Specific criteria included fever of >100°F, muscle weakness, altered mental status, meningeal signs, or cerebrospinal fluid parameters indicative of a viral etiology (i.e., protein concentration of ≥40 mg/dl or leukocyte count of ≥5/ml and negative Gram staining and culture results) (25).

Enhanced Passive Surveillance

Passive surveillance was conducted through weekly facsimile alerts that were sent to the New York City medical community to provide updates on the outbreak as it unfolded and to remind physicians to report suspected cases of viral meningoencephalitis and to provide appropriate clinical specimens for laboratory testing.

These alerts included a standardized reporting form. Reports were received at the New York City Department of Health by facsimile or telephone and were immediately entered into a computerized database.

Active Surveillance

Active physician-based surveillance was expanded to include nine specialty areas at all 70 hospitals (including adult and pediatric general wards; adult and pediatric infectious disease, neurology, and intensive care specialists; and infection control) to ensure complete and rapid case ascertainment. Weekly telephone calls were made to these specialists to query them directly regarding any new patients who met the clinical criteria for reporting.

Laboratory and Retrospective Surveillance

Two additional surveillance systems were implemented: (i) a laboratory-based system to collect cerebrospinal fluid with hematologic and biochemical markers indicating a viral infection to identify cases that did not meet the clinical case definition or that were not reported by clinicians and (ii) a retrospective system that used hospital discharge data to determine if the outbreak had started prior to the onset of the earliest known case. Beginning on 1 October, laboratory-based surveillance was implemented in New York City at 10 sentinel hospitals in four boroughs (Brooklyn, Bronx, Manhattan, and Queens). These laboratories were asked to freeze all cerebrospinal fluid specimens with parameters suggestive of viral infection (negative by Gram staining and culture, protein concentration of $\geq$40 mg/dl, leukocyte count of $\geq$5 cells/ml) for weekly collection by the New York City Department of Health and serologic testing for arboviral antibodies by immunoglobulin M (IgM)-capture enzyme-linked immunosorbent assay (ELISA) at the Centers for Disease Control and Prevention. In addition, retrospective surveillance was implemented by using hospital discharge databases at 10 area hospitals in New York City. The hospitals were queried for patients with discharge diagnoses of viral encephalitis, viral meningitis, or GBS occurring after 1 July 1999. The laboratories at these hospitals were contacted to determine if any cerebrospinal fluid or serum specimens from these patients were still available. If none existed, the patients were contacted by telephone, and a convalescent-phase blood specimen was requested and obtained through a visit to the patient's home by a phlebotomist.

These enhanced, active surveillance efforts identified additional cases of viral meningoencephalitis than had been reported historically through a passive surveillance system. During the 3 months of the outbreak investigation, 589 cases of meningoencephalitis were reported among New York City residents; this is compared to the 180 cases that are usually reported each year. Each individual with a suspect case was evaluated and prioritized for laboratory testing. Serum and cerebrospinal fluid samples were requested from all patients who met the clinical case criteria. The New York City Department of Health worked closely with the city's Office of the Chief Medical Examiner to ensure that autopsies were performed for all individuals with clinically suspect fatal cases. All specimens were picked up by

the New York City Department of Health and sent to the Centers for Disease Control and Prevention for West Nile virus testing by IgM-capture and IgG ELISAs of cerebrospinal fluid and serum, PCR testing (of cerebrospinal fluid and autopsy tissue), or immunohistochemical staining (of autopsy tissues). The results for specimens positive by serology were confirmed by virus-specific plaque reduction neutralization testing (PRNT).

EPIDEMIOLOGIC AND CLINICAL FINDINGS DURING THE 1999 HUMAN OUTBREAK

Reports on 719 suspected cases of meningitis and encephalitis were received by health departments in New York City (n = 589), Nassau County (n = 70), and Westchester County (n = 60). For 62 (8.6%) of the 719 case patients, there was laboratory evidence of recent West Nile virus infection. Three of these patients experienced illness that consisted only of fever and headache and that did not result in hospitalization. In New York City, 4 of the 45 hospitalized patients were identified by retrospective surveillance of hospital discharge data. Laboratory-based surveillance did not detect any additional cases.

The descriptive epidemiology presented here focuses on the 59 patients who were hospitalized during the 1999 West Nile virus outbreak. The median age of the hospitalized patients was 71 years (range, 5 to 90 years), and most (88.1%) were aged ≥50 years. The overall rate of West Nile virus clinical infection during the 1999 outbreak was at least 6.5 cases per million population. The rate of clinical West Nile virus infection increased sharply with age (Fig. 4), and attack rates among persons aged ≥50 years were significantly higher than attack rates among those aged <50 years (rate ratio, 19.6; 95% confidence interval, 17.9 to 21.6). The onset of illness ranged from 2 August through 24 September 1999, with the peak of the outbreak occurring in mid- to late August. Of the 59 case patients, 44 resided

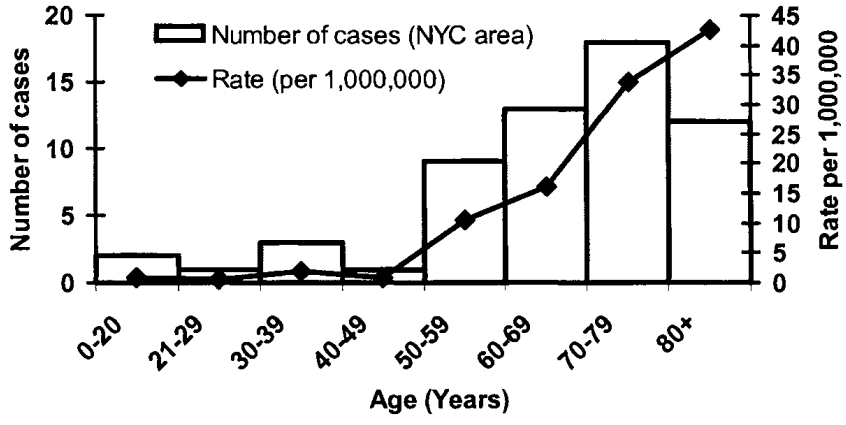

Figure 4. Number and attack rate of clinical West Nile virus infections (n = 59) in the New York City (NYC) area, 1999, by age group.

in New York City and 14 lived in two adjacent counties of New York State; 1 was a Canadian tourist who arrived in New York City on 1 September and who became ill on 5 September. The highest number of cases (n = 32) and the highest rate of hospitalization (16.4 per million population) occurred in the New York City borough of Queens, the epicenter of the outbreak (Fig. 2).

Clinical presentations included encephalitis with muscle weakness (n = 20; 34%), encephalitis without muscle weakness (n = 17; 29%), meningitis (n = 17; 29%), or milder illness characterized by fever and headache (n = 5; 8%). There were seven deaths (case fatality rate, 12%). Older adults experienced more severe clinical disease (encephalitis [median age, 73 years] versus viral meningitis [median age, 58 years]). Age (≥75 years) was the strongest predictor of death (relative risk adjusted for the presence or absence of diabetes mellitus, 8.5; 95% confidence interval, 1.2 to 59.1), followed by diabetes mellitus (age-adjusted relative risk, 5.1; 95% confidence interval, 1.5 to 17.3). Cerebrospinal fluid parameters were consistent with a viral etiology: mean leukocyte count of 38 cells/ml (range, 0 to 525 cells/ml) and mean protein concentration of 104 mg/dl (range, 38 to 899 mg/dl). More detailed clinical characteristics of the patients involved in this outbreak have been published elsewhere (25).

EVALUATION OF HUMAN SURVEILLANCE

In New York City, patients presenting with encephalitis with onset in August 1999 were more likely to be seropositive (52%), especially when the encephalitis was accompanied by muscle weakness (positive predictive value, 63%). By contrast, the positive predictive value for suspected cases with viral meningitis was relatively low (16%). There was an even lower yield (0.9% seropositivity) among persons identified through retrospective surveillance, but this may have been due to a high completeness of retrospective reporting of clinically suspected cases. However, age was a stronger predictor of seropositivity among patients who met the clinical case definition. The positive predictive values were 2.5% among those aged 0 to 19 years, 4.3% among those aged 20 to 39 years, 12.5% among those aged 40 to 64 years, and 33% among those aged 65 years and over. No serologically positive cases were detected by laboratory-based surveillance, but this system was not initiated until October, and by that time the outbreak had subsided.

The available epidemiologic surveillance data indicate that West Nile virus is a substantial threat to older persons but is less of a threat to younger persons (Fig. 4). In addition, no data from the New York area experience indicate that newborns, infants, or children are at greater risk for manifesting clinical signs of West Nile virus infection after becoming infected. Public health messages regarding protective measures for avoidance of West Nile virus infection and West Nile disease surveillance should therefore be particularly targeted at older persons.

MOSQUITO SURVEILLANCE

With the assistance of entomologists from the Centers for Disease Control and Prevention and the New York State Department of Health, surveillance for mos-

quitoes was initiated on 10 September by setting mosquito traps (for larval, adult, and adult gravid mosquitoes) citywide to monitor as well as to attempt virus isolation and determine the viral infection rates in various species. The geographic distribution of West Nile virus-positive mosquitoes correlated strongly with that of human cases in New York City, with the greatest concentration of viral activity occurring in northern Queens and the south Bronx. West Nile virus-positive mosquitoes were primarily *Culex pipiens* and *Culex restuans,* which are common peri-residential urban mosquitoes (24).

AVIAN SURVEILLANCE

Avian surveillance focused on tracking of the number of dead birds and the West Nile virus positivity rate among dead birds reported by the public. Dead birds were collected and examined for pathologic evidence of encephalitis and were then forwarded to the Centers for Disease Control and Prevention for West Nile virus testing. In addition, the Centers for Disease Control and Prevention, in collaboration with the U.S. Geological Survey's National Wildlife Health Center, conducted surveys to estimate the seroprevalence of West Nile virus antibodies among wild, live birds.

Avian seroprevalence and the geographic distribution of infected dead birds also correlated with West Nile virus activity among humans (12). However, West Nile virus activity in birds was much more geographically widespread (11), with infected dead crows found in areas where no human cases were detected, including eastern Long Island, the lower Hudson Valley, eastern New Jersey, southern Connecticut, and Baltimore, Md. Although thousands of birds were estimated to have died from West Nile virus infection in 1999, with over 23 species affected (9a, 27), the highest percentage of avian fatalities (88%) occurred among crows.

SEROPREVALENCE SURVEY CONDUCTED IN NORTHERN QUEENS, 1999

To better assess the overall infection rate and the spectrum of illness associated with the outbreak, the New York City Department of Health and the Centers for Disease Control and Prevention conducted a household-based seroepidemiologic survey in October 1999 in a 3-square-mile area around the epicenter in northern Queens. The seroprevalence in the surveyed area (population, 46,220) was estimated to be 2.6% (95% confidence intervals, 1.2 to 4.1%) (23a). This infection rate was similar to that found in an outbreak in 1996 in Romania, an area where West Nile virus was previously nonendemic (28). In New York City, an estimated 134 persons were infected with West Nile virus for every 1 patient who was hospitalized with more severe neurologic disease. This ratio was higher among older adults; in persons older than 65 years, approximately 1 severe neurologic case occurred per 50 persons infected with West Nile virus. Twenty percent of seropositive persons reported a mild febrile illness during the late summer or fall that could be attributed to West Nile virus infection. Among those persons, predominant

symptoms included myalgia (100%), headache (89%), fatigue (87%), and arthralgia (76%).

THE EXPERIENCE 1 YEAR LATER

In northern Europe, new introductions of West Nile virus into regions with temperate climates, such as Romania in 1996, have tended to be short-lived, with decreasing viral activity in the years following a major outbreak (9). To determine whether West Nile virus had persisted in New York City after the 1999 outbreak, surveillance was implemented during the winter of 1999–2000 for infected adult female *Culex* sp. mosquitoes overwintering in underground areas of northern Queens. Although very few mosquitoes were found, 3 of the 69 pools tested were positive for West Nile virus RNA by PCR testing, and infectious West Nile virus was isolated from one of these (5–7).

These findings increased concerns that the virus would reemerge during the summer of 2000 and underscored the need to maintain intensified efforts to reduce the potential for a recurrent outbreak of West Nile virus infections. The New York City Department of Health and public health authorities throughout the eastern United States implemented comprehensive arthropod-borne disease surveillance and mosquito control programs that emphasized reduction of mosquito breeding sites, extensive application of larvicides, and public health education regarding personal protective measures (8).

In 2000, only 21 persons were diagnosed with West Nile virus infection. Of these, 19 required hospitalization and 2 died. The timing of the outbreak in 2000 was similar to that in 1999, with most cases occurring in mid- to late August. Although most cases occurred in Staten Island (53% of cases; $n = 10$), the geographic area of the outbreak expanded nearly twofold in 2000, with human cases occurring in five New Jersey counties. In Staten Island, the number of cases was smaller, but the rate of infection during 2000 (22.2 per million population) was higher than that in Queens during 1999 (16.4 per million population). As in 1999, encephalitis was the most common clinical presentation, and both severe neurologic disease and fatal outcome were more common among older patients. No infants or children were diagnosed with West Nile virus infection in 2000.

Most impressive was the dramatic geographic spread of the West Nile virus epizootic in 2000, with involvement of 12 states and the District of Columbia (5). West Nile virus-infected birds were identified as far north as Vermont and as far south as North Carolina, representing a threefold increase in the geographic area affected by the epizootic compared with the area affected in 1999. Surveillance throughout the Northeast and the mid-Atlantic states identified approximately 4,300 infected birds, involving 78 species (although most [80%] were crows), and 480 infected mosquito pools, involving 14 species, with the majority (>90%) being *Culex* spp. Of most concern was the finding of infected mosquito species that are more likely to bite humans (including *Oclerotatus triseriatus, O. japonicus, O. vexans, Aedes albopictus,* and *Culex salinarius*). The epizootic also severely affected horses; 63 equine cases of West Nile virus infection occurred in seven states, with a case fatality rate of 39%. Avian mortality and mosquito surveillance data

consistently detected West Nile virus in an area before the occurrence of human illness.

FUTURE SURVEILLANCE FOR WEST NILE VIRUS IN THE UNITED STATES

Arboviral activity in areas of endemicity tends to be unpredictable from year to year, making prediction of future West Nile virus activity in the United States difficult. Although West Nile virus was not detected in any overwintering mosquitoes during the 2000–2001 winter season, the rapid spread of this virus within just 1 year of its introduction into the United States indicates that West Nile virus could eventually become established throughout much of the United States. The risk for recurrent human outbreaks in particular places depends on such factors as the effectiveness of local vector control activities in diminishing adult mosquito populations, increasing immunity in the avian population, the degree of amplification of the virus in local mosquito and avian reservoir hosts during the summer season, local weather conditions, and compliance with recommendations for personal protective measures (especially by older adults).

Health care providers in the United States, especially those along the eastern seaboard, should remain alert for possible cases of viral encephalitis during the peak months of the adult mosquito season. Suspected viral encephalitis cases should be reported immediately to local or state health departments, especially when they occur in older adults or if they are associated with severe muscle weakness.

Physicians should also consider West Nile virus in the differential diagnosis of viral meningitis, especially among older patients presenting during the summer months. Neurologic involvement is more common among older adults infected with West Nile virus than it is among younger persons. Children with viral meningitis are more likely to have enteroviral infections, especially in the late summer and early fall. Since severe muscle weakness and flaccid paralysis were notable findings during the 1999 outbreak, physicians should also consider West Nile virus infection in the differential diagnosis of GBS, particularly in association with atypical features such as fever, altered mental status, or a pleocytosis.

LABORATORY TESTING FOR WEST NILE VIRUS

Laboratory confirmation of West Nile virus infection is based on the following criteria (26): (i) isolation of West Nile virus from or demonstration of West Nile virus antigen or genomic sequences in tissue, blood, cerebrospinal fluid, or other body fluid; (ii) demonstration of IgM antibody to West Nile virus in cerebrospinal fluid by ELISA; (iii) demonstration by PRNT of a fourfold serial change in PRNT titer of antibody to West Nile virus in paired acute- and convalescent-phase serum samples (obtained at least 2 weeks apart); or (iv) demonstration of both West Nile virus-specific IgM and IgG antibodies in a single serum specimen by ELISA and PRNT methods (8).

The most sensitive screening test for West Nile virus is the IgM-capture ELISA for both cerebrospinal fluid and serum. Among the 1999 and 2000 case patients

for whom cerebrospinal fluid specimens were available, approximately 95% were positive for West Nile virus IgM antibody. IgM reactivity in the cerebrospinal fluid was often present soon after clinical symptoms began, with 90% of specimens obtained within 8 days of illness onset testing positive. The infections in the remaining patients were diagnosed on the basis of West Nile virus IgM reactivity in the serum, and all serologic diagnoses were confirmed by virus-specific PRNT. Testing for viral RNA by real-time PCR (TaqMan) was not as sensitive as testing by serologic assays, with only 57% of cerebrospinal fluid specimens and 14% of serum specimens testing positive for West Nile virus nucleic acids in 1999. In 2000, PCR testing was also less sensitive, as only 1 (8%) of 13 cerebrospinal fluid specimens tested was positive for West Nile virus nucleic acid.

Substantial serologic cross-reactivity exists among flaviviruses. Persons who have ever received yellow fever vaccine or persons previously or currently infected with closely related viruses, such as dengue virus or St. Louis encephalitis virus, might have a false-positive result by an IgM and IgG ELISA for West Nile virus. Virus-specific PRNT should be performed to determine the specific viral etiology.

CONCLUSIONS

The introduction of West Nile virus into New York City in 1999 and the rapid spread of the epizootic throughout most of the Northeast by 2000 illustrate how vulnerable we remain to imported disease threats. Clearly, the emergence of West Nile virus in the Western Hemisphere has important public health implications, demonstrating the ease with which pathogens can move between continents and infect immunologically naïve human and animal populations, with potentially devastating effects.

The emergence of such diseases must be detected quickly. The West Nile virus outbreak described here highlights the need for physician reporting in recognizing unusual disease clusters. West Nile virus was detected because an astute infectious disease doctor immediately reported her concerns to a public health agency that rapidly acted on that call. However, this outbreak could easily have been missed. At the time that the epidemiologic investigation started in late August 1999, 19 patients who were later diagnosed with West Nile virus infection were hospitalized in New York City. However, 15 (80%) patients had not yet been reported to the New York City Department of Health. Therefore, if the one infectious disease physician had not reported the initial cluster, it is unclear when or if this outbreak would have been detected.

Public health agencies cannot be aware of infectious disease events in their jurisdictions unless health care providers report their concerns. The ability to recognize an outbreak that affects patients at more than one institution requires that each provider report all notifiable diseases and any unusual cases to the public health authorities in that jurisdiction. To improve our ability to detect and respond to emerging infectious disease threats nationwide, we need to improve the public health infrastructure in the United States and actively foster strong relationships among local and state public health agencies and the medical community throughout the United States.

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Emerging Infections 5
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Chapter 3

Q Fever: Queries Remaining after Decades of Research

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Q fever is a zoonosis caused by *Coxiella burnetii.* It has been reported from six continents, occurring in epidemic or sporadic forms (67). The infection in humans is variable in its clinical expression, in its severity (varying from asymptomatic to fatal), and in its natural course, i.e., acute or chronic. As an acute disease it can manifest as fever, pneumonia, hepatitis, exanthema, myocarditis, pericarditis, meningitis, or encephalitis. In some hosts acute infection can lead to chronic infection, and if the patient is pregnant (at the time of the infection) stillbirth or prematurity can result. Endocarditis may occur in patients with valve lesions, or cancer (84), and chronic endovascular infection may be the manifestation of chronic Q fever in patients with vascular prosthesis or aneurysm (Table 1). Because of the wide spectrum of clinical manifestations and the nonspecific nature of the symptoms, the prevalence of Q fever in a specific area reflects the interest and index of suspicion of local physicians for Q fever and the quality of the laboratory diagnostic services. Human infections result mainly from aerosols, but cases have been associated with the consumption of raw milk (31). Aerosols are frequently generated by farm animals such as cattle, goats, and sheep and are transported long distances by the wind (109). Pets, including pigeons, cats, rabbits, and dogs, have more recently been implicated in the spread of Q fever. In mammals, parturition is important in the transmission of Q fever, as the bacteria are concentrated in the placenta. Because the bacterium is highly resistant, it can remain infectious in the environment, and because of the long incubation period (2 to 3 weeks), the source of sporadic cases is frequently unknown. *C. burnetii* could be transported on infected dust by the wind, and therefore the source of contamination could be distant and unidentified by the patient (31). *C. burnetii* is extremely infectious; a single organism can

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Table 1. Conditions that predispose to chronic infection in human beings and in laboratory animals

Condition	Predisposal to infection in:			
	Mice	Rabbits	Guinea pigs	Human beings
Cancer				+
Corticosteroid treatment	+		+	+
Cyclophosphamide	+			
Irradiation	+		+	+
Valve lesion		+	+	+
Pregnancy	+	+		+
Vascular lesion				+

cause an infection, which explains why it has been considered a potential weapon for biological warfare (4, 58).

There has been a recent increase in interest in the disease in several countries, leading to an increase of reported cases in the literature. Because of the lack of true active surveillance, it is difficult to determine whether this is due to an increase in interest in the disease and hence detection of previously unrecognized cases or whether these are really new cases that represent the introduction of Q fever into areas that were previously free of this infection. However, Q fever is not currently a major topic in the literature, as indicated by the fact that a Medline search of the 10 last years yielded only 409 citations. When the diagnosis of Q fever is considered, it is extremely easy to confirm it by serological testing using microimmunofluorescence, the reference method for the diagnosis of rickettsial diseases. Furthermore, this technique can distinguish between acute and chronic infections and therefore help the physician in planning treatment.

A SHORT HISTORY OF Q FEVER

In August 1935, E. H. Derrick, a pathologist at the Queensland Health Department, Brisbane, Australia, was asked to investigate an outbreak of an undiagnosed febrile illness among workers at the Cannon Hill abattoir in Brisbane (69). Derrick first carefully described the clinical illness. He noted that it lasted 7 to 24 days and was characterized by fever, headache, malaise, anorexia, and myalgia. Blood cultures were negative and serum samples had no antibodies to influenza virus or organisms causing typhus, leptospirosis, typhoid, or paratyphoid. He named the illness Q (for query) fever (22).

Derrick inoculated blood or urine from his febrile patients into guinea pigs, but he was unable to isolate a microorganism from the guinea pigs despite the occurrence of fever in the animals. At this point, October 1936, he sent a saline emulsion of infected guinea pig liver to Frank Macfarlane Burnet, a virologist (and later a Nobel laureate) who was working at the Walter and Eliza Hall Institute in Melbourne. Burnet and his research associate Mavis Freeman found oval areas about the size of a nucleus in sections of spleen from a mouse that had been inoculated with Derrick's specimen. When these smears were stained by Castaneda's method,

"bodies which appeared to be of rickettsial nature were found, sometimes in enormous numbers" (12).

In the spring of 1935, Laurence Humble, a laboratory attendant at the Rocky Mountain Laboratory in Hamilton, Mont., collected 200 *Dermacentor andersonii* ticks and brought them to Gordon Davis, who was working at the laboratory on various aspects of Rocky Mountain spotted fever (69). Davis was able to isolate an organism (which he called Nine Mile agent because the ticks were collected near Nine Mile Creek) from these ticks. By 1938 it was apparent that the tick agent and the Q fever agent were identical. Herald Rea Cox, a Rocky Mountain Laboratory scientist, worked with Davis and characterized the Nine Mile agent. He was able to cultivate this agent in embryonated eggs.

Rolla Dyer (director of the National Institutes of Health from 1942 to 1950) visited Cox to review his data. Dyer left convinced about the validity of the results. A few days later he developed fever and retroorbital headache. The Q fever agent was isolated from his blood (69).

As is often the case in the nomenclature of microorganisms, there has been a series of names for this agent: initially it was called *Rickettsia diaporica* (*diaporica* is Greek for "having the property or ability to pass through," describing the filterability of this agent), and then *Rickettsia burnetii* was proposed but later was elevated to a subgenus, *Coxiella* (69). The agent is now known as *Coxiella burnetii*. Cox and Burnet both died in 1986.

The next key event was the recognition of outbreaks of Q fever among British troops stationed in Italy, Greece, and Corsica during World War II. At about the same time outbreaks of this infection were also reported among American troops returning from the Mediterranean area (69). In 1946 and 1947, outbreaks of Q fever were observed at meatpacking plants in Amarillo, Tex., and Chicago, Ill. Epidemiological studies of the Chicago outbreak led to the conclusion that Q fever was transmitted by aerosols (69). In a series of studies in California, Lennette showed that sheep and goats were reservoirs of *C. burnetii* and that the organism could be isolated from the air of premises housing the infected animals (69). In 1948 the organism was isolated from milk, and in the same year it was demonstrated that aureomycin was an effective treatment for patients with Q fever (69). In 1950 Luoto and Huebner isolated *C. burnetii* from the placentas of parturient cows, and in 1956 Stoker and Fiset described antigenic phase variation of *C. burnetii* (69).

Over the next 40 years additional reservoirs of *C. burnetii* were delineated and the various manifestations of Q fever were described. In particular, the clinical picture of acute Q fever was differentiated from that of chronic Q fever. Laboratory scientists began to dissect this fascinating microorganism and demonstrated a spore-like stage to its development. Even now in the era of molecular biology, much remains to be explained about the intracellular adaption of this pathogen.

In the 1990s, Raoult and others have begun to dissect the host response to infection with *C. burnetii* with the ultimate aim of elucidating why only a small percentage of all those infected with this microorganism develop chronic Q fever (14, 45, 70).

It is perhaps fitting that the new millennium begins with another sentinel observation from workers in Australia (43). These workers showed that *C. burnetii* can persist for years in humans with post-Q fever fatigue syndrome and that the bone marrow is the probable source from which the liver and peripheral blood mononuclear cells are seeded (43).

THE BACTERIUM

C. burnetii is a small, pleomorphic, obligate intracellular bacterium. Phylogenically, based on 16S rRNA sequencing, it belongs to the gamma subgroup of proteobacteria in a clade including *Legionella* spp. and *Francisella tularensis*.

As an organism, *C. burnetii* exhibits several distinct characteristics. It is highly resistant to physicochemical stress. This property has been attributed to the observation of a sporelike structure by electronic microscopy (68). *C. burnetii* resists a temperature of 60°C for 60 min and survives exposure to 0.5% formalin for 4 days. In Marseille, France, we isolated *C. burnetii* from a formalin-fixed heart valve from a patient with Q fever endocarditis who had a valve replacement in Paris, 800 km away (unpublished data). The organism can survive several months on walls and in milk. This is the reason why it remains viable in dust, several weeks after environmental contamination (31).

C. burnetii in culture undergoes phase variation, with the virulent phase I changing to an avirulent, lipopolysaccharide (LPS)-deficient phase II. Apparently phase II is a deletion mutant of phase I (112), whose fitness, in vitro, is superior to that of phase I. In animals or human beings the opposite is true (i.e., phase I is more fit than phase II). Surprisingly, antibodies observed after the primary infection are directed mainly against the avirulent phase II. Patients with chronic infection exhibit high titers of antibodies to both phases I and II.

C. burnetii is the single species of its phylum. There is a low degree of genetic heterogeneity among strains. This has been studied by pulsed-field gel electrophoresis, and 20 restriction groups were found (106). Several studies based on gene sequencing (95) confirmed that the genotype is associated with the geographical origin. *C. burnetii* strains have a variable genome size (it may be linear), ranging from 1.5 to 2.4 Mb (115). Some of the strains contain a plasmid structure of various sizes. Their specific functions are unknown, as some isolates have the same genes integrated in the chromosome rather than on the plasmid (57). The role of plasmids in virulence, and specifically in the causation of chronic disease, has not been confirmed (67) (Table 2).

PHYSIOPATHOLOGY OF THE DISEASE

The current understanding of the natural history of Q fever is that a nonimmune patient enters into contact with *C. burnetii,* which causes a primary infection that could be asymptomatic or symptomatic. The symptomatic primary infection is acute Q fever. The spontaneous evolution of acute infection is usually a complete recovery in the normal host. In some hosts *C. burnetii* can multiply despite an antibody response following primary infection (symptomatic or not). In these cases,

Table 2. Immunological profile of patients with Q fever

Immunological parameter	Profile for patients with Q fever categorized as:		
	Chronic	Relapsing	Acute
Immunoglobulin titers			
IgG to phase I	High (≥1,600)	Increasing	Low (<1,600)
IgA to phase I	High (≥100)	Increasing	Low (<100)
IgM	Variable	Increasing	High (≥100)
Lymphocyte subsets			
CD4	Decreased		
CD8	Decreased		
CD4/CD8	Decreased		
Microbicidal effect of monocytes	Decreased	Decreased	Normal
Plasma cytokines			
TNF	Increased	Increased	
IL-6	Increased	Increased	
IL-1β	Normal	Normal	
Soluble receptors[a]			
IL-1Ra	Normal	Increased	
sTNF-RI	Normal	Normal	
sTNF-RII	Increased	Increased	
sCD14 (LPS receptor)	Normal	Normal	
sCD25 (IL-2R)	Increased	Increased	
sCD23	Increased	Normal	
Cytokine production by monocytes			
IL-10	Increased	Increased	
TGF-β1	Increased		
TGF-β2	Increased		
TNF-α	Increased		
IL-1β	Increased		
TNF-RI	Normal		
TNF-RII	Increased		
C. burnetii in organ lesions	Many	Rare or absent	
Granuloma formation in lesions	Rare or absent	Many	

[a] The prefix "s" stands for "soluble."

because the immune system is unable to control the infection, chronic infection develops. This hypothesis is supported by all available data from human beings as well as from animal models (reviewed in reference 67).

In rabbits, acute infection that resolves spontaneously follows challenge with *C. burnetii*, but pregnant females abort. To develop a rabbit model of Q fever endocarditis, a valve lesion has to be induced, because rabbits without a valve lesion do not develop endocarditis. In otherwise healthy mice the disease is usually asymptomatic, but tissue lesions are observed when mice are sacrificed 3 to 7 days postinfection. The route of infection (aerosol or intraperitoneal) determines the specific organ that is involved (lung or liver, respectively). Immunocompromised animals can suffer a relapse of infection, following corticosteroid treatment or whole-body irradiation, or exhibit chronic infection (athymic mice). This relapse can be associated with endocarditis in animals receiving cyclophosphamide. *C.*

burnetii-infected pregnant mice develop chronic infection associated with miscarriage, premature birth, and endocarditis. Guinea pigs have been extensively used for Q fever studies. The route of infection, as observed in mice, is correlated with specific organ lesions. Moreover, a large inoculum is associated with myocarditis.

HOST-BACTERIUM RELATIONSHIPS

The immune control of Q fever is T cell dependent, but it does not lead to *C. burnetii* eradication, and immunosuppression can induce relapse of infection in apparently cured patients or laboratory animals (97, 98). Recently, Harris et al. (43) showed that PCR could demonstrate *C. burnetii* DNA in circulating monocytes and bone marrow months or years after acute Q fever, and we showed that DNA could be recovered from dental pulp of apparently cured guinea pigs (1).

It was observed very early that in vitro, *C. burnetii* phase I, the virulent form, was partially resistant to phagocytosis (31). This was associated with a more rapid multiplication of phase II, the avirulent form, in nonphagocytic cells used to cultivate *C. burnetii*. This explains why in vitro phase II forms predominate, and, after several passages, become the major variant. In vivo, the avirulent form did not survive in myeloid cells and intramonocytic killing of phase II but not phase I occurred (14, 19). In nonphagocytic cells, vacuoles containing virulent or avirulent forms of *C. burnetii* exhibit all the features of mature phagolysosomes (44), which can fuse with other intracellular compartments (111). In phagocytic cells, phagolysosomal fusion is delayed in J774 cells infected by virulent *C. burnetii* (46). In human monocytes, the generated virulent vacuole form is not able to acquire cathepsin D, thus demonstrating a blockade in late events of phagolysosomal fusion (39). This may explain why only the virulent form is obtained from infected animals and why the avirulent form is predominant in vitro.

The different steps of phagocytosis of *C. burnetii* have recently been studied. The adhesion process within monocytes generated ruffles when incubated with virulent forms but not with avirulent forms (70). The attachment of virulent forms to monocytes is mediated by $\alpha_v\beta_3$ integrin only (14), whereas the interaction of avirulent forms with monocytes requires $\alpha_v\beta_3$ and CR3 integrins. Therefore, the virulent variant largely inhibits the internalization mediated by CR3, likely through the impairment of the cross talk between these two integrins.

Together, these data suggest that a high-frequency avirulent mutant is positively selected in cells and embryonated eggs because of its more effective entry in cells. In monocytes, this leads to destruction of the avirulent mutant by complete phagolysosomal fusion; therefore, phase I is positively selected by monocytes. The in vitro infection of monocytes with virulent *C. burnetii* affects the monocytes' function. The transendothelial migration of monocytes infected by virulent *C. burnetii* is impaired, whereas that of monocytes infected by the avirulent form is not (21). Moreover, virulent *C. burnetii* elicits monocytes' production of tumor necrosis factor (TNF) through a mechanism involving both $\alpha_v\beta_3$ integrin and the interaction with the bacterial LPS (20).

The immunologic response, including monocyte function and cytokine production, in patients with either acute or chronic Q fever has been investigated. A major

finding was that *C. burnetii* survives in monocytes from patients with chronic infection and not in patients with acute Q fever and seronegative controls (19). These patients are unable to clear *C. burnetii* by themselves. When patients with endocarditis were cured, their monocytes were able to kill *C. burnetii*. Apparently the bactericidal impairment of the monocytes was related to a dysregulation of the cytokine network. TNF contained in monocyte supernatants from patients depressed the microbicidal activity of monocytes (19). The secretion of one of the soluble TNF receptors (TNF-Rs), TNF-R75, is increased in these patients and may be critical for the microbicidal defect (39). Finally, the monocyte microbicidal defect is associated with interleukin 10 (IL-10) overproduction, since neutralizing anti-IL-10 antibodies restored the microbial activity of monocytes in vitro (40). The upregulation of one of its soluble receptors (TNF-R75) by monocytes seemed to be critical in this effect (39). A study of T-lymphocyte subsets in patients with Q fever endocarditis showed that the number of CD4 lymphocytes was markedly decreased, resulting in a reduction of the CD4/CD8 ratio (91).

Incubation of monocytes from patients with Q fever endocarditis demonstrated an increase in the secretion of IL-10, which was also increased in patients who had relapsed after treatment of Q fever endocarditis (15). We feel that this could be a good indicator of relapse (15). Transforming growth factor β1 (TGF-β1) and TGF-β2 are also increased, but the increase is not related to the severity of the disease. The production of TNF-α and IL-1β is also increased in patients with endocarditis but is not related to relapse (40). The rates of secretion of cytokines in the plasmas of patients with acute and chronic Q fever have been compared. TNF-α, IL-6, and TNF-RII secretion increased in both groups. IL-1 receptor (IL-1R) antagonist was selectively increased in plasma of patients with acute Q fever, and soluble CD23 secretion was increased in plasma of patients with chronic Q fever. This last finding is in agreement with the large increase in the concentration of immunoglobulins that is seen in patients with Q fever endocarditis (13) (Table 2). The immunoglobulin G1 (IgG1) subclass is selectively increased during endocarditis as well as specific IgG and IgA antibodies directed against phase I of *C. burnetii*, the titers of which can reach as high as 10^6.

Biopsy specimens obtained from patients with acute Q fever show very few microorganisms and a strong cellular reaction. For example, *C. burnetii* is rarely obtained from cerebrospinal fluid samples of patients with meningitis and rarely detected in liver or bone marrow biopsy specimens. In bone marrow and hepatic biopsy specimens, a strong immune reaction is noted, with a typical granuloma surrounded by a fibrinoid ring. In contrast, during chronic infections, biopsy specimens (liver or heart valve) show many bacteria and a moderate cellular immune response (67). These findings can be compared to those in the different clinical forms of leprosy. In the tuberculoid form of leprosy, granulomas are noticed but few organisms are found. During lepromatous leprosy a high humoral response is associated with a large number of bacteria and a lack of cellular immune response. The latter form is associated with high secretion of IL-10, which is also found during Q fever endocarditis.

Together, these data show that Q fever endocarditis in human beings and in animals is associated with immunosuppression and/or damaged valves (Table 1).

Therefore, patients with acute Q fever who are immunocompromised or who have a cardiac valve lesion are particularly at risk to develop chronic infection and especially endocarditis.

At the opposite end of the immunological spectrum, some patients with acute Q fever apparently have an exaggerated immune response, including the formation of hepatic granulomas, and eventually exhibit autoantibodies and various abnormalities, including anti-smooth muscle and antimitochondrial antibodies and circulating anticoagulant. These patients frequently do not respond to antibiotic therapy and remain febrile until corticosteroids are used.

THE DISEASE

Q fever has such a wide spectrum of clinical manifestations that cases are found only when systematic testing is performed, and this is why it is said that "Q fever follows rickettsiologists" (83). Following primary infection, 60% of patients seroconvert without clinical manifestations (Table 3), and only 2% are hospitalized. In some hosts, a chronic infection develops, affecting less than 1% of all hosts who are acutely infected. However, as the majority of these patients are later hospitalized, the percentage of diagnosed cases is larger than that of acute infection in countries where Q fever is recognized; i.e., there is an ascertainment bias. The currently identified clinical manifestations represent only a partial spectrum of the disease. For example, pericarditis (59) and myocarditis (35) were not previously considered to be typical manifestations of Q fever, so only a few cases of Q-fever-related pericarditis and myocarditis have been reported in the literature. However, recently we reported a series of 1,070 cases of acute Q fever (90) with 13 cases of pericarditis and 11 cases of myocarditis. In a series of 1,127 patients from England and Wales (79) reported at the same time as our series, there were 18 patients with pericarditis and 9 with myocarditis. This shows that these two major clinical syndromes were not clearly identified until recently. Recently we have noted a high prevalence of Q fever during pregnancy in the small town of Martigues, near Marseille. The index patient was a nurse working in an intensive care unit, who became sick during the 3rd month of her pregnancy. She was diagnosed

Table 3. Clinical manifestations of Q fever

Type of infection	Prevalence or associated clinical condition(s)
Primary	
Asymptomatic	60%
Acute, self-limited	38%
Acute, hospitalized	2%
Acute, during pregnancy	Rare
Chronic	0.2–0.5%
Patients with valve lesions and/or cancer	Endocarditis
Patients with vascular aneurysm or prosthesis	Vascular infection
Patients with cancer	Osteoarticular infection
Pediatric patients	Osteoarticular infection

as suffering from Q fever, was treated for 3 weeks, and, after she was apparently cured, aborted a few weeks later. The obstetrician who performed the uterine curettage developed Q fever (89). *C. burnetii* was isolated from the placenta and fetus. This sequence of events was a shock to the nurse's colleagues at this hospital. Since then, any patient with a miscarriage or abnormal pregnancy is tested for Q fever. This practice has resulted in the largest known series of Q fever cases during pregnancy to date (103).

Acute Q Fever

Based on a study of 477 patients suffering from acute Q fever, the following symptoms were found (90): the onset is usually abrupt, and patients present with high fever (91%), headaches (51%), myalgias (37%), arthralgias (29%), and cough (34%) and less frequently present with a rash (11%) or a meningeal syndrome leading to a spinal tap (4%). Laboratory investigations show thrombocytopenia (35%), elevated liver enzymes (62%), and an elevated erythrocyte sedimentation rate (55%). The chest X ray is abnormal in 27% of patients (Table 4).

The clinical presentation varies from country to country in that one of the three major manifestations, i.e., isolated fever, hepatitis, or pneumonia, dominates. No clear reason for such variations has been identified. Four hypotheses have been proposed: a specific local interest in a clinical form, strain variability, route of infection (aerosol versus raw milk [i.e., oral route]), and host specificity. The last

Table 4. Clinical spectrum of Q fever

Parameter	Datum for:	
	France	United Kingdom
Authors	Raoult et al.	Pebody et al.
Reference	90	79
No. of cases	1,383	1,117
No. of cases of acute infection	1,070	
Prevalance of:		
Isolated fever	14%	
Pneumonia[a]	63%	47%
Hepatitis	60%	5%
Pericarditis	1%	2%
Myocarditis	1%	2%
Meningoencephalitis	1%	
Meningitis	0.5%	0.3%
Peripheral neuropathy	0.2%	
Erythema nodosum	50 cases	0.1%
Q fever during pregnancy	20 cases	
Chronic infection	293 cases	
Endocarditis	78%	50 cases
Vascular infection	9%	
Chronic hepatitis	3%	
Osteoarticular infection	2%	
Other infection	8%	

[a]Patients could have both pneumonia and hepatitis.

was recently substantiated in a large study (90) in which patients with either isolated fever, pneumonia, hepatitis, or a combination were compared. In this study patients with isolated fever were more likely to be male, while those with hepatitis were younger, had more direct contact with animals, and were less frequently immunosuppressed. Those with pneumonia were much older and more likely to be immunocompromised. To the best of our knowledge, this was the first report of an association of acute Q fever clinical symptoms with specific host conditions.

Clinically isolated fever, without pneumonia or hepatitis, is usually associated with severe headache, and in some patients it persists long enough to meet the criteria for fever of unknown etiology. Fever lasts longer in older patients (16), and it is more frequently associated with a rash (20%) than the other manifestations of Q fever. Pneumonia is the major clinical presentation of Q fever in Nova Scotia (Canada), the Basque region of Spain, and the United Kingdom. In our experience these patients have lower fever and fewer headaches, as well as a lower rate of thrombocytopenia and less elevation of the sedimentation rate than patients with other forms of acute Q fever. Hepatitis is the most common form worldwide, including in France and Australia. We observed a higher rate of complaints of various pains (headaches, myalgias, and arthralgias) in Q fever hepatitis patients than in others, and we noticed thrombocytopenia as well as an elevated sedimentation rate more frequently. Usually hepatitis is detected on the basis of increased liver enzymes; however, a few patients present with jaundice and/or hepatomegaly. When a liver biopsy is performed, it shows inflammatory granulomas. Typically, they are organized in the form of a doughnut; i.e., the granuloma has a lipid vacuole in its center and is surrounded by a fibrinoid ring (67). Q fever hepatitis patients frequently exhibit autoantibodies, including anti-smooth muscle antibodies, antinuclear antibodies, and antiphospholipid antibodies (77). Because of the presence of this "lupus anticoagulant," all patients with suspected Q fever hepatitis who are going to have a liver biopsy should have a platelet count, an international normalized ratio (for anticoagulant monitoring), a prothrombin time test, and a test for antiphospholipid antibodies prior to the biopsy.

Neurological manifestations of Q fever which occur in 1% of large series include meningitis, meningoencephalitis, and peripheral neuropathy (23). In our study (90), neurological involvement was frequently associated with occupational exposure and direct contact with animals. In fact, we were surprised that several patients were shepherds, a very rare profession in our area. We speculated that they would have been subjected to a larger inoculum or repeated infections.

Cardiac involvement occurs in 2% of acutely ill patients. This includes myocarditis (35), which is a major cause of death in patients with Q fever. The reason why these patients have such clinical features is unknown, but experimental data suggest that a large inoculum is more likely than lower infecting doses to result in myocarditis (56). Pericarditis is frequently nonspecific. *C. burnetii* has recently been demonstrated to be the most frequently identified cause of pericarditis in Marseille (59), and it is a major cause of pericarditis in Spain and England. Ten percent of persons with Q fever pericarditis develop chronic pericarditis and have recurrences for unidentified reasons.

Chronic fatigue following acute Q fever has been described in Australia and the United Kingdom (6, 80). Ayres et al. interviewed 71 patients 5 years after infection and compared them to controls (6). They found that the patients were more likely to have increased sweating, breathlessness, blurred vision, and undue tiredness. It was reported that patients with post-Q fever fatigue syndrome (20%) have moderate cytokine dysregulation (80). This may be related to geographic specificity, as it is not observed everywhere. In Marseille (unpublished data), among 80 patients from Martigues with acute Q fever, none exhibited asthenia lasting 6 months after the disease, compared to 37% in the United Kingdom (100). However, convalescent asthenia was noticed in 25% (unpublished data).

Q fever in immunocompromised hosts has been reported in patients with cancer (84) as well as in those with human immunodeficiency virus infection (88). Given the experimental data, these patients should be considered at high risk of developing relapses or chronic infection.

Long-term cardiovascular complications of Q fever have been reported by a Swiss team monitoring a large outbreak (62). These authors reported an increased mortality, mainly from stroke and cardiac ischemia, in these patients during a 12 year follow-up. The Swiss authors speculated that *C. burnetii* could be another infectious cause of atherosclerosis, as *Chlamydia pneumoniae* is postulated to be. This analysis has been criticized (114) because smoking could be a risk factor for Q fever, potentially biasing the study.

Twenty-three cases of Q fever during pregnancy have been reported (103). Among those, the majority have had complications, including fetal or newborn death (11 cases) and premature birth (7 cases); only five subjects in this study had a normal pregnancy. Half of the patients develop a serological profile of chronic Q fever during pregnancy, and, as in other mammals, *C. burnetii* can be isolated from the milk, the placenta, and the vaginal discharge. Multiple premature births have been reported in such cases (103), and these patients should probably receive prolonged treatment to eradicate the bacteria as they are subject to relapses during later pregnancies. Endocarditis has been documented as a complication of Q fever during repeated pregnancies in mice (102), but this has not been documented in humans. Patients who have had Q fever infection before pregnancy apparently do not experience relapse during pregnancy.

Patients with a valve lesion, arterial aneurysm, or arterial prosthesis who have an epsiode of acute Q fever are at very high risk for chronic infection (90). In a recent study, 38% of patients with acute infection and a valve lesion developed endocarditis within 2 years (27). This evolution to chronic Q fever was not prevented by doxycycline treatment alone but was prevented by treatment with a combination of doxycycline and hydroxychloroquine. This study emphasized the importance of recognizing Q fever in patients with valve lesions and of identifing valve lesions in patients suffering from Q fever. In the general population, it is believed that 1 to 2% have a valve lesion (27). In such patients, if one-third develop endocarditis, this means that 0.3 to 0.6% of those who have symptomatic or asymptomatic Q fever will develop endocarditis. This may explain why in the large Swiss survey (62) an increased risk of endocarditis could not be identified, as only 1 or

2 out of 451 infected patients would be expected to have endocarditis; hence, the power of the study to detect this rate of endocarditis was too low.

Chronic Q Fever

The major clinical form of chronic Q fever, and the best known, is endocarditis. A minimum of 800 cases were reported from 1949 to 2000 (8, 25, 90, 93, 99). The places where the disease is most frequently reported are Great Britain and Ireland (277 cases), France (264 cases), Spain (62 cases), Israel (35 cases), Switzerland (21 cases), Australia (18 cases), and Canada (10 cases). In France, Q fever causes 5% of endocarditis cases, with an estimated prevalence of 1 per 10^6 inhabitants per year, which is close to what has been observed in Israel and Switzerland. In comparison to other patients with endocarditis (9), patients with Q fever endocarditis are younger (mean age, 48 years). They nearly all have a previously identified valve lesion and frequently have a prosthetic valve.

When we compiled data from the five most recent series (Table 5), 53% of 118 patients had a prosthetic valve when diagnosed and 99% had a valvular abnormality. The clinical presentation of patients with Q fever endocarditis varies according to the delay in diagnosis. With a long time to diagnosis, the frequency of visceral involvement increases. For example, in a series of 15 patients that one of us presented in 1987 (86), 12 had hepatomegaly, 7 had splenomegaly, 7 had increased transaminases, and 7 had thrombocytopenia, the diagnostic delay being estimated at 18 months. Among the last 13 patients (with a 3-month median delay in diagnosis) that the same author recently studied, 1 patient had hepatomegaly, 1 had splenomegaly, 3 had increased transaminases, and 3 had thrombocytopenia (unpublished data). The typical clinical and laboratory findings in Q fever endocarditis are shown in Table 6.

The diagnosis of Q fever endocarditis is easy if it is considered as part of the differential diagnosis, but frequently a diagnosis of endocarditis is not made for these patients. In fact, they are usually afebrile or suffer low-grade, intermittent fever. The echocardiography is frequently inconclusive and fails to identify vegetations. Therefore, in patients with no fever and no vegetations, the diagnosis of endocarditis could easily be missed. The clue to diagnosis is a patient having a

Table 5. Recent series of cases of Q fever endocarditis (1997 to 2000)

Yr of publication	Reference	Country	No. of cases	Mean age (yr)	No. of patients with:			Mortality (%)
					Cardiovascular predisposition	Valvular prosthesis	Valve replacement	
1997	99	Israel	35	52	35	19	14	5
1998	25	Switzerland	21	47	21	6	15	2
1999	8	Ireland	7	53	6	2	5	1
1999	87	France	35	47	35	20	20	2
2000	93	Spain	20	42	20	15	14	8
Total[a]			118	48	99%	53%	58%	15

[a]Units for totals are the same as those given in the table column head unless otherwise indicated. The total value given for the mean age column is the overall mean for the 118 cases studied.

Table 6. Clinical symptoms and signs and laboratory findings in Q fever endocarditis patients[a]

Finding	% of patients
Clinical	
Male sex	76
Valve involved	
Aortic	33
Mitral	50
Both	17
Fever	68
Cardiac failure	67
Hepatomegaly	56
Splenomegaly	55
Clubbing of digits	37
Purpuric rash	19
Arterial embolism	21
Death	57
Biological	
Leukocytosis	25
Leukopenia	15
Increased transaminase levels	40–83
Thrombocytopenia	26–56
Anemia	40–55
Increased creatinine level	65–73
Elevated sedimentation rate	88
Increased gamma globulin	94
Circulating immune complexes	90
Antinuclear antibodies	35
Rheumatoid factor	60
Smooth muscle antibodies	40

[a]Adapted from reference 67.

known valve lesion with unexplained illness (fever, hepatitis, weakness, digital clubbing, weight loss, stroke, or renal insufficiency) or an elevated sedimentation rate, increased transaminases, or thrombocytopenia. In such cases serology for Q fever should be ordered, and then the diagnosis is easy to confirm using modified Duke criteria (33).

The prognosis of chronic Q fever has changed dramatically over the past few years. The mortality rate was 37% among 76 patients from seven series compiled in 1987 (86), and it is now 15% among 116 patients reported from 1997 to 2000 (Table 6). In our experience, among those diagnosed during the past 7 years, the mortality rate is under 5% (87). This is probably related to earlier diagnosis and prolonged treatment with a combination of antibiotics (99) as well as more-careful follow-up.

Vascular infection is the second most commonly identified form of chronic Q fever. We have diagnosed 25 cases in our laboratory (34, 90), and 6 more were reported from other centers, in countries including England, the United States, Switzerland, and Australia (25, 26, 28, 72). An aortic aneurysm may be infected and therefore can be complicated by an aortoenteric fistula or spondylitis. Vascular prostheses can also be infected by *C. burnetii*. The prognosis is poor when the

infection is untreated. The treatment of an infected blood vessel is the same as that for endocarditis except that removing the infected prosthesis is probably necessary for cure.

Other manifestations of chronic Q fever include osteomyelitis (17), chronic hepatitis diagnosed in patients with alcoholism (90), pseudotumor of the spleen, pseudotumor of the lung, and infection of a ventriculoperitoneal drain (61). Recently, a case of vasculitis and pulmonary amyloidosis (48) following acute Q fever and resulting in the death of the patient was reported.

DIAGNOSIS

The diagnosis of Q fever is currently based on serology. Antibodies are detectable usually 2 to 4 weeks after the onset of the infection. The diagnosis of acute Q fever is confirmed by seroconversion (a fourfold or greater increase in antibody levels between acute- and convalescent- [10 to 20 days later] serum sample or the presence of an anti-*C. burnetii* IgM) (36).

With chronic Q fever, a single serum sample is diagnostic of both Q fever and of chronic disease when high titers of antibody to phase I antigen are detected. The complement fixation test lacks sensitivity in our hands and should be replaced by microimmunofluorescence, the reference method. This allows testing for antibodies to both phases I and II and determining the IgG, IgM, and IgA antibody titers. Titers of phase II IgG and IgM antibodies of ≥200 and ≥50, respectively, are diagnostic of acute Q fever. A high titer of IgG (≥1,600) against phase I but also against phase II antigen has a high predictive value for chronic infection (108). In this form of the disease, IgM-specific antibodies are frequently lacking and an increase in IgA antibodies against both phases is present. In order to avoid false-negative and false-positive results, IgG should be removed prior to testing for IgM and IgA antibodies (108). An enzyme-linked immunosorbent assay has also been used in the serological diagnosis of acute Q fever, especially for the detection of IgM antibodies (30) (Table 7).

Microimmunofluorescence serology is also convenient for the follow-up of patients with chronic infection. In patients with Q fever endocarditis, when IgM

Table 7. Diagnostic tests for Q fever

Clinical presentation	Serology (ELISA[a] for IgM)	Titer as determined by microimmunofluorescence						Culture	PCR
		Anti-phase II			Anti-phase I				
		IgG	IgM	IgA	IgG	IgM	IgA		
Acute infection	+	≥200	≥50	NT[b]	<800	≥50	NT	Blood before treatment	Questionable
Endocarditis	+/−	≥1600	+/−	≥50	≥1600	+/−	≥50	Blood, valve	Valve

[a]ELISA, enzyme-linked immunosorbent assay. Results: +, positive; +/−, indeterminate.
[b]NT, not tested.

antibodies are present at the beginning of the treatment they disappear first, usually within 6 months of treatment. IgA decreases next, and IgG (87) decreases later but never disappears.

Culture of *C. burnetii* must be done in biosafety level 3 laboratories. A recently developed cell microculture system, the shell vial assay designed for virus isolation, is especially efficient (36). One of us (D.R.) isolated 102 *C. burnetii* strains by using this system (unpublished data) (Color Plate 1 [see color insert]). Human embryonic fibroblasts are grown on a coverslip within a vial and inoculated with either blood or ground-up biopsy specimens. *C. burnetii* is detected by immunofluorescence and/or PCR, 6 days after inoculation. *C. burnetii* was isolated from the blood of 15% of patients with acute Q fever, when the blood was sampled early in the disease and prior to antibiotic treatment, and from 53% of patients with endocarditis prior to treatment.

Genomic amplification using PCR is an attractive diagnostic technique. Several genes have been used to generate specific primers, including those encoding 16S rRNA, 23S ribosomal DNA, superoxide dismutase, plasmid-based sequences, and the IS*1111* multicopy insertion sequence (36). The amplification of *C. burnetii* DNA from tissues (e.g., placenta and heart valve vessels) is usually easy and efficient. The amplification of *C. burnetii* DNA from serum samples is less reproducible, and we have failed to reproduce in our laboratory published results from other centers (36). *C. burnetii* can also be detected in biopsied tissue by immunohistochemistry (Color Plate 2 [see color insert]). This is especially useful because it allows the retrospective study of paraffin-embedded valves (10).

EPIDEMIOLOGY

Reservoirs

Q fever is a worldwide zoonosis. The reservoir is very large and includes mammals, ticks, and birds. Mammals are often asymptomatic. They are infected through aerosols, by drinking raw milk, and possibly by sexual intercourse (67). During pregnancy the infection is reactivated and *C. burnetii* multiplies in the uterus, the placenta, and the mammary glands. This can result in abortion, low birth weight, and prematurity for the fetus (31). After delivery, the placenta is a major source of contamination, as it contains 10^9 organisms per g (7). When interviewing a patient with suspected Q fever, one should ask about recent contact with parturient mammals. Domestic ungulates (cattle, goats, and sheep) represent the major identified sources of infection for humans. This explains why, in many places, annual reporting of cases of Q fever parallels the time when livestock give birth (31, 109). In developed countries, direct contact with livestock is diminishing, and therefore this risk should be associated with a decrease in prevalence of Q fever, as observed in California (31). However, in many areas, students visit farms and exhibitions involving livestock as part of school expeditions, and Q fever occurs. This has happened recently in France (unpublished data) and in Quebec, Canada. In Europe, sheep are transported to mountain pastures in the spring with their lambs and return

in the autumn through towns and villages, where contaminated aerosols infect the inhabitants (Table 8).

Mammalian pets, including dogs, cats, and rabbits, especially at the time of parturition, represent a newly identified source of infection (Table 8). Recently, the wild brown rat was implicated in transmitting infection between farm animals and cats (113). Birds shed *C. burnetii* in their feces and therefore can cause Q fever, as was recently demonstrated when a family outbreak of Q fever was related to the presence of infected pigeon feces (104).

Ticks can be infected with *C. burnetii* but apparently do not represent a major source of infection for human beings.

Transmission

The major route of transmission of Q fever to humans is by aerosol. Inhalation of infected dust could result directly from parturient fluids. The organism can adhere to wool and dust and can be spread by the wind to distant places (31). We recently demonstrated that, in an area near Marseille, the temporal distribution of cases of Q fever resulted from the association of lambing and windy days (109). Cases of Q fever occurred miles away from the lambing but in the direction that the winds blew.

Ingestion of milk and milk products is a risk factor for transmission of Q fever. Raw milk from cows was considered a risk factor for Q fever in California, and the decrease in the prevalence of Q fever in this state could be partly related to cessation of the practice of drinking raw milk (31). The consumption of goat cheese made from raw milk was also reported as a risk factor in France (32). More re-

Table 8. Recently reported outbreaks of Q fever

Source	Yr reported	Country	No. of cases	Specificity	Reference
Sheep	1981	United States	81	Medical school	71
	1982	England	14	Laboratory	42
	1983	Switzerland	415	Alpine pasture	24
	1993	Italy	58	Alpine pasture	64
	1996	Germany	45		27
	1996	Germany	18		94
	1996	France	204	Abattoir related	5
Cattle	1982	United States	25	University	42
	1996	Poland	25		110
Cats	1984	Canada	13	Family	53
	1988	Canada	12	Poker players	55
	1989	United States	15	Family	81
Goats	1992	France	40	Cheese	
	1998	Slovakia	113	Aborting goats	54
	2000	Canada	62	Cheese	32
Rabbits	1986	Canada	4		65
Pigeons	2000	France	4	Family	102
Dogs	1996	Canada	3	Family	11

cently, in Canada, an outbreak of Q fever was related to the consumption of pasteurized goat cheese (51).

Among recent outbreaks, seven were described following contact with sheep (Table 8). Interestingly, three outbreaks followed the use of infected sheep in a laboratory, faculty, or university in England or the United States and three occurred in association with sheep migration.

Although many cases are suspected to be related to goats, only three outbreaks have been reported, and two of these were associated with cheese consumption. In the last goat-related Q fever outbreak, in Canada, one of the risk factors was consumption of pasteurized cheese. This is disappointing, as pasteurization was considered a safe way to decontaminate milk. New sources have been demonstrated with two outbreaks related to parturient cats in Canada and the United States, as well as one associated with dogs, one with rabbits, and one with pigeons.

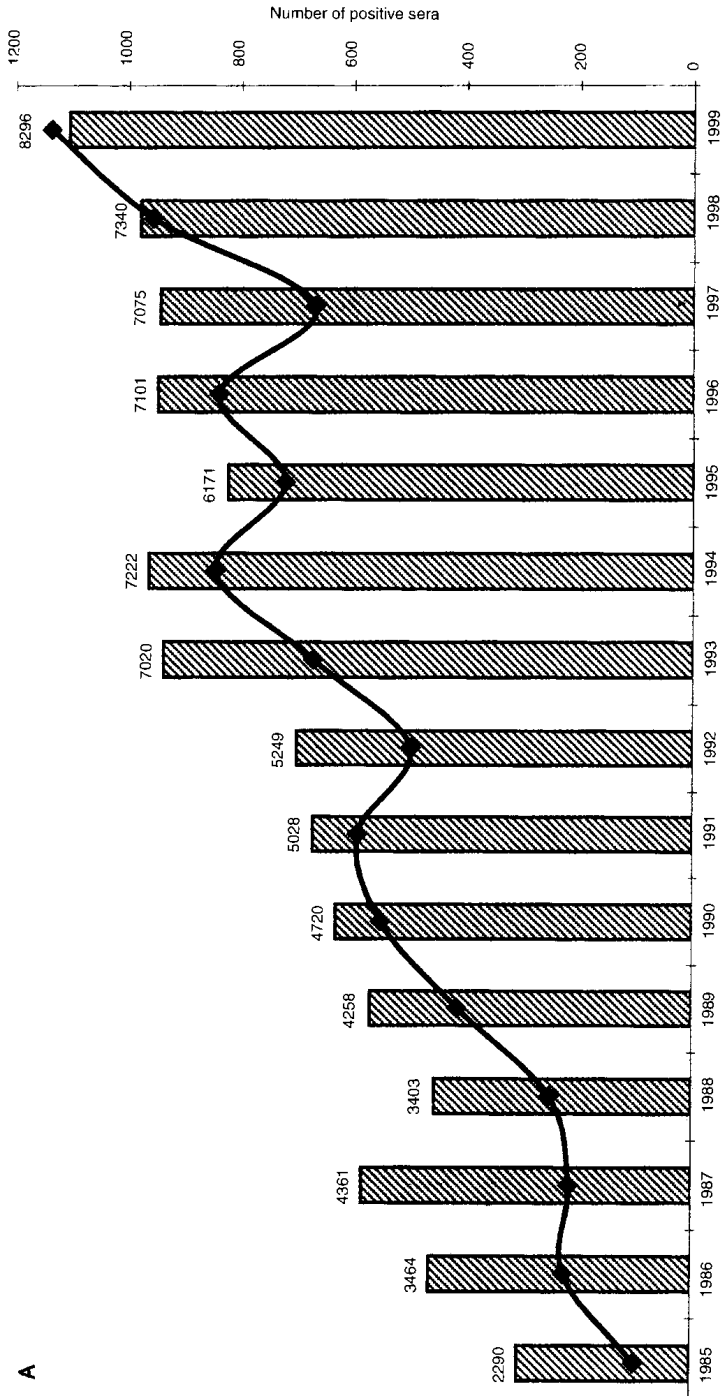
Age and Sex

The risk of exposure to a contaminated source should determine the prevalence of Q fever. However, in any study of Q fever, the disease is most common in middle-aged males (62, 90). In our study children under 9 years of age had a lower relative risk of infection and adults between 50 and 59 years of age had a higher risk. The risk of infection as evaluated by seroprevalence studies in the same area does not reflect the same bias (109). Males are more frequently diagnosed with Q fever in France, with a male/female ratio of 2.45, but the seroprevalence is not higher in males (109). These data apparently show that there is a increased risk of being symptomatic and/or diagnosed as having Q fever in males and in middle-aged patients when exposed to *C. burnetii* but not an increased risk of exposure.

Geographical Repartition

The disease is prevalent on six continents and in any country where testing for Q fever has been carried out, with the exception of New Zealand (49). In North America, Q fever is prevalent in Canada; a few cases have been recently reported in the United States (Maine [81], Texas [72], and North Dakota [41]), but no active surveillance is carried out there. Few data are available from Latin America, but the disease is prevalent in Cuba (49), Uruguay (49), El Salvador (49), Mexico (92), and Argentina (unpublished data).

In Europe outbreaks and series of cases have been reported from France (1,383 cases) (90). In the Marseille National Reference Center, the number of annually detected cases rose from 99 to 1,138 from 1984 to 1999. A total of 8,681 patients over 15 years had IgG antibodies of $\geq$200, as shown by a microimmunofluorescence test. Of those, we had clinical information allowing us to diagnose 1,269 cases of acute Q fever and 334 cases of chronic Q fever (annual report of the National Reference Center, Marseille, 2000) (Fig. 1). A large series of 1,117 cases was reported from the United Kingdom (79). Q fever has also been reported from Ireland (79), Switzerland (25), Italy (7), Spain (93), Greece (101), Slovakia (96), Poland (110), Germany (27), and Sweden (63). In Bulgaria, following dramatic political and economic changes, the epidemiology of Q fever changed (96). An


A
Number of positive sera
1200
1000
800
600
400
200
0
8296
7340
7075
7101
6171
7222
7020
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3403
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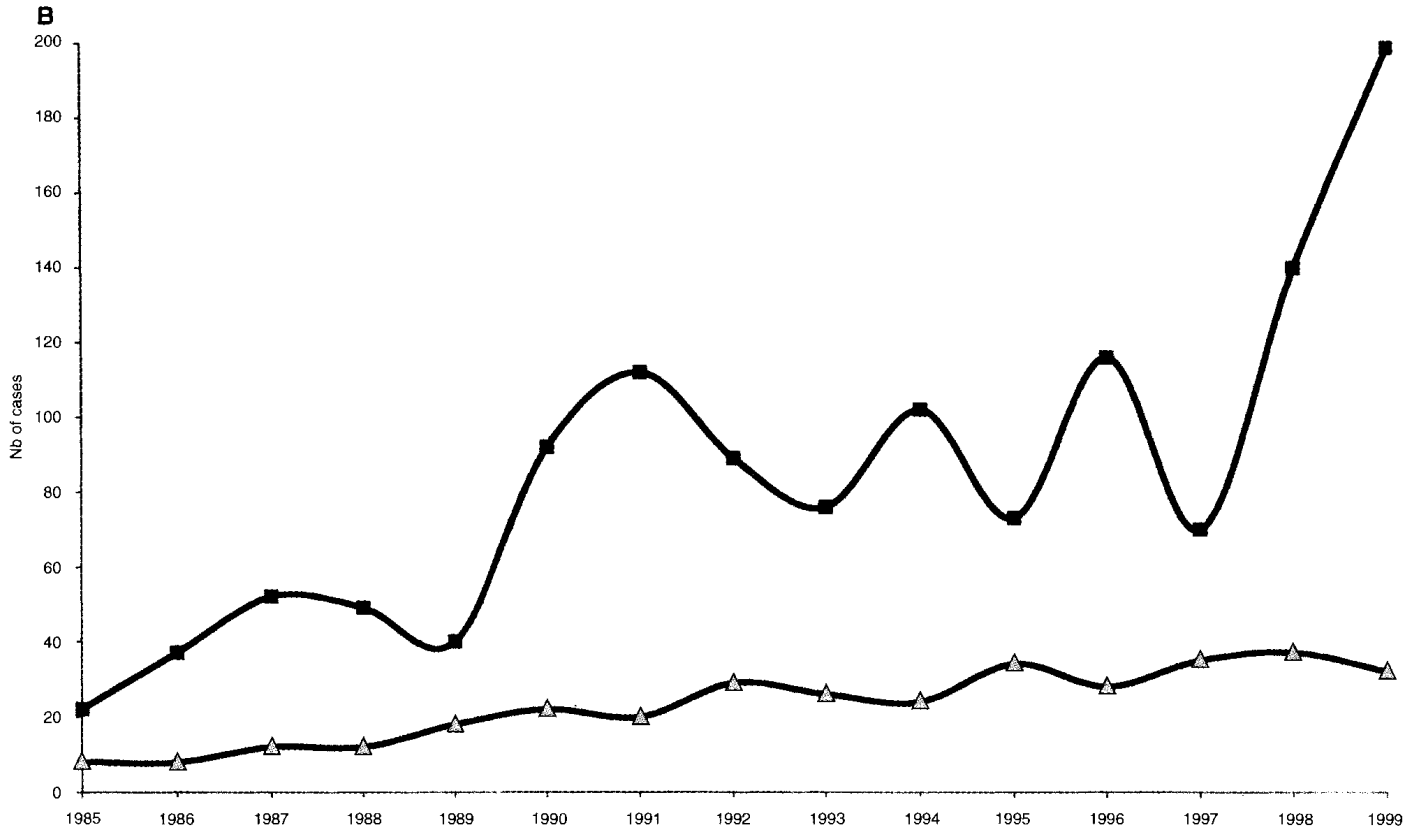

Figure 1. Number of cases of Q fever in France as diagnosed at the National Reference Center (1985 to 1999). (A) Number of submitted sera (bars) and number of positive sera (IgG titer of $\geq 1/200$ by immunofluorescence) (diamonds). (B) Number of recorded observations of acute (squares) and chronic (triangles) Q fever.

increase in the incidence of Q fever followed an increase in the the number of goats from 430,000 to 1,000,000 and the massive domestic consumption of unpasteurized milk.

In Asia, cases have been reported from Israel (99), Saudi Arabia (29), Taiwan (52), Japan (47), and Malaysia (105).

In Africa, recent studies emphasize the presence of Q fever in Morocco (73), Tunisia (76), Algeria (unpublished data), Zimbabwe (50), Nigeria, Tanzania (3), Zambia (75), and Mauritania (74) and in Angola, Burkina Faso, the Central African Republic, Cameroon, Congo, Mali, and the Ivory Coast (107). Together, the seroepidemiological findings suggest that these countries have the highest prevalence of Q fever in the world despite the lack of reported cases. It has been observed in Africa that the prevalence of Q fever is partly coincident with the time of an Islamic holiday (107) because of the contact with sheep, once a year, for the annual religious festival.

In Australia, 202 to 860 cases of Q fever are reported annually. A total of 2,635 cases were reported from 1991 to 1994 (38). The national notification rate was 4.9/100,000 inhabitants in 1993. Abattoir workers in Australia are vaccinated against Q fever (2).

TREATMENT

The goal of the specific treatment varies with the various clinical syndromes of Q fever. During acute Q fever in the normal host, when an antibiotic regimen inhibits bacterial multiplication (bacteriostatic compound), it allows the immune system to rapidly control the infectious process. In patients with acute Q fever and an exaggerated immune response, such as those with hepatitis and autoantibodies, antibiotics usually fail to control the disease, which is rapidly cured by the addition of corticosteroid therapy.

Patients with impaired immunity, pregnancy, or valvular or vascular lesions are usually unable to control the multiplication of *C. burnetii*. Therefore, antibiotic treatment should be either bacteriostatic and prescribed for the duration of the immune defect, including lifelong therapy for vascular lesions and for the duration of the pregnancy, or, ideally, bactericidal. Studies with human beings showed that several antibiotic regimens failed to eradicate *C. burnetii* and therefore relapses occurred when treatment was interrupted. Moreover, study of heart valves removed from patients receiving long-term antibiotic therapy showed that *C. burnetii* was still present. A model of in vitro infection demonstrated that no antibiotic compound was bactericidal (66, 85). The reason for this lack of efficacy of antibiotics was suspected to be the low pH of the cell vacuole where *C. burnetii* resides and where the antibiotic has to act. Such a low pH (4.8) inhibits the activity of most antibiotics, with the exception of rifampin, in vitro. The increase of the intracellular pH by chemical lysosomotropic agents, such as chloroquine, restored the bactericidal effect of doxycycline. The combination of doxycycline with chloroquine is the only bactericidal regimen that can be used safely in humans and therefore is theoretically the best regimen for patients who need a bactericidal regimen.

Current Recommendations

Acute Q fever often resolves spontaneously. It should be treated when the patient is diagnosed during the acute phase. Frequently, the diagnosis is confirmed when the patient has already undergone spontaneous cure, which explains why so few studies have been performed to evaluate the benefit of antibiotic therapy for Q fever (82). However, a single study demonstrates the superiority of treatment with tetracyclines over no treatment. Therefore, doxycycline (200 mg/day for 2 to 3 weeks) is the recommended treatment for adults.

Acute Q fever with major inflammatory response is treated by adding a 7-day course of prednisone (40 mg/2 days) to doxycycline (60). Prednisone may also be indicated when patients fail to respond to 7 days of doxycycline and remain febrile (18, 37, 60).

Acute Q fever during pregnancy cannot be treated with doxycycline; therefore, either rifampin or co-trimoxazole, each of which is a bacteriostatic agent relatively well tolerated during pregnancy, can be used. We have used co-trimoxazole until delivery for six pregnant women, all of whom were successfully treated; however, four had a premature infant. This contrasts with seven abortions or stillbirths among eight women who had Q fever that was not treated during pregnancy. Q fever during pregnancy should be treated with co-trimoxazole (800 mg of sulfamethoxazole/25 mg of trimethoprim daily) for the duration of the pregnancy. The red blood cell count should be monitored during the pregnancy, and eventually the patient should receive folic acid and folinic acid if co-trimoxazole-related macrocytic anemia is noticed.

Acute Q fever in patients with valvular lesions should be treated, as approximately one-third to one-half of these patients will develop endocarditis in the following months or years. We recently demonstrated that doxycycline alone did not prevent subsequent endocarditis in these patients. Therefore, in this setting we recommend treatment for 1 year with doxycycline and hydrochloroquine. Our preliminary results have been satisfactory (see above).

Chronic Q fever endocarditis has had a poor prognosis, and the best results have been obtained from prolonged therapy with a combination of antibiotics (25, 87, 93, 99). The combination of doxycycline and either fluoroquinolones, rifampin, or co-trimoxazole for 3 years or more and the combination of doxycycline and hydroxychloroquine for 18 to 36 months are effective (87). In any case treatment should not be interrupted until the serology returns to a nonchronic profile, i.e., an anti-phase I IgG titer of $\leq$800 and an IgA titer of $\leq$25. Currently, we have considerable experience with long-term treatment with doxycycline (200 mg daily) plus hydroxychloroquine. The dosage of hydroxychloroquine is adjusted on the basis of levels in plasma, which should be 1 $\pm$ 0.2 μg/ml. Usually, we start with 600 mg daily, and the dosage should be reduced 3 to 12 months after the onset of therapy. This treatment along with alternative treatments carries a major risk of photosensitization, and patients should avoid any sun exposure. Retinal accumulation of hydroxychloroquine should also be monitored every 6 months (87). In our experience valvular surgery is required only for hemodynamic reasons. The removed valve should be sent to a special laboratory to detect the presence of *C.*

burnetii by immunohistochemistry, culture, or PCR amplification. A negative result allows the stopping of treatment. The patient should be monitored for years after completion of treatment, as early and late relapses do occur.

VACCINATION AGAINST Q FEVER

Vaccination is a logical strategy to try to prevent Q fever among humans at high risk for this infection and among animals. A formalin-inactivated Q fever vaccine (Q-Vax; Commonwealth Serum Laboratories), prepared from phase I *C. burnetii* Henzerling strain, was approved for use in Australia in March 1989. Prevaccination testing is necessary to ensure that those who are to be vaccinated are seronegative and skin test negative. Even with this precaution up to 40% of subjects receiving the vaccine reported local effects consisting of tenderness or soreness at the site of the injection or, less commonly, severe pain (78). Two of more than 5,000 vaccinees in Australia developed an abscess at the site of inoculation (78). A single dose of 30 μg of the Australian vaccine confers immunity by 10 to 15 days after administration, and immunity lasts at least 5 years (78). In areas where Q fever is endemic, the following groups should be offered vaccination: abattoir workers; those who work with cattle, sheep, or goats; and dairy workers.

Vaccination of cattle, sheep, and goats in these areas is also indicated, although few data are available to indicate the effectiveness and cost benefit of such a strategy in eliminating Q fever. A strategy that could be implemented is to select only seronegative animals as breeding stock and immunize these animals. In addition, animals that are to be shipped from one geographic area to another in a country or between countries should be tested for antibodies to *C. burnetii*, and only seronegative animals should be shipped. Before shipping, these animals should be vaccinated.

CONCLUSIONS

Q fever is a worldwide zoonosis caused by *C. burnetii*. The main reservoirs are pets and ungulates. Due to its nonspecific clinical presentation, only active surveillance can provide information on its incidence in a specific geographical area. Q fever is a protean disease with a wide spectrum of manifestations. Acute Q fever is the primary infection, and it may be asymptomatic or may present as prolonged fever, pneumonia, or hepatitis. More rarely it could present as encephalitis, mycorditis, or pericarditis. In the normal host, it is a self-limited illness which resolves in a few days, with the exception of some severe cases and some cases with prolonged fever associated with an exaggerated immune response. In other hosts (those who are immunocompromised or pregnant or have valvular or vascular lesions), Q fever may become chronic. In pregnant women it may cause abortion, in patients with valvular or vascular lesions it can cause endocarditis, and in patients with cancer it may cause endocarditis or prolonged fever. Chronic Q fever endocarditis was frequently fatal in the past, and a major benefit has been found with combination antibiotic therapy. The combination of hydroxychloroquine and doxycycline is currently the most effective therapy. The diagnosis of Q fever is easy when it is

considered as part of the differential diagnosis, and the main diagnostic test for Q fever is microimmunofluorescence serology.

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Emerging Infections 5
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Chapter 4

Emerging from Obscurity: Understanding Pulmonary and Extrapulmonary Syndromes, Pathogenesis, and Epidemiology of Human *Mycoplasma pneumoniae* Infections

Deborah F. Talkington, Ken B. Waites, Stephanie B. Schwartz, and Richard E. Besser

Few bacteria have ever generated more confusion and controversy than have mycoplasmas. Because they lack a cell wall and are very small for bacteria, mycoplasmas historically were misidentified as viruses and "L-form" variants of other bacteria without cell walls. Their temporary classification as viruses and fastidious growth requirements set the stage early for the use of diagnostic techniques better suited to viral detection and for the well-known difficulties of diagnosing infections in the clinical laboratory. For decades, human infections with *Mycoplasma pneumoniae* have been considered by many in the medical practice and teaching communities to be infrequent, confined to the respiratory tract, and largely self-limiting. A review of the literature provides ample evidence that it is time to change these misconceived notions about human mycoplasma infections, and new detection assays provide much better tools for the clinician as well as tremendous potential for unlocking the secrets of mycoplasma pathogenesis.

In this chapter we explore the fascinating biology and history of human mycoplasma infections with a focus on disease caused by *M. pneumoniae*. Clinical symptoms and advances in diagnosis, treatment, and prevention of *M. pneumoniae* infections are discussed, along with evidence that extrapulmonary complications due to *M. pneumoniae* are more common and potentially serious than generally perceived. We also address the association of *M. pneumoniae* infections with asthma.

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BACKGROUND

The first mycoplasma to be cultured was the bovine pleuropneumonia agent (now known as *Mycoplasma mycoides* subsp. *mycoides*) in 1898 (149). Because they were able to pass through traditional bacterial filters but not viral filters, the few mycoplasmas identified in the early 20th century were considered to be viruses until Klieneberger in the 1930s introduced the concept that mycoplasmas were L forms of bacteria lacking cell walls, living symbiotically with other walled bacteria (162). Thus began a spirited debate pitting those who believed that mycoplasmas were unique species against those who believed that mycoplasmas were wall-less variants of other known bacterial species undeserving of unique taxonomic representation (162). This controversy was not completely settled until the 1960s when G+C content assays and hybridization assays showed that mycoplasmas were indeed unique species.

The term "mycoplasma" (Gr. *mykes*, fungus; Gr. *plasma*, formed) emerged in the late 1950s (53) as a less linguistically challenging replacement for the term "pleuropneumonia-like organisms." The allusion to a fungus-like growth pattern in the name happens to describe only the growth of *M. mycoides*, but the term was nevertheless adopted. As the single order of mycoplasmas grew to three orders by the late 1960s, mycoplasmas were elevated to a class named *Mollicutes*, which derives from Latin words meaning soft ("mollis") and skin ("cutis"). The term *Mollicutes* is now used in general to refer to the current four orders and over 150 species of known mycoplasmas. Thirteen species in the genus *Mycoplasma* are associated with human colonization or infection (Table 1).

A brief history of the identification of *M. pneumoniae* illustrates the struggle to understand the taxonomy of mycoplasmas. In 1944, Eaton et al. succeeded in isolating a filterable agent from the sputum of a patient with primary atypical pneumonia using tissue culture (52). Tests on volunteers and field studies conducted in

Table 1. Members of genus *Mycoplasma* known to colonize or infect humans[a]

Species	Site(s) of localization (incidence)
M. pneumoniae	Oropharynx, nasopharynx, lower respiratory tract, many extrapulmonary sites (varies)
M. hominis	Urogenital (common), respiratory tract (uncommon)
M. genitalium	Urogenital, respiratory tract (uncommon?[b])
M. orale[c]	Oropharynx (common)
M. salivarium[c]	Oropharynx (common), urogenital (uncommon)
M. fermentans	Urogenital, systemic (uncommon?)
M. pirum	Systemic, urogenital (uncommon)
M. penetrans	Systemic (uncommon)
M. primatum	Oropharynx, urogenital (uncommon)
M. faucium	Oropharynx (uncommon)
M. buccale	Oropharynx (uncommon)
M. lipophilum	Oropharynx (rare)
M. spermatophilum[c]	Urogenital (rare)

[a]Adapted from reference 112.
[b]Uncertain whether incidence is uncommon.
[c]Found as commensal flora in healthy individuals.

the 1950s and 1960s provided definitive evidence that the Eaton agent caused lower respiratory tract infections in humans (31, 32, 35, 124). During this time the Eaton agent was considered to be a virus until it became clear that antibiotics could be effective against it, and in 1961 Marmion and Goodburn postulated that the Eaton agent was a pleuropneumonia-like organism (129). In 1962 Chanock and colleagues succeeded in culturing the Eaton agent on cell-free medium and proposed that it be assigned to the order *Mycoplasmataceae* (33), and in 1963 the taxonomic designation *Mycoplasma pneumoniae* was proposed (34).

Mycoplasmas have intriguing biologic idiosyncrasies. They represent the smallest self-replicating organisms that are capable of cell-free existence. The cells of *M. pneumoniae* are only 1 to 2 μm long and 0.1 to 0.2 μm wide, compared with a typical bacillus of 1 to 4 μm long and 0.5 to 1.0 μm wide (205). Accordingly, the *M. pneumoniae* cell volume is typically less than 5% of that of a typical bacillus. The small size and volume of mycoplasma cells allow them to pass through 0.450-μm-pore-size filters commonly used to filter sterilize media.

The small mycoplasma cell is accompanied by a small genome. Because *Mycoplasma genitalium* has one of the smallest genomes of any bacterium (580,020 bp and about 500 predicted genes), its genome was one of the first bacterial genomes to be completely sequenced (71). At 816,394 bp and about 700 predicted genes, the genome of *M. pneumoniae* was completely sequenced in 1996 (87). In contrast, *Escherichia coli* weighs in at 4.6 million bp and about 4,300 genes (167). The small mycoplasma genome sparked another debate in the 1970s concerning evolutionary heritage. Did the small genome size reflect a primordial state that was a stepping stone to larger genomes and walled bacteria, or was the small genome the result of a gradual reduction in size from a larger ancestral genome under strong but unknown evolutionary pressures? Studies of 16S rRNA sequences suggest that mycoplasmas and members of the gram-positive lactobacillus group (including *Bacillus*, *Streptococcus*, and *Lactobacillus* species) evolved from a common ancestor, thus favoring the latter hypothesis (127). The term "degenerative evolution" has been applied to the process by which mycoplasmas lost genetic material and lost complexity (127).

Mycoplasmas in general have a low ratio of G+C to A+T in their genomes and a high rate of mutation (127, 171). Another unique genomic characteristic in *Mycoplasma, Ureaplasma,* and *Spiroplasma* species is that the mRNA codon UGA is translated as tryptophan instead of as a stop codon as it is in other eubacteria (92, 171).

As one might assume from the presence of a relatively low number of genes, mycoplasmas as a group have less synthetic capacity and a much greater requirement for nutrients in artificial media for adequate growth than do other bacteria. Given the fact that most mycoplasmas have a parasitic relationship with eukaryotic cells, the obvious source of the many nutrients that cannot be synthesized is the host cell. To obtain nutrients from host cells, *M. pneumoniae* and many other mycoplasmas have developed specialized tip organelles that mediate extracellular attachment (Fig. 1). In some cases mycoplasmas may invade the cell and become intracellular, but they are not obligate intracellular pathogens as are chlamydiae, for example (discussed in greater detail later).

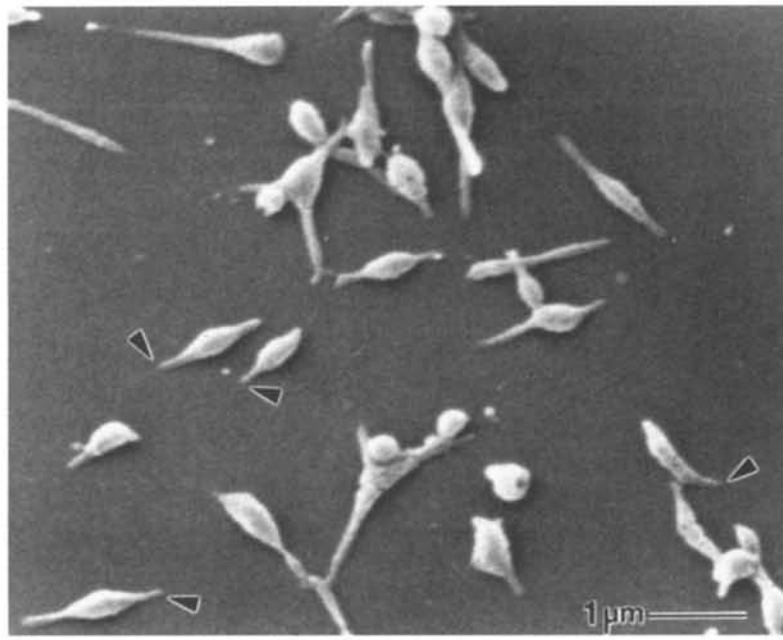

Figure 1. Scanning electron micrograph of whole *M. pneumoniae* cells. Arrowheads indicate terminal structures (tip organelles). Reprinted from reference 112 with permission.

Of the more than 100 known species of the genus *Mycoplasma,* only a few are associated with disease in humans (recently reviewed in reference 12). Principal among these is *M. pneumoniae*. Two species, *Mycoplasma salivarium* and *Mycoplasma orale*, are normal flora in the throat and nasopharnyx of most healthy humans. This normal colonization by these species must be carefully considered when designing methods of detection for *M. pneumoniae* based upon nucleic acid. Although mycoplasmas are much better known to the medical community for causing disease in humans, they are ubiquitous in nature, causing disease in animals, plants, and insects. In laboratory settings, their predilection for chronically infecting cell cultures and adulterating experiments makes them well known, if not well understood.

The strategy for serologic diagnosis of *M. pneumoniae* was strongly influenced by early assumptions that mycoplasmas were viruses. This led to the development of complement fixation (CF) tests for *M. pneumoniae* diagnosis. The sensitivity and specificity of CF tests for viral infections are due in part to the relatively low number of viral antigens, which reduces the chances of cross-reactivities. Because of the much larger complement of bacterial antigens in *M. pneumoniae*, the CF test for *M. pneumoniae* suffers from decreased sensitivity and specificity, limiting its usefulness in serologic testing. In addition, *M. pneumoniae* has complex requirements for growth on artificial media that few non-research laboratories could easily produce. Therefore, it was not until the 1970s that specialized mycoplasma

commercial media became widely available. Advances in culture and detection continue, and we now know that *M. pneumoniae* escapes the respiratory tract and enters the bloodstream to seed a variety of tissues and cause a variety of extrapulmonary complications. Evidence is also accumulating that *M. pneumoniae* infections are a factor in initiation and progression of asthma and can produce long-lasting detrimental effects as noted in pulmonary function tests.

RESPIRATORY TRACT INFECTIONS

M. pneumoniae is perhaps best known as the primary cause of walking or atypical pneumonia, but the most frequent clinical syndrome is that of tracheobronchitis or bronchiolitis, often accompanied by upper respiratory tract symptoms (28). Mild infections are common, although there is some degree of protective immunity against reinfections (39). Symptoms can persist for weeks to months and include hoarseness, fever, a cough which is initially nonproductive but later may yield small to moderate amounts of nonbloody sputum, sore throat, headache, chills, coryza, myalgias, and general malaise (39, 157). Patients may actually complain of chest soreness due to protracted coughing. Dyspnea may be evident in more severe cases, and the cough may take on a pertussis-like character (39). The throat may be inflamed with or without cervical adenopathy, and myringitis sometimes occurs (4). Bronchopneumonia involving one or more lobes develops in 3 to 10% of infected persons (28). Chest auscultation may show scattered or localized rhonchi and expiratory wheezes. Since the alveoli are usually spared, rales and frank consolidation are fairly uncommon unless atelectasis is widespread. In uncomplicated cases, the acute febrile period lasts about a week, while the cough and lassitude may persist for 2 weeks or even longer (4). The duration of symptoms and signs generally shortens if antimicrobial treatment is initiated early in the course of illness (27, 39). Recent evidence indicates that this organism is second only to *Streptococcus pneumoniae* as a cause of community-acquired bacterial pneumonia requiring hospitalization in elderly persons in some populations (131). *M. pneumoniae* pneumonia can cause death, even in previously healthy persons (174, 190). Children with sickle-cell disease (179) and immunosuppressed patients (70) may be at risk of developing more fulminant forms of mycoplasmal pneumonia.

Patients experience extrapulmonary complications at various times after onset of, or even in the absence of, respiratory illness (26). About 7 to 17% of patients hospitalized with *M. pneumoniae* infections develop significant extrapulmonary manifestations (4, 26). Extrapulmonary manifestations most commonly include skin rashes (erythema multiforme), hemolytic anemia, arthritis, meningoencephalitis, peripheral neuropathy, and pericarditis (26). Other nonspecific complications include nausea, vomiting, and diarrhea (142). Severe and sometimes fatal cases of *M. pneumoniae* pneumonia have been associated with respiratory failure as well as extensive involvement of other organs, including disseminated intravascular coagulation (30, 49, 120, 174). Extrapulmonary complications are reviewed in greater detail below.

Autoimmune reaction may be responsible for many of the extrapulmonary complications associated with mycoplasmal infection. However, the presence of *M.*

pneumoniae in extrapulmonary sites such as synovial fluid, cerebrospinal fluid (CSF), pericardial fluid, and skin lesions has been documented by PCR as well as culture, and direct invasion must always be considered. The frequency of direct invasion of these sites is unknown because the organism is rarely sought.

Clinical Presentation

The clinical presentation of *M. pneumoniae* respiratory disease is similar to what is seen with other atypical bacterial pathogens such as *Chlamydia pneumoniae* and in some cases infections with *S. pneumoniae* and various respiratory viruses. *M. pneumoniae* may be present in the respiratory tract concomitantly with other pathogens (59, 131), and a chronic carrier state may be apparent in the absence of overt disease (64).

Clinical laboratory findings are seldom diagnostic. About one-fourth to one-third of persons with lower respiratory tract infections due to *M. pneumoniae* have leukocytosis and/or an elevated erythrocyte sedimentation rate. Sputum Gram stain may show mononuclear cells or neutrophils and normal flora. There are no hepatic or renal abnormalities typical of mycoplasmal infection, although some patients may develop a hemolytic anemia as one of the numerous extrapulmonary manifestations, and this may be reflected in the hemogram.

The radiographic findings in *M. pneumoniae* pneumonia can be extremely variable and mimic a wide variety of lung diseases, but sometimes the radiologic appearance coupled with clinical manifestations can give clues to a mycoplasmal etiology. The inflammatory response elicited by *M. pneumoniae* causes interstitial mononuclear inflammation in the lungs that may appear radiographically as bronchopneumonia of the perihilar regions or lower lobes, usually with a unilateral distribution, and hilar adenopathy. However, lobar consolidation has been described, and the degree of consolidation may exceed what would be expected based on the clinical presentation. Among patients with *M. pneumoniae* pneumonia who require hospitalization, over 10% will require mechanical ventilation (130). Pleural effusions and diffuse alveolar damage (19, 160, 174) sometimes occur in association with more severe cases, and long-term sequelae such as bronchiectasis and pulmonary fibrosis have been reported (101, 207). In some patients, the organism may persist for weeks to months in the lower respiratory tract following acute pneumonia, resulting in prolonged reduced pulmonary clearance and airway hyperresponsiveness (168, 178). Clinicians should remember that mycoplasma pneumonia can be very severe in otherwise healthy people.

Although a few case reports of *M. pneumoniae* infections in pediatric AIDS patients have appeared in the literature (23, 96) and there has been speculation about a possible role for *M. pneumoniae* as a cofactor in AIDS (60), it is not known if the incidence or severity of pulmonary or extrapulmonary *M. pneumoniae* infections in AIDS patients is increased. Hypogammaglobulinemic patients do appear to be at increased risk of developing joint and respiratory infections (85, 115, 144, 156, 164, 177, 192, 194, 206), but how the immunosuppressed state specifically affects host resistance to *M. pneumoniae* infections is unclear. There have been reports of transplant recipients becoming susceptible to infection with other species

of mycoplasmas in a variety of sites, and because *M. pneumoniae* may not be killed in donor tissues by antibiotics (193), there is the possibility that infection could be acquired by transplant recipients on immunosuppressive drugs (reviewed in reference 12).

M. pneumoniae and Asthma

Because of their potential for pulmonary infection and damage, it is not surprising that a number of reports find that *M. pneumoniae* infections are associated with some exacerbations of asthma (42, 133, 146, 166, 176), although this finding is not universal (41).

The more important question is whether mycoplasmas are a primary cause of asthma, or if mycoplasma infection is at least a cofactor in the development of asthma. The suggestion that *M. pneumoniae* may play a role beyond simple, acute exacerbation comes from multiple lines of evidence. First, *M. pneumoniae* can be recovered more often from the airways of patients with chronic, stable asthma than from matched control patients (73, 108, 210). Clarithromycin treatment of asthma patients with mycoplasma infections can result in significant improvement in pulmonary function tests in comparison with asthma patients not infected with mycoplasmas. Mycoplasmas have also been detected by PCR in airways even when cultures and serologic results were negative, suggesting that low numbers of *M. pneumoniae* may evade detection by the immune system (M. Kraft, G. H. Cassell, L. B. Duffy, T. Metze, J. Pak, and R. J. Martin, *Abstr. 5th Int. Conf. Macrolides Azalides Streptogramins Ketolides Oxazolidinones*, abstr. 11.20, 2000). Second, chronic reductions in lung function tests 1 year (128), 3 years (168), and up to 9 years or more (136) after an episode of mycoplasma respiratory tract infection have been reported, establishing the ability of mycoplasmas to induce chronic to permanent lung damage long after resolution of respiratory tract symptoms. Similarly, bronchial hyperreactivity to methacholine challenge was present in 50% of patients 12 weeks after recovering from mycoplasma pneumonia compared with none in controls (208). Third, *M. pneumoniae* is known to induce a number of inflammatory mediators implicated in the pathogenesis of asthma. Mediators such as immunoglobulin E (IgE), substance P, and neurokinin 1 are induced at high level by mycoplasma infection and may play a role in exacerbations, which often include wheezing (38, 89, 175, 178, 196, 209).

Although one study suggests that a delay in initiation of proper antibiotic therapy may be a risk factor for potentially permanent reductions in lung function (128), much more research is needed to determine the true role of *M. pneumoniae* in the initiation and chronicity of asthma.

Antibiotic Therapy and Resistance

Clinical isolates of *M. pneumoniae* are susceptible to macrolides, azalides, tetracyclines, and newer fluoroquinolones such as levofloxacin, gatifloxacin, sparfloxacin, and moxifloxacin (204). Clindamycin is effective in vitro, but limited reports suggest it may not be active in vivo and should not be considered a first-line treatment (39). Naturally occurring antimicrobial resistance in *M. pneumoniae*

against the antibiotic classes listed above is not thought to be common (27). However, clinical treatment failures in respiratory infections have sometimes been documented in immunocompetent hosts, and the organism may be shed in respiratory secretions for weeks after treatment with drugs such as erythromycin or tetracycline. Because current antibiotic regimens do not always kill the organisms, host defenses appear to be very important in eliminating infection (27, 39, 193). Accordingly, therapy in immunodeficient individuals may need to be prolonged (12, 193).

Experimental observations on laboratory-derived erythromycin-resistant mutants of *M. pneumoniae* have suggested that macrolide resistance in this organism can be due to point mutations in the 23S rRNA gene that reduce the affinity of these antibiotics for the ribosomes (125). The extent to which this occurs under natural circumstances or by selection after antibiotic exposure in vivo is not known, although high-level macrolide-resistant strains have been isolated following treatment with erythromycin (187).

DETECTION

Difficulties in detecting mycoplasma infections are not often fully appreciated. Due to their complex nutritional requirements and small genome sizes, cell-free medium for their culture requires a nutritional mix of serum, yeast extract, antibiotics, and other ingredients needed to sustain growth. One medium used successfully for culture of many mycoplasma species is SP-4 (where "SP" stands for "spiroplasma") (200, 205). Some species, such as *Mycoplasma hominis*, grow fairly rapidly, and visual evidence of growth is usually present 2 to 3 days after culture. In contrast, *M. pneumoniae* primary culture requires 3 weeks and in some cases up to 2 months. Regardless of choice of medium, culture of *M. pneumoniae* is insensitive, laborious, and expensive, requiring serial blind passes and long incubation periods. Therefore, serologic evidence of infection has most often been used to detect a current infection. However, serologic methods are themselves fraught with difficulties. Such methods often require paired sera (acute and convalescent) taken 2 to 3 weeks apart, and the results must be interpreted very carefully (reviewed recently in reference 205).

More so than with other more easily cultured pathogens, the accurate diagnosis of *M. pneumoniae* infection will often require the use of modern molecular detection assays. This in itself is a challenge in specific primer design because of the small genome size of this organism. A detailed discussion of molecular methods for detecting *M. pneumoniae* is beyond the scope of this chapter, but a review of recent PCR and other detection assays is available (205). Refinements to traditional PCR assays such as real-time detection using specific probes with matched internal controls to evaluate polymerase inhibition will make detection and quantitation of this pathogen much easier, particularly for acute and subclinical infections (M. Zhang, W. L. Thacker, and D. F. Talkington, *Abstr. 98th Gen. Meet. Am. Soc. Microbiol.*, abstr. G-3, 1998; M. Zhang, B. P. Holloway, W. L. Thacker, and D. F. Talkington, *Abstr. 12th Congr. Int. Org. Mycoplasmol.*, abstr. A.21, 1998). Only

with rapid and sensitive diagnostics can the true incidence and involvement of this pathogen in respiratory illness and other complications be evaluated accurately.

PATHOPHYSIOLOGY OF INFECTIONS

As discussed earlier, mycoplasmas are unique among prokaryotes in that they lack the ability to synthesize cell walls as a result of gene deletion during their evolution from gram-positive ancestors, and they have complex nutritional requirements that must be met in order for them to survive and replicate. These biological characteristics strongly influence the morphology and physiology of mycoplasmas, the manner in which they replicate and interact with eukaryotic host cells, and the mechanisms by which they produce disease and the clinical manifestations of the disease. Appreciation for the complex microecological relationship of *M. pneumoniae* with eukaryotic cells in the human host and for new disease associations is increasing as a result of the availability of the organism's complete genomic sequence and powerful molecular biological techniques such as PCR.

Cytadherence

Evidence accumulated since the 1960s through studies using animal models and in vitro cell and organ culture systems indicates that attachment of *M. pneumoniae* to the respiratory epithelium is a prerequisite for later events that culminate in production of disease (72, 88). The close interaction between the mycoplasma and host cells protects it from removal by the mucociliary clearance mechanism. *M. pneumoniae* possesses a specialized tip structure consisting of a central core with a dense central filament surrounded by a lucent space that is enveloped by an extension of the organism's cell membrane. The tip structure is actually a network of interactive proteins and adherence accessory proteins that cooperate structurally and functionally to mobilize and concentrate adhesins at the tip of the organism, permitting mycoplasmal colonization of mucous membranes and eukaryotic cell surfaces. This specialized tip structure is the leading point in the movement of the organism. The host-cell ligands for mycoplasmal adhesins have not been characterized conclusively, though sialoglycoconjugates and sulfated glycolipids have been implicated (113, 163).

The P1 adhesin is a 170-kDa protein that is now known to be the major structure responsible for interaction of *M. pneumoniae* with host cells (11, 12, 13, 15, 40, 44, 45, 75, 88, 109, 199). Loss of P1 activity through spontaneous mutation or by trypsin treatment results in reduced adherence of mycoplasmas to eukaryotic cells and avirulence (15, 75, 88). Spontaneous reversion to the cytadhering phenotype is accompanied by the reappearance of the implicated proteins, restoration of structurally and functionally intact tip organelles, and return of full infectivity (15). Further support for the functional role of P1 as an adhesin was obtained by the demonstration that monoclonal antibodies to P1 block adherence in a hamster model of mycoplasma respiratory disease (111). Randomly produced antibodies against other proteins from *M. pneumoniae* had no effect on attachment. However, other studies suggest that P1 protein expression alone is not sufficient to mediate

adherence of *M. pneumoniae* to certain host cells and cause disease (14, 110, 111). P30 is one additional protein that has been implicated in the adherence process based on the demonstration that antibodies developed against P30 can block the adherence of *M. pneumoniae* to erythrocytes (14, 43, 44, 140). Other adhesion structures produced by *M. pneumoniae* have also been studied as possible mediators in cytadherence, both in *M. pneumoniae* and in other human mycoplasmas that manifest similar attachment tip structures, such as the MgPa protein of *M. genitalium* (199).

The P1 protein is the target of many of the antibodies that are produced in response to *M. pneumoniae* infection and has also served as a target for development of both serologic and PCR assays. It has been postulated that the immunodominant, variable epitopes of the *M. pneumoniae* adhesins differ from the highly conserved adherence-mediating domains of the adhesins, and thus one reason for the lack of protective immunity against reinfection in the midst of a serologic response could be that antibody directed against the variable domains does not block cytadherence (94).

Cytotoxicity

Cytadherence in the respiratory tract is the initiating event in disease production by *M. pneumoniae*. It is not known precisely how *M. pneumoniae* injures the respiratory epithelial cell after attachment, but a number of biochemical and immunologic properties of the organism that are likely to be involved have been described. Close approximation of the organism to the host cells facilitated by the adhesin proteins appears to be important in facilitating localized tissue disruption and cytotoxicity. Hydrogen peroxide and superoxide radicals synthesized by *M. pneumoniae* act in concert with endogenous toxic oxygen molecules generated by host cells to induce oxidative stress in the respiratory epithelium (199). Consistent with the small genome, *M. pneumoniae* apparently lacks superoxide dismutase and catalase, as well as iron-containing cytochromes. It also has minimal abilities to produce other enzymes for biochemical reactions that are common in other bacteria (199).

Hydrogen peroxide production in *M. pneumoniae* occurs as a result of a flavin-terminated electron transport chain (199). Hydrogen peroxide production is known to be an important virulence factor in *M. pneumoniae*, because hydrogen peroxide is the molecule that confers hemolytic activity (182). The ultrastructural effects of peroxide on host cells such as erythrocytes include loss of reduced glutathione, denaturation of hemoglobin, peroxidation of erythrocyte lipids, and eventually lysis of the cells. Superoxide anions produced by *M. pneumoniae* may also act to inhibit catalase in host cells, thereby reducing the enzymatic breakdown of peroxides produced endogenously and by the mycoplasmas and thus rendering the host cell more susceptible to oxidative damage (5). *M. pneumoniae* hemadsorption and lysis of guinea pig erythrocytes (low in endogenous catalase) are also mediated by peroxide (199). Hemadsorption has long been used as a diagnostic test to presumptively distinguish *M. pneumoniae* from other commensal mycoplasmas commonly found

in the human respiratory tract that do not produce hydrogen peroxide and therefore do not hemadsorb in this manner.

Host cell lactoferrin acquisition by *M. pneumoniae* is yet another possible means by which local injury may occur. This takes place through generation of highly reactive hydroxy radicals resulting from the introduction of iron complexes in a microenvironment rendered locally acidic by cellular metabolism that also includes hydrogen peroxide and superoxide anion (198).

Mammalian cells parasitized by *M. pneumoniae* exhibit a number of cytopathic effects that may occur as a result of the local damage mediated biochemically following cytadherence. *M. pneumoniae* infection leads to deterioration of cilia in the respiratory epithelium, both structurally and functionally. Cells may lose their cilia entirely, appear vacuolated, and show a reduction in oxygen consumption, glucose utilization, amino acid uptake, and macromolecular synthesis, ultimately resulting in exfoliation of all or parts of the infected cells (39, 40). These subcellular events can be translated into some of the clinical manifestations of respiratory tract infection that are associated with this organism.

Inflammatory Effects

Evidence from animal models and human cases suggests that the pulmonary infiltrates in mycoplasmal pneumonia are related to mononuclear cell migration into the airways. Alveolar macrophages undergo chemotactic migration to the site of infection. Following opsonization of the mycoplasmas by complement or antibodies, macrophages can phagocytize and become activated (159). Afterwards, the macrophages begin to release cytokines and a mononuclear cell inflammatory response develops. $CD4^+$ T cells, B cells, and plasma cells infiltrate the lung (30, 150), and this is followed by further amplification of the immune response, proliferation of lymphocytes, production of immunoglobulins, and release of proinflammatory cytokines (150, 199). Cytokine production and lymphocyte activation may either minimize disease through the enhancement of host defense mechanisms or exacerbate disease through immunologic lesion development (161). However, evidence suggests that the more vigorous the cytokine- and cell-mediated immune response, the more severe the pulmonary injury is (93, 160, 191). Examination of histopathologic specimens from fatal cases of *M. pneumoniae* pneumonia has shown peribronchial mononuclear infiltrates with luminal exudates consisting of mononuclear, polymorphonuclear, and sloughed epithelial cells. Lymphoplasmacytic bronchiolar wall infiltrates and bronchiolitis obliterans have been described in open lung biopsy specimens from patients with nonfatal respiratory failure due to *M. pneumoniae* (30, 120). Immunosuppressed persons with *M. pneumoniae* infection may lack pulmonary infiltrates (70), further attesting to the importance of the host immune response in lesion development.

Molecular Mimicry and Autoimmunity

The extensive homology of the *M. pneumoniae* adhesin proteins and glycerophospholipids with mammalian tissues is a well-known example of molecular mimicry that may trigger autoimmune disorders through formation of antibodies against

substances such as myosin, keratin, fibrinogen, brain, liver, and lung tissues (39). Mycoplasmal adhesins also exhibit amino acid sequence homologies with human CD4 and class II major histocompatibility complex lymphocyte proteins, which could generate autoreactive antibodies and trigger cell killing and immunosuppression (165). Also, mycoplasmas may serve as B-cell and T-cell mitogens and induce autoimmune disease through the activation of antiself T cells or polyclonal B cells. Circulating immune complexes occur during acute phases of diseases (39).

A well-known autoimmune effect of *M. pneumoniae* formerly used to aid in diagnosis of infections is the development of IgM antibodies against the I antigen of human erythrocytes, which causes their agglutination at 4°C. These antibodies are produced 1 to 2 weeks after initial infection in about 50% of *M. pneumoniae* infections and persist for several weeks.

Antigenic Variation

Many mycoplasmal species that infect animals or humans are known for their ability to induce chronic disease states. Therefore, they must have developed means by which they can successfully evade the host immune response. One way of achieving this may involve high-frequency phase and antigenic variation of surface adhesin proteins, made possible by DNA rearrangements in truncated and sequence-related copies of the adhesin genes that are dispersed throughout the genome (188, 189). Such recombinational events promote diversity and altered specificities and affinities and maximize the coding potential of the limited mycoplasma genome (12).

Intracellular Localization

Whether pathogenic mycoplasmas actually enter and survive within mammalian cells under natural conditions has been debated for many years. The organisms exist in intimate contact with mammalian cells and may logically be capable of initiating fusion with host cells through their cholesterol-containing unit membranes. Recent reports of intact mycoplasmas within the cytoplasm and the perinuclear regions of cells from infected humans and in cell cultures, along with evidence that species including *M. pneumoniae* are capable of long-term intracellular survival and even replication, offer an additional dimension to the pathogenic potential of mycoplasmas (13, 16, 46). An intracellular existence that sequesters *M. pneumoniae* could facilitate the establishment of latent or chronic states, circumvent mycoplasmacidal immune mechanisms, and impair efficacy of some drug therapies (16, 199), thereby explaining some of the clinical features that are well known in infections caused by this organism. Studies demonstrating the intracellular survival and replication of *M. pneumoniae* were performed in an artificial cell culture system, and the extent to which such processes actually occur in vivo is not known.

EXTRAPULMONARY INFECTIONS

Reports of apparent extrapulmonary *M. pneumoniae* complications have appeared in the literature for the last 60 years. Although the complications are

grouped roughly by organ system, it is important to realize that often multiple organ systems are involved, and hematogenous spread to many organ systems in the same patient has also been reported (9, 106, 169). It is also important to realize that extrapulmonary complications can be seen before, during, or after pulmonary manifestations or can occur in the complete absence of any respiratory symptoms (26).

Neurologic Complications

Since the early 1940s, neurologic complications associated with *M. pneumoniae* infections have been consistently reported in the literature (24). Such complications have included encephalitis, cerebellar syndrome and polyradiculitis (183, 186), meningitis or meningoencephalitis (158), acute disseminated encephalomyelitis, Guillain-Barré syndrome (18), and cases of acute psychosis secondary to encephalitis (48, 74). A number of extrapyramidal (primarily motor) deficiencies have also been described (6, 181). These associations were initially based upon serology and isolation of mycoplasmas from the respiratory tract rather than demonstration of direct invasion of the central nervous system (CNS). The lack of clear evidence that mycoplasmas were actually in neurologic tissues led to theories that damage to brain tissue was from cross-reacting or autoimmune antibodies elicited by *M. pneumoniae* (57, 62), and even to concern that neurologic infections by other bacterial pathogens were causing false-positive mycoplasma serologic results (105). Studies of immunologic sequelae of *M. pneumoniae* infection that can lead to neurologic complications continue (147, 152), but the availability of PCR for *M. pneumoniae* in the 1990s (1, 50, 91, 145, 197) confirmed and extended reports of *M. pneumoniae* in the CNS that were documented by positive cultures of CSF (1). *M. pneumoniae* has been detected in the CSF and has been reported to directly invade the brain parenchyma (116). PCR was used to detect *M. pneumoniae* in the CSF of a patient who had CSF hypoglycorrhachia and whose early azithromycin treatment did not abrogate subsequent CNS infection (M. A. Neill, J. Friedman, R. Crausman, S. B. Schwartz, and D. F. Talkington, *Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am.*, abstr. 517, 1998; S. B. Schwartz, M. Zhang, W. L. Thacker, and D. F. Talkington, *Abstr. 99th Gen. Meet. Am. Soc. Microbiol.*, abstr. G-11, p. 324, 1999).

Neurologic infections usually resolve completely, but they can result in chronic debilitating deficits in motor or mental function (181). The presence of peripheral neurologic sequelae such as radiculitis and transverse myelitis has been identified as a risk factor for chronic CNS sequelae (25, 158). A number of studies have reported that approximately 6 to 7% of hospitalized patients with serologically confirmed cases of *M. pneumoniae* pneumonia experience neurologic complications of varying severity (107, 137, 154, 183). High-dose steroid therapy has sometimes been effective in reversing neurologic symptoms (83), and some clinicians use steroids in combination with an antibiotic such as doxycycline or chloramphenicol, which can penetrate the CNS (50). Most patients with neurologic complications experience them 1 to 2 weeks after the onset of respiratory signs, but 20% of

patients or more have no preceding or concomitant diagnosis of respiratory infection (157). This figure may be higher yet for children (195).

Neurologic complications from respiratory *M. pneumoniae* infection may ultimately result from direct infection of brain tissues as well as secondary destruction of tissue caused by the generation of autoimmune antibodies (61, 152), but only recently have assays evolved to reliably identify low numbers of mycoplasmas in tissues so that this issue can be properly addressed (190).

In a multiyear Centers for Disease Control and Prevention program designed to uncover infectious disease diagnoses for patients with unexplained serious illnesses in the United States, *M. pneumoniae* was responsible for cases characterized by primarily neurologic symptoms as well those with respiratory symptoms (R. A. Hajjeh, D. Relman, P. R. Cieslak, A. N. Sofair, D. Passaro, J. Flood, J. Johnson, J. K. Hacker, W.-J. Shieh, R. M. Hendry, S. Nikkari, S. Ladd-Wilson, J. Haddler, J. Rainbow, J. W. Tappero, C. Woods, L. Conn, S. Reagan, S. Zaki, B. A. Perkins, and the Unexplained Deaths and Critical Illness Working Group, submitted for publication). The frequency of mycoplasma invasion into CSF and neurologic tissue needs continuing evaluation using more sensitive assays such as PCR when opportunities arise involving patients with neurologic sequelae.

Renal Complications

Renal complications associated with *M. pneumoniae* respiratory infections were first reported in the 1970s. Renal diseases such as acute glomerulonephritis and renal failure (sometimes secondary to rhabdomyolysis), tubulointerstitial nephritis, and IgA nephropathy (48, 98, 106, 151, 173, 203) as well as others have been reported sporadically. Attempts to demonstrate mycoplasma antigen in damaged renal tissue with immunohistochemical techniques have not been uniformly successful, once again leading to theories that an antibody-mediated pathogenesis and not direct infection may be responsible for some cases of renal disease (98, 204). A recent attempt to use PCR to identify mycoplasmas in renal tissue from four young patients with acute nephritis with serologic evidence of recent *M. pneumoniae* infection also failed (169). This finding is in contrast with repeated successful attempts to identify *Mycoplasma fermentans* in the renal tissue of AIDS patients with AIDS-associated nephropathy (2, 17). Although renal complications of *M. pneumoniae* infection are rare (169), additional attempts to identify mycoplasmas in kidney tissue using PCRs are needed to clarify whether direct or indirect damage to tissue is occurring.

Arthropathies

Three basic patterns of joint disease associated with *M. pneumoniae* infection have been described: transient polyarticular pain or discomfort centered primarily on the large joints coincident with acute illness, polyarthropathy affecting primarily medium-sized joints that can persist for a year or more after acute illness (4), and septic arthritis reported most often in hypogammaglobulinemic patients (85, 115, 144, 156, 177, 192, 194, 206). However, in only a few cases had mycoplasmas been cultured from joint fluid (95, 164) until the mid- to late 1980s, when reports

of *M. pneumoniae* isolations from joint fluids from immunocompetent patients with polyarthritis appeared (47). The difficulties of isolating mycoplasmas from affected joints of patients who fit clinical criteria for mycoplasma pneumonia invite suggestions that *M. pneumoniae* and other mycoplasmas may be cofactors in arthropathies such that direct invasion of joints may not be necessary (153). Nonseptic arthralgia is present in 14% of patients or more, but septic arthritis is uncommon in immunocompetent patients (26). It is anticipated that increased use of PCRs to better screen for mycoplasmas from joint fluid and tissues will establish how often mycoplasmas are really present in joints but not cultivable. This will help clarify the role direct invasion plays in arthritis associated with *M. pneumoniae* pulmonary or systemic infection.

Cardiac Complications

Reports of cardiac complications from *M. pneumoniae* infection began to appear in the literature in the mid- to late 1960s (81, 114). A variety of cardiac complications have since been reported, including pericarditis, myocarditis, and pericardial effusion with and without cardiac tamponade (23, 56, 99, 102, 132, 155, 157, 172, 181). Of note, a prospective study of pericardial effusions uncovered two cases of *M. pneumoniae* infection confirmed by isolation of mycoplasmas from pericardial effusion or tissue. An additional two cases of pericarditis due to *Mycoplasma hominis* and one due to *Ureaplasma urealyticum* have also been diagnosed (203).

In general, cardiac complications are relatively uncommon, but involvement has been reported in 1 to 8.5% of patients with serologic evidence of infection, primarily in adult patients (132). A large 1979 study reported that 4.5% of patients with serologically confirmed cases of *M. pneumoniae* pneumonia had cardiac complications, and almost half of these patients had evidence of symptoms or heart abnormalities an average of 16 months later (155).

Dermatologic Complications

A variety of dermatologic abnormalities have been associated with *M. pneumoniae* infection in up to 25% of patients (26). Among the more common are erythematous maculopapular and vesicular rashes or exanthems. Most cases associated with pneumonia are self-limiting within about 7 days and for unknown reasons are most often seen in males (36). More severe mucocutaneous syndromes have also been reported, including severe conjunctivitis, generalized ulcerative stomatitis, and bullous exanthems (36, 118). Mild to severe forms of Stevens-Johnson syndrome have also been associated with *M. pneumoniae* infections, most often presenting as cases of erythema on the trunk with stomatitis and sometimes conjunctivitis rather than the severe toxic reactions with widespread epidermal necrosis more commonly seen with drug reactions (119, 186; S. Fourneir, S. Bastuji-Garin, H. Mentec, J. Revuz, and J. C. Ronjeau, Letter, *Eur. J. Clin. Microbiol. Infect. Dis.* **14:**558–559). The value of using steroids to treat Stevens-Johnson syndrome caused by *M. pneumoniae* has not been clearly established (119).

Pediatric patients with pleuropneumonia are more likely to present with dermatologic manifestations than are those with uncomplicated pneumonia (143). Cli-

nicians should keep in mind that the presence of erythematous maculopapular rashes in *M. pneumoniae* patients can also be caused by a number of antibiotics commonly given to such patients (126).

Hematopoietic Complications

Ever since the 1940s, hemolytic anemia has been recognized as a rare but severe complication of mycoplasma pneumonia (69). Numerous reports have documented this complication, found in higher incidence among children (10, 37, 55, 123, 180). Hemolytic anemia in cold agglutinin disease and autoimmune hemolytic anemia have been attributed to *M. pneumoniae*-induced cross-reacting antibodies to the I antigen of human erythrocytes (39, 55, 123, 201).

A recent report suggests that cases of thrombotic thrombocytopenic purpura sometimes seen in patients with *M. pneumoniae* infection may be the result of cross-reactive antibodies inactivating the plasma von Willebrand factor-cleaving protease, resulting in unusually large von Willebrand factor multimers that later on initiate the cascade of thrombotic thrombocytopenic purpura (9). Two pediatric cases of aplastic anemia associated with *M. pneumoniae* also have been reported (184). If subclinical forms of hemolytic anemia and intravascular coagulation are considered, over 50% of patients with *M. pneumoniae* infections may be affected (29).

Other Extrapulmonary Infections

A variety of less common extrapulmonary infections and sequelae have been reported for *M. pneumoniae*. Although rare, ocular manifestations have been reported, primarily in pediatric cases. Ocular manifestations include conjunctivitis, anterior uveitis, optic neuropathy, retinitis and retinal hemorrhages, iritis, and optic disc swelling, with or without permanent degradation of vision (134, 170).

Up to one-third of patients may have nonspecific ear symptoms, in addition to otitis externa and interna (142, 186). Rarely, hepatitis and pancreatitis have been associated with respiratory infections (7, 20, 180, 202), and *M. pneumoniae* has been isolated from the urogenital tracts of males and females and has been cultured from a tubo-ovarian abscess (76). Given the apparent ability of the organism to invade the bloodstream, infections in almost any organ system would seem to be possible.

A recent report raises the intriguing possibility that *M. pneumoniae* may colonize atherosclerotic plaques and possibly play a synergistic role with *C. pneumoniae* in causing plaque ruptures and emboli leading to myocardial infarction (86). Atherosclerotic plaques are rich in cholesterol, a necessary mycoplasma nutrient. More studies are needed to verify this preliminary finding and investigate whether there is any association of mycoplasmas with atherosclerotic disease (86).

EPIDEMIOLOGY OF MYCOPLASMA INFECTIONS

M. pneumoniae epidemiology has not been studied extensively beyond North America, Europe, and Japan, but climate and geography are not known to play an

important role in disease. In the United States, there is no national surveillance system tracking mycoplasma infections; much of what we know about rates of endemic disease comes from population-based studies performed in the 1960s and 1970s in Seattle and Michigan (66, 67, 68, 82, 138, 139). The epidemiology of *M. pneumoniae* infections is characterized by both endemic and epidemic disease.

Seasonality of Endemic Disease

Historically, endemic disease transmission has been punctuated with cyclic epidemics every 4 to 5 years (though this pattern may be changing) (122). This has been seen in various parts of the United States (66), Denmark (121), Iceland (84), and Great Britain (148). Interestingly, epidemics may occur during different years within various parts of a country (66). Although the incidence of disease does not appear to vary by season, the proportion of patients with *M. pneumoniae* pneumonia is greatest in the summer because of the low incidence of other respiratory pathogens (3, 131).

Age Distribution

The incidence of *M. pneumoniae* pneumonia is greatest among school-aged children and declines after adolescence (3, 68). Rates of disease among children less than 5 years old have been reported to be low, but these studies were conducted at a time when out-of-home child care was less common, and population-based epidemiologic studies of nonhospitalized patients have not been done since then. It is likely that the rate among preschool children is higher than once thought because of the ease with which young children share respiratory secretions.

Although most mycoplasma infections occur among outpatients, *M. pneumoniae* is a significant bacterial cause of hospitalizations for pneumonia in the United States, especially among the elderly (131). Most reports of severe complications and deaths from mycoplasma infection occur in this age group. Children may also represent an asymptomatic reservoir of infection for outbreaks in families (51).

Transmission in Families

Mycoplasma infections are frequently spread among family members (8, 21, 51, 65, 97). In a recent study in The Netherlands, *M. pneumoniae* could be isolated from the nose or throat of 15% of family members of *M. pneumoniae*-positive index patients within 4 weeks of the index case (51). The frequency of transmission within families was the same regardless of the appropriateness of the antibiotic used for treatment of the index patient. The frequency of transmission reported in this study probably represents a marked underestimate of the risk of transmission within families, given that other studies have shown that several cycles of 3 to 4 weeks may be necessary before intrafamily transmission is complete. In a study in Seattle, 39% of family contacts eventually became infected, many asymptomatically (65).

Epidemics in Closed or Semiclosed Settings

Epidemics of *M. pneumoniae* infections can occur in the community or in closed or semiclosed settings. Military bases (54, 59, 77, 78, 135), hospitals (63, 100, 104), religious communities (117, 141), and facilities for the mentally or developmentally disabled have been particularly hard hit (90, 103, 185). Unlike endemic disease which has no seasonality, outbreaks in the United States tend to occur in the summer or early fall (22, 29, 59, 90, 103).

New recruits on military bases are at particularly high risk of acquiring *M. pneumoniae* infection (77). In the 1970s, more than half of all recruits acquired an acute mycoplasma infection in some companies (54). Although the majority of infections are mild, *M. pneumoniae* is a leading cause of bacterial pneumonia among both outpatient and hospitalized military personnel (78, 80).

Outbreaks in closed or semiclosed settings can be very difficult to control for many reasons, including delayed outbreak detection, difficulty of implementing effective infection control practices, and a long incubation period. Attack rates can be quite high, with reports ranging from 25 to 71% in some settings (3, 22, 59, 103). Although long-term morbidity is uncommon, these outbreaks can be very disruptive and can consume large amounts of resources. Strategies to control these outbreaks have included cohorting and using antibiotic therapy for symptomatic persons. A number of trials of prophylactic antibiotics have been tried with varied success (J. Ackelsberg, C. Carlyn, and S. Wong, *Abstr. 37th Annu. Meet. Infect. Dis. Soc. Am.*, abstr. 560, 1999; J. A. Schillinger, K. E. Arnold, E. C. Mokulis, H. B. Lipman, G. P. Melcher, R. F. Breiman, B. Schwartz, and M. J. Dolan, *Abstr. 35th Intersci. Conf. Antimicrob. Agents Chemother.*, abstr. K60, 1995). Many of these studies were longitudinal in nature, making it difficult to determine whether the interventions were effective or whether all susceptible persons had been exhausted. In the most recent study, a placebo-controlled trial of azithromycin prophylaxis in a psychiatric hospital, therapy demonstrated a protective efficacy of 75% against illness resulting in physician visits among hospital employees (90). In selected circumstances, the use of prophylactic antibiotics may be an effective tool for control of mycoplasma outbreaks in closed settings.

SUMMARY

M. pneumoniae research and a complete understanding of its pathogenic potential have been historically hampered by the organism's misidentification as a virus, a low growth rate in the laboratory stemming from complex medium requirements, a lack of sensitive and specific serologic assays, and a mistaken impression by many in the medical community that all infections are almost always mild and self-limiting. However, *M. pneumoniae* is second only to *S. pneumoniae* as a cause of community-acquired pneumonia in some populations. Also, an extensive body of literature suggests that extrapulmonary infections are not uncommon and are often serious. A number of important research questions need renewed attention to provide a better understanding of pathogenesis. These include the role of autoimmunity induction in a variety of mycoplasma syndromes, the existence and importance of

an asymptomatic carrier state, and a much better understanding of the role of direct invasion in extrapulmonary syndromes. Continued improvement in both serologic and antigen detection assays is needed in this regard, and this should allow more efficient diagnoses of pulmonary and extrapulmonary infections as well as improved epidemiologic and antibiotic therapy trials. The development of an effective vaccine is a long-term goal and is dependent on continued advances in understanding the pathogenesis of this enigmatic bacterium.

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Emerging Infections 5
Edited by W. M. Scheld, W. A. Craig, and J. M. Hughes

Chapter 5

Community-Onset Oxacillin-Resistant *Staphylococcus aureus* Infection

Julie Louise Gerberding and Henry F. Chambers

The emergence of antibiotic-resistant strains of staphylococcus with the capacity to produce outbreaks—and even epidemics—of suppurative disease among hospital patients, and to spread from them into the community, is a relatively new problem, but one that already aroused universal concern.

Dr. L. E. Burney, Surgeon General (4)
September 1958

The osteomyelitic lesions of Egyptian mummies, the wound infections of medieval soldiers, and contemporary outbreaks of neonatal sepsis all point to one fact: *Staphylococcus aureus* is as old as medicine and is an extremely successful pathogen, capable of causing invasive disease at all stages of life (4). *S. aureus* possesses an impressive array of adhesins, exotoxins, and other virulence factors that no doubt account for its success. Coincident with the development and use of new antimicrobials, *S. aureus* has demonstrated an even more formidable characteristic—resistance to multiple antimicrobial agents, including virtually all beta-lactams. Oxacillin-resistant *S. aureus* (ORSA) (also known as methicillin-resistant *S. aureus* [MRSA]) emerged in hospitals and currently accounts for more than 40% of hospital-onset staphylococcal infections in the United States. (Regarding the terms ORSA and MRSA, oxacillin is the antimicrobial drug most often used to detect resistance; hence, ORSA is now the preferred term for *S. aureus* resistant to antistaphylococcal beta-lactams.) Community outbreaks of ORSA infections among otherwise healthy adults and children with no direct link to health care have been increasing over the past 5 years. If the patterns observed with penicillin-resistant *S. aureus* hold true, we can expect the prevalence of ORSA to steadily increase in the community over the next decade. In 1997, the first report of ORSA with reduced

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susceptibility to vancomycin was publicized, portending even more challenges ahead (6). The story of *S. aureus* is remarkably consistent: development of resistance to every antimicrobial class; only the time it takes for resistance to emerge and spread in specific human populations seems to vary.

EMERGENCE AND SPREAD OF PENICILLIN-RESISTANT *S. AUREUS*

Penicillinase-producing strains of *S. aureus* were first detected in hospitalized patients almost immediately after the drug was introduced (2, 22). The prevalence of penicillin resistance rapidly increased thereafter. For example, 85% of 516 *S. aureus* isolates derived from patients in Bellevue Hospital, New York, N.Y., between 1953 and 1954 were resistant to penicillin (23). By 1956, the problem had reached a level of impact sufficient to prompt the first national meeting on the subject, the Symposium on Staphylococcal Infections supported by the New York Academy of Medicine. Two years later, Congress appropriated $325,000 to the Public Health Service for laboratory services, training, and epidemic aid and an additional $1 million to the National Institutes of Health to expand staphylococcal research (4). In 1958, the U.S. Public Health Service Communicable Disease Center and the National Academy of Science convened an international conference that included 59 professional organizations to fashion a coordinated national plan and stimulate state and local actions to combat penicillin-resistant *S. aureus*.

Until the early 1960s, community isolates of *S. aureus* were almost always penicillin susceptible, though surveillance for resistant strains was rare. Spread to household contacts of patients with hospital-acquired penicillin-resistant strains was recognized as one notable exception, but the capacity for community transmission was largely ignored. In 1969, a comprehensive surveillance study of *S. aureus* blood culture isolates obtained in Denmark was published (20). More than 85% of hospital-acquired isolates were penicillin resistant, but 65 to 70% of community-acquired isolates also were resistant to penicillin. Of note, the hospital-acquired penicillin-resistant isolates usually were resistant to other antimicrobial classes, whereas the community-acquired isolates often were resistant only to penicillin. Similar rates of penicillin resistance were noted in the United States by the 1970s. No distinct subpopulations at risk were apparent; 75 to 80% of isolates from urban, rural, pediatric, and adult populations were resistant (13, 19, 27).

In summary, penicillin-resistant *S. aureus* was reported shortly after penicillin was introduced; within less than a decade, more than 25% of hospital strains were resistant, and within 2 decades, more than 75% were resistant (12, 14). This rapid increase in the prevalence in hospitals was followed a decade or so later by a similar rate of increase in the community. By the early 1980s, roughly 4 decades after penicillin was widely available for civilian use, the prevalence of penicillin resistance in health care and community settings was virtually identical and exceeded 85%, a situation which persists today.

EMERGENCE AND SPREAD OF ORSA

In 1961, *S. aureus* first developed resistance to methicillin, almost simultaneous with the drug's release. The genotypic changes responsible for this trait confer

cross-resistance to virtually all beta-lactam antimicrobials, including oxacillin and the other antistaphylococcal beta-lactams that are now most often prescribed. Over the last 3 decades, the proportion of oxacillin resistance among *S. aureus* causing infections in hospitalized patients has steadily increased in the United States. The National Nosocomial Infections Surveillance data collected by the Centers for Disease Control and Prevention (CDC) indicate that the prevalence is highest in intensive care units and in larger teaching hospitals but is not much lower in many smaller facilities in less urban locales. In 2000, 55% of *S. aureus* infections in intensive care settings and 46% of infections elsewhere in the hospital were caused by oxacillin-resistant strains (Fig. 1). Risk factors for ORSA colonization or infection in the hospital include prior antimicrobial therapy, admission to an intensive care unit, surgery, and exposure to an ORSA-colonized patient (3, 32).

Until 1980, cases of community-onset ORSA infection almost always were attributable to a history of recent hospitalization, close contact with a person who had been hospitalized, or similar exposures (25, 26; S. Gross-Schulman, D. Dassey, L. Mascola, and C. Anaya, Letter, *JAMA* **280:**421–422, 1998; F. L'Heriteau, J. C. Lucet, A. Scanvic, and E. Bouvet, Letter, *JAMA* **282:**1038–1039, 1999). In addition, some cases of community-onset infection were actually due to acquisition of ORSA in long-term care settings, an environment that also promotes emergence and spread of resistant staphylococci. However, cases of community-onset ORSA (MRSA) infection occurring in 1980 and 1981 that were reported from Detroit provided the first suggestive evidence that ORSA could spread in communities independent of direct health care exposure. Most of the affected patients were injection drug users, and sharing needles was the most likely mode of spread (28, 29). Previous antimicrobial (especially cephalosporin) use was an important risk for infection with the outbreak strain. Among the injection drug users, hospitalization in the past 4 months was not an independent risk for ORSA infection, but the outbreak strain was the same phage type as the *S. aureus* isolate causing infections in burn unit patients elsewhere (10). Among non-injection drug users, recent hospitalization was associated with ORSA infection.

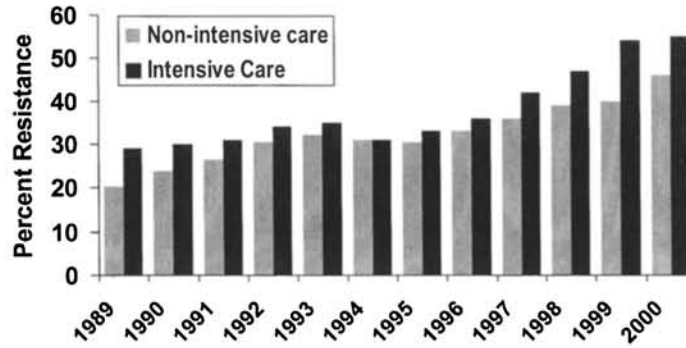

Figure 1. Proportion of hospital-onset *S. aureus* infections resistant to oxacillin (based on data from the National Nosocomial Infections Surveillance System, 1989 to 2000).

Population-based surveillance of community isolates of *S. aureus* does not yet exist, and the true prevalence of community-acquired ORSA is not known. The number of published reports of ORSA colonization and infection in community settings suggests that the problem is emerging in many countries. In the 15 years between 1981 and 1995, only 12 reports from the United States and 5 reports from other countries described community-acquired ORSA and made a reasonable effort to exclude linkages with health care delivery or other traditional risks. In the next 5 years, 18 domestic and 16 international reports describing community-acquired ORSA were published.

Perusal of these publications illustrates an important obstacle to understanding the epidemiology of ORSA in the community: most *S. aureus* surveillance data are hospital based, and there is a tendency to assume community acquisition if the infection does not meet the traditional criteria for nosocomial infection (i.e., onset of infection 72 or more hours after hospitalization and *S. aureus* neither incubating nor present at the time of hospitalization). In other words, anything that is not hospital onset is assumed to be community acquired. The fallacy of this assignment strategy became evident when the complex relationship between the risks for *S. aureus* colonization and infection that are present throughout the delivery system, the dynamics of patient movement from one health care setting to another, and the high probability for prolonged staphylococcal colonization in many patients was better appreciated. A staphylococcal infection may be nonnosocomial with reference to the index hospital admission, but the organism causing that infection may well have been acquired during a remote hospitalization or during care received elsewhere in the delivery system. Creating more precise definitions of the epidemiological parameters describing infections in health care and community settings is an essential step toward effective prevention programs. For this reason, the CDC has proposed new terminology that more clearly differentiates the place where the organism is actually acquired (e.g., community-acquired ORSA, long-term-care-acquired ORSA) from the place where infection has its onset (e.g., community-onset *S. aureus* infection, intensive care unit-onset ORSA infection). From this perspective, some hospital-onset ORSA infections may be caused by community-acquired organisms, and some community-onset ORSA infections may be caused by hospital-acquired organisms.

Several factors seem to distinguish the community-onset ORSA infections not attributable to health care exposure from those that are health care associated. Community-acquired ORSA tends to cause infections that occur in clusters or small outbreaks that affect unique populations such as young children, Australian Aborigines, Native Americans, Alaskan Natives, prisoners, and college athletes. Recent antimicrobial exposure is less likely to be reported as a risk for resistant infections in the community than in health care settings. Most infections are mild and limited to skin and soft tissues, though invasive infections do occur and may serve as "sentinels" that first bring a more widespread community problem to the attention of clinicians and public health personnel. Perhaps most intriguing, the antimicrobial susceptibility patterns observed for community ORSA strains are unusual. Unlike hospital strains, which typically are resistant to multiple antimicrobials and share a common genotype with other hospital isolates, community-

acquired strains tend to be susceptible to non-beta-lactams and have genotypes distinct from hospital isolates in the same community (1, 5, 15).

A recent study of methicillin-resistant *S. aureus* carriage in children attending day care centers suggests one potential community reservoir for ORSA (1). In children attending two day care centers in Dallas, Tex., each of which had an index case of MRSA infection, 3 and 24% of children in the respective centers were colonized. The isolates generally were susceptible to most non-beta-lactam antimicrobials. Forty percent of the colonized children had no direct or indirect (via a household member) contact with a health care facility within the prior 2 years. In a hospital-based study of Chicago children, the number of children admitted to the hospital with an ORSA infection who lacked an identifiable risk factor for prior colonization increased by 25-fold (15). As with the Dallas ORSA isolates, these strains were usually susceptible to non-beta-lactam antimicrobials and had pulsed-field gel electrophoresis patterns that were distinct from those of the common hospital isolates.

The deaths of four children from rural Minnesota and North Dakota caused by community-onset ORSA infection in 1999 focused national attention on this emerging problem (5). These children, like those in Dallas and Chicago, lacked established risks for ORSA infection. In addition, their infections were caused by isolates susceptible to most non-beta-lactam antimicrobials and had identical genotypes distinct from those of ORSA isolates from local hospitals.

These reports of infection and colonization by strains of MRSA in children provide compelling evidence that MRSA strains, like penicillinase-producing strains almost 30 years ago, have gained a foothold in the community and are emerging as important outpatient pathogens (J. M. Boyce, Editorial, *JAMA* **279:** 623–624, 1998). Based on the experience with penicillin-resistant strains, their prevalence is likely to rapidly increase within the next 5 to 10 years, unless prompt action is taken. The CDC has initiated pilot programs to conduct population-based surveillance of community-acquired ORSA in several states. Hopefully these and similar investigations will more clearly define the risk factors for emergence in specific locales and suggest effective control measures that will attenuate the rate of spread.

ORIGINS OF COMMUNITY-ACQUIRED ORSA

The origins of community-acquired ORSA are not established. Three main hypotheses have been proposed to account for their appearance. First, they may actually be health care-acquired pathogens, but the direct link to health care was not detected in the relevant investigations. This hypothesis is consistent with some early investigations of community-onset infections, but several recent studies of community ORSA failed to detect health care risks despite detailed investigations. Another hypothesis to account for the emergence of ORSA is that these isolates are direct descendents of health care-acquired strains that are now circulating in the community. The density of health care-acquired ORSA in the community has increased over the past decade, as the prevalence of colonized patients discharged to home or other community settings increased. Hence, there are more opportunities

for spread to household contacts or to others in close proximity to patients in health care settings. The CDC is currently conducting studies to evaluate the extent to which transmission of ORSA to health care personnel and household contacts of patients and health care personnel occurs. However, this hypothesis is inconsistent with the phenotypic (e.g., antimicrobial susceptibility) and genotypic (e.g., genotype pattern) differences between community-acquired and health care-acquired isolates.

A final hypothesis to explain the appearance or ORSA in communities is that the *mecA* gene, the genetic determinant necessary for the expression of oxacillin resistance, has been transferred to one or more previously oxacillin-susceptible strains of *S. aureus* that occupy traditional community niches. This possibility does account for the distinct phenotypic and genotypic characteristics of community-acquired ORSA. Horizontal transmission of the plasmid-borne penicillinase gene responsible for penicillin resistance likely played the dominant role in emergence of community-acquired penicillin-resistant *S. aureus*. *S. aureus* plasmid genes easily are transferred by transduction or conjugation, a fact which may explain the enormous genetic diversity among penicillin-resistant oxacillin-susceptible *S. aureus*.

The *mecA* gene codes for penicillin-binding protein 2a (PBP 2a), a unique transpeptidase with low affinity for beta-lactams. *mecA* is located on the staphylococcal cassette chromosome *mec* (SCC*mec*), a highly conserved resistance island present in all resistant strains (16–18, 21, 24), and is not plasmid borne. In addition to carrying *mecA*, SCC*mec* may include genetic determinants for resistance to bleomycin, aminoglycosides (tobramycin and amikacin), macrolide-lincosamide-streptogramin B, and spectinomycin (18). Horizontal transfer of SCC*mec* is thought to be relatively rare; only a handful of ancestral strains account for all clinical isolates worldwide (24). Ribotyping (a genotyping scheme that uses Southern blot analysis to identify DNA restriction enzyme polymorphisms of the five or six rRNA genes distributed throughout the *S. aureus* chromosome) and cluster analysis indicate that SCC*mec* has integrated into at least three distinct methicillin-susceptible chromosomal backgrounds, A, B, and C (16, 17). SCC*mec* itself is polymorphic; three types have been identified: I, II, and III. These polymorphs differ in number of base pairs, genetic organization, number of insertion sequences, and resistance determinants. All three SCC*mec* types have been found integrated into ribotype cluster A. Type II SCC*mec* has also integrated into cluster B and C ribotype backgrounds. Thus, only five distinct ancestral clones of ORSA have been identified worldwide since the first strain was isolated in 1961; even if more clones were identified, the relatively low number pales in comparison to the large number of distinct clones of methicillin-susceptible strains.

The recent whole-genome sequencing of *S. aureus* strongly supports the concept that horizontal gene transfer is a common evolutionary mechanism in *S. aureus*, even though SCC*mec* transfer itself may only rarely occur; hence, horizontal transfer of *mecA* or SCC*mec* is a plausible hypothesis to account for emergence of community-acquired ORSA isolates that are dissimilar to health care setting-acquired isolates (18). The relatively long time lag from the appearance of ORSA in hospitals to its emergence in community settings, unlike the situation with pen-

icillin resistance, may in part be due to the low frequency of horizontal chromosomal gene transfer compared to the plasmid transduction and conjugation that efficiently spread penicillinase.

The mechanism by which SCC*mec* is mobilized and transferred is under intense investigation. Two genes, *ccrAB* (cassette chromosome recombinase genes A and B), are homologous to DNA recombinases of the invertase-resolvase family and play an important role (21). The proteins encoded by these genes catalyze precise excision and site-specific and orientation-specific integration of SCC*mec* into the *S. aureus* chromosome. In this manner, SSC*mec* is analogous to the pathogenicity islands found in gram-negative bacilli, except that this locus encodes resistance determinants instead of virulence factors. How an element as large as SCC*mec* is transferred from donor to recipient is unknown.

Regardless of the origins, which are likely to become obscured as clones move back and forth between health care patients and community populations over time, emergence of ORSA within the community is a major threat. Although most of the current isolates are sensitive to other drugs, this will not remain the case for long. The clinical implications are obvious: treatment failure with accompanying complications or death may result if an antistaphylococcal beta-lactam antibiotic is used for serious infections and the infecting strain proves to be resistant; infections caused by oxacillin-resistant strains may be more difficult to manage or more expensive to treat, in part because vancomycin is inherently less efficacious (9, 11, 31, 32); and the increasing prevalence of ORSA will inevitably increase vancomycin use, adding further to the problem of antimicrobial-resistant gram-positive bacteria.

EMERGENCE OF VANCOMYCIN RESISTANCE?

In 1997, a report of severe infection with an ORSA isolate with reduced susceptibility to vancomycin was reported from Japan (6). Since then several more reports of serious infections with vancomycin-intermediate *S. aureus* have been published from seven U.S. states and several other countries, suggesting that strains capable of expressing this phenotype are already widely distributed (7, 8). If anything has been learned about staphylococci in the antimicrobial era, it is that resistance will follow use of virtually every class of drugs. The appearance of vancomycin-intermediate *S. aureus* portends the near-term loss of the first-line drug as the treatment of choice for serious ORSA infections. Only immediate and widespread action to reduce vancomycin use is likely to halt the emergence of fully resistant strains, increasing prevalence of these strains in health care settings, and then their eventual emergence in community settings.

CONCLUSIONS

S. aureus is a formidable foe; not only does it posses a diverse array of virulence factors that can produce severe invasive infections and toxic shock, but it also is capable of developing resistance to antimicrobial agents faster than the pharmaceutical industry can create them. Resistance typically is first manifest in hospitals,

but ultimately resistant organisms or resistance factors spread to the community at large. Defining the factors that promote emergence and spread of resistance determinants may be facilitated by new research that is now possible since the staphylococcal genome has been sequenced. Novel targets for effective treatment or prevention may be identified in the near future. Nevertheless, given the inexorable march of this increasingly resistant organism, time is of the essence.

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Emerging Infections 5
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Chapter 6

Electron Transport-Deficient *Staphylococcus aureus* Small-Colony Variants as Emerging Pathogens

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SMALL-COLONY VARIANTS OF STAPHYLOCOCCI

A pathogenic organism can be seen as an "emerging" pathogen in several ways. It may be a newly discovered organism, an organism that has acquired new virulence factors or antibiotic resistance, or a well-known pathogen for which newly discovered subpopulations of the parent strain are able to produce disease. *Staphylococcus aureus* small-colony variants (SCVs) fall into the last category.

A variant subpopulation of *S. aureus* has been characterized that is defective in electron transport (73–79). These organisms grow slowly and produce colonies <10% the size of the parent strain grown on the same medium for the same amount of time. While SCVs have been appreciated for decades, we found that many of the clinical isolates were defective in electron transport because of defects in the pathways leading to the biosynthesis of electron transport chain components. Most of these clinical isolates cannot synthesize menadione or hemin, and this results in a block of electron transport at the level of menaquinone or the cytochromes (Fig. 1).

At first glance, staphylococcal SCVs might seem to be less virulent because of their slow growth and decreased production of coagulase and alpha-toxin. However, while the production of some virulence factors is sacrificed, SCVs gain an advantage by being able to persist inside of host cells and to resist antibiotics. The intracellular location shields them from host defenses (6, 50, 75, 106), their slow growth reduces the efficacy of cell wall-active antibiotics (73, 77), and a decreased membrane potential protects them from positively charged antimicrobials (e.g., ami-

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noglycosides and some cationic bactericidal proteins) (54, 73). Hence, they represent a subpopulation of *S. aureus* that can cause persistent and recurrent infections.

ILLUSTRATIVE CASES OF STAPHYLOCOCCAL SCV INFECTIONS

A case illustrating a recurrent infection was seen in a 69-year-old woman who had osteomyelitis of the right thigh in childhood and developed pain in her leg 53 years later (78). X-rays showed a mass, which was thought to be a malignant tumor, but a biopsy and culture revealed a hemin-auxotrophic *S. aureus* SCV. She denied any history of drainage, fevers, pain, or other symptoms during the 53-year disease-free interval before recurrence. An example of a patient with persistent infection is a grandfather who had a systemic infection with a menadione-auxotrophic staphylococcal SCV (78). He had get-well cards from his grandchildren that spanned November to February. His daily temperatures were persistently above 38.5°C, and his blood cultures remained positive throughout this 12.5-week interval. The colonial morphology showed a mix of normal and SCV organisms. Susceptibility testing showed that the large-colony forms were susceptible to methicillin, yet intravenous therapy with apparently active antibiotics had failed. Finally, the patient was given high-dose vitamin K and trimethoprim-sulfamethoxazole was added to his regimen. The vitamin K (menadione) had been shown to reverse the SCV phenotype and increase the activity of antibiotics. A large inflammatory mass in his right sternoclavicular joint resolved, and his fevers cleared within 36 h of addition of vitamin K. We hypothesize that reversal of the SCV phenotype released the organisms from their intracellular location and increased their growth rate in vivo, thereby increasing the activity of the antibiotics. We discuss the clinical situations where SCVs have been isolated and their potential mechanisms for disease causation below.

SCVs ARE RECOVERED FROM PATIENTS

SCVs have been reported to occur among a wide range of gram-positive and gram-negative organisms, including *S. aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, a *Shigella* sp., *Brucella abortus*, *Escherichia coli*, *Lactobacillus acidophilus*, *Serratia marcescens*, and *Neisseria gonorrhoeae* (1, 3, 4, 6, 7, 11, 14, 17–19, 21, 22, 27, 28, 40–43, 46–48, 48–51, 55, 57, 58, 60, 65–68, 77, 80–84, 86, 90, 92–103, 105, 107–109, 111–113, 115, 118). The common phenotypic trait is slow growth, which leads to the development of microcolonies, usually defined as being >10-fold smaller than the normal colonies. Several other features are often found, including auxotrophies for hemin, thiamine, or menadione (*S. aureus*, *S. epidermidis*, and *S. enterica* serovar Typhimurium) (1, 11, 17, 29, 47, 72, 78, 81–83, 89, 93, 94, 104, 115); decrease in pigment formation (carotenoids in *S. aureus* and pyocyanin in *P. aeruginosa*) (7, 8, 20, 27, 51, 72, 96, 113; I.-M. Jonsson, C. von Eiff, R. A. Proctor, G. Peters, and A. Tarkowski, *9th Int. Symp. Staphylococci Staphylococcal Infect.*, abstr. 198, 2000); decrease in fermentation of sugars (a *Shigella* sp. and *S. aureus*) (20, 116); ability to revert to the parental phenotype (a *Shigella* sp., *S. aureus*, and

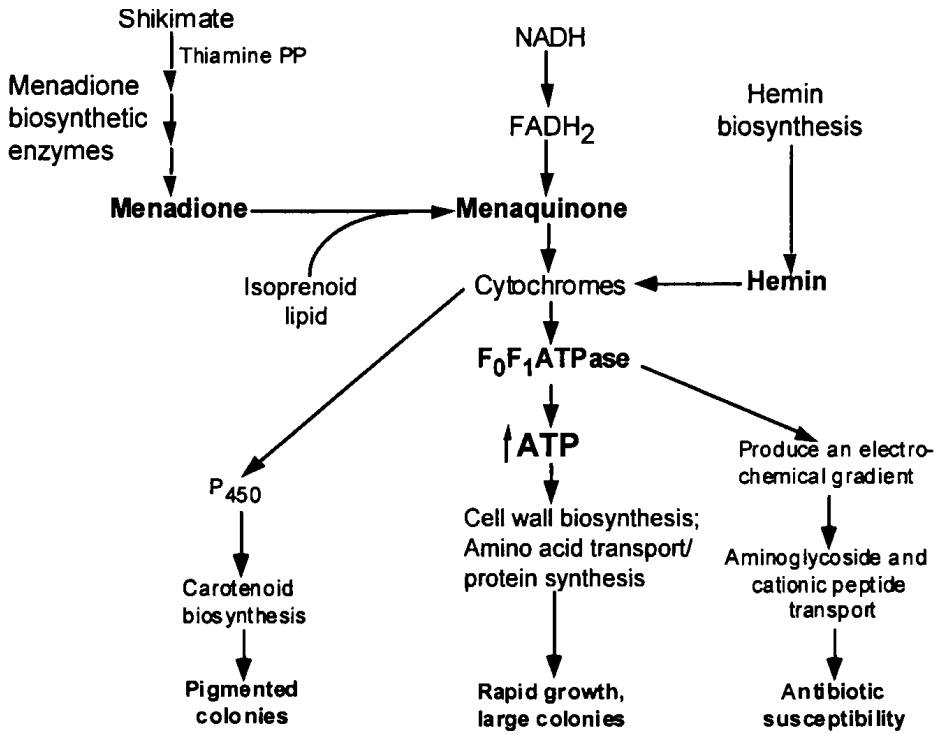

Figure 1. Relationship between electron transport and SCV phenotype in *S. aureus*. Clinical SCV isolates are frequently found to grow more rapidly when supplemented with thiamine, menadione, or hemin. Each of these substances is a component of the quinone or the cytochromes that constitute part of the electron transport chain. Intact electron transport provides ATP through the biosynthetic enzyme, the F_0F_1 ATPase; creates an electrochemical gradient across the cell membrane; and is involved in carotenoid biosynthesis. Interruption of electron transport because of the loss of one of the components in the chain or due to anaerobic growth causes multiple phenotypic changes. The organisms grow slowly, have reduced pigmentation, and are more resistant to antibiotics. Cell wall-active antibiotics are less effective because of the low rate of bacterial growth. The smaller negative membrane charge in bacteria deficient in electron transport results in decreased binding of positively charged antibiotics to the membrane. $FADH_2$, reduced flavin adenine dinucleotide.

S. epidermidis) (17, 20, 21, 68, 80, 97, 113, 117, 118); and decreased respiration (a *Shigella* sp. and *S. aureus*) (21, 116). The largest number of phenotypic characterizations and studies of SCVs is available for staphylococcal species, and these organisms will be emphasized in the rest of this review. Of interest, several phenotypic characteristics vary simultaneously when *S. aureus* organisms become SCVs; e.g., *S. aureus* is frequently nonhemolytic (6, 14, 41, 50, 73–80, 106, 113), fails to produce pigment (6, 7, 20, 50, 51, 72, 96, 113), produces only small amounts of coagulase (6, 41, 50, 72, 80, 113), shows decreased mannitol fermentation (6, 7, 20, 48, 50, 53, 68, 92), and displays resistance to aminoglycoside

antibiotics (1, 6, 7, 11, 14, 18, 20, 24, 25, 50, 57, 58, 65, 66, 68, 72, 73, 78, 80, 95, 105–107, 115).

S. aureus SCVs fulfill Koch's postulates. They have been recovered, sometimes in pure culture, from abscesses (1, 39, 42, 57, 90, 92, 99), blood (1, 53, 78, 81, 95, 112, 113), bones and joints (1, 11, 45, 78, 105, 107), the respiratory tract (17, 50, 95, 112, 113), and soft tissues (17, 39, 57, 58, 78, 91, 99, 112). In animal studies, *S. aureus* SCVs cause disease and can be reisolated in pure culture (20, 66, 68, 72, 80, 113). However, some animal models suggest that *S. aureus* SCVs may be less virulent than normal strains (66, 68, 72, 80, 97, 113), as measured by lethal doses and fatality rates. This may in part be due to decreased resistance in serum (113) and/or decreased rate of growth (1, 3, 4, 6–8, 11, 14, 17, 18, 20–22, 29, 39–41, 45–47, 50, 51, 55, 57, 58, 60, 66–68, 78, 80–84, 86, 90, 92–97, 99, 104, 109, 111–113, 115, 118). In spite of this apparent decreased virulence, *S. aureus* SCVs are able to persist as well as the parent strains in these animal models (3, 39, 61, 68, 80, 113). More recently, we have found that *S. aureus* SCVs show increased virulence with regard to joint destruction in a murine model (Jonsson et al., *9th Int. Symp. Staphylococci Staphylococcal Infect.*). This probably relates to increased production of proteases found in the hemin auxotrophic strain used in this study (Jonsson et al., *9th Int. Symp. Staphylococci Staphylococcal Infect.*).

Antibiotics, especially aminoglycosides, but also sulfonamides and β-lactam antibiotics, select for SCVs (1, 7, 11, 14, 17, 18, 20, 50, 54, 57, 58, 61, 68, 72, 73, 78, 86, 95, 104–108, 115). Although many SCVs are unstable, i.e., revert to a rapidly growing phenotype, stable SCVs are most frequently isolated from patients and animals receiving long-term antibiotics (1, 8, 11, 17, 18, 50, 57, 58, 75, 78, 88, 95, 107, 108) or from organisms cultured in the presence of antibiotics (5–7, 17, 20, 51, 60, 68, 72, 80, 85, 99, 111, 113, 115, 118). SCVs may be more resistant to antibiotics due to decreased uptake of drugs (aminoglycosides) (15, 16, 54, 59, 65, 66, 73, 74, 107, 108) or because of their low rate of growth (beta-lactams). SCVs selected by sulfonamides are most frequently thymidine auxotrophs, which are a different class of SCVs than are those considered herein (50). Nevertheless, SCVs have been isolated from patients who have not received antibiotics (11, 45, 80, 113) and in vitro by repeated subculture or direct isolation from aged cultures (22, 40, 45, 96, 97). There is also the possibility for selection of SCVs within mammalian cells by cationic peptides that are found in tissues, as these compounds are known to exert a selective pressure in vitro (55; R. A. Proctor, unpublished data [on protamine]).

Phagocytosis of *S. aureus* may favor the selection of staphylococcal SCVs. *S. epidermidis* (3) and *S. aureus* (18, 66) SCVs have been harvested from rats and rabbits by using the endocarditis model. In these cases, antibiotic selection may have played a role, but we also have found that infecting cultured endothelial cells with wild-type *S. aureus* leads to an enrichment for SCVs (104). This selection occurs in the absence of antibiotics within the intracellular milieu, because lysostaphin was continuously present in the culture medium (6, 102). In this tissue culture model, the number of organisms inoculated was 5×10^7, yet five relatively stable *S. aureus* SCVs were obtained, which is orders of magnitude higher than would be predicted from the rate of isolation of SCVs in broth or on solid medium.

The factor(s) in the host cells that causes this shift to SCVs is unknown, but the presence of host cationic proteins may select for SCVs (55, 73).

SCVs ARE EASY TO MISS DUE TO THEIR ATYPICAL PHENOTYPE FOR *S. AUREUS*

Slow growth, atypical colony morphology, and an unusual biochemical profile make clinical laboratory personnel likely to miss or misidentify SCVs. Such an error is important, because this subpopulation is more resistant to antibiotics than the parent population from which they arose. When an infection is particularly resistant to therapy, persists for a long period of time, or fails apparently adequate antimicrobial therapy, then the clinician and clinical laboratory should consider special efforts to search for SCVs.

The presence of SCVs in clinical specimens may be masked by the standard laboratory routines used to culture bacteria (49, 50, 74–79, 105, 107, 108). Slow growth allows SCVs to be rapidly overgrown by normally growing organisms in liquid medium (division time of 180 versus 20 min), and SCVs may be missed on agar plates because the SCV colonies appear after 48 h, i.e., at a time when the plates may have already been discarded (49, 50). Also, SCVs tend to be unstable (17, 20, 49, 68, 75, 78, 80, 97, 105, 107, 108, 113, 117, 118). Even a small percentage of reversion at an early point in time would allow overgrowth by the more rapidly proliferating forms. Hence, only pure or stable cultures of SCVs would be likely to be found unless special efforts are made, e.g., primary plating of specimens onto solid medium, incubating cultures for at least 72 h, and reporting colonial variants to the physician. In our experience, SCVs are found as mixtures of normal and SCV colony types. When the SCVs are further characterized and found to be the same as their more rapidly growing counterparts, laboratory personnel may discard the plates and not report these variants. Other factors that make SCVs difficult to culture and identify are atypical colony morphology (e.g., nonhemolytic, translucent colonies), unusual biochemical profiles (e.g., holding the test for up to 24 h before it shows coagulase positivity or decreased sugar fermentation), and fastidious requirements for rapid growth (e.g., auxotrophy for menadione, thiamine, CO_2, or hemin) (1, 6, 11, 39, 47, 50, 51, 72, 81, 83, 90, 92–95, 99, 109, 115). Unless a tube coagulase test is performed and the test is incubated for 24 h, *S. aureus* SCVs may be reported to be coagulase negative. Nevertheless, two studies are available that suggest that *S. aureus* SCVs are found in 1 to 2% of clinical isolates (1, 113). The major drawback to these studies is that no primary plating was performed on these clinical isolates, resulting in the recovery of only relatively stable and/or pure cultures of SCVs. Thus, the frequency of SCV infections may have been underestimated in these two studies, and overall, such infections may be easily missed in the clinical laboratory because of atypical colony morphology, atypical biochemical profile, and slow, fastidious growth.

Over the past 8 years, we have described *S. aureus* and *S. epidermidis* SCVs in studies of patients with osteomyelitis, bacteremia, cystic fibrosis, pacemaker infection, and cellulitis. Most of these SCVs had deficits in either menaquinone or hemin biosynthesis (49, 50, 78, 105, 107, 108). Some patients had infections that persisted

for prolonged periods, e.g., 27 and 53 years (78), while others continued to have fever or other signs of active infection in spite of prolonged, intensive antibiotic therapy (51, 78, 105, 107, 108). Frequently, both normal and SCV phenotypes were isolated, but they were shown to be clonal by field inversion gel electrophoresis (51, 78, 105, 107, 108). Thus, clinical *S. aureus* SCV isolates with defects in electron transport cause persistent infections that resist antibiotic therapy. In previous reports, *S. aureus* or *S. epidermidis* has been found to cause persistent, resistant, and recurrent infections in animal models (18, 66, 80, 113) and in human disease (3, 17, 39, 45, 53, 68, 113).

The basis for the persistent and antibiotic-resistant infections may be related to the ability of *S. aureus* SCVs to reside within cultured cells (6). Persistence is thought to be due to the reduced production of alpha-toxin (6, 106). The importance of alpha-toxin production for endothelial cell lysis has been previously established with a site-directed mutant of the alpha-toxin gene (102). The alpha-toxin-negative mutants, but not the parent strain, were able to persist within cultured endothelial cells. While the mechanism for decreased production of alpha-toxin by *S. aureus* SCVs is unknown, most laboratory and clinical *S. aureus* SCVs produce nonhemolytic colonies (6, 14, 41, 50, 59, 66, 68, 72, 78, 80, 104, 106, 107), whereas almost all normally growing *S. aureus* clinical isolates are strongly hemolytic.

METABOLIC BASIS OF SCVs

Reduced alpha-toxin production in *S. aureus* SCVs shows a strong correlation between a disrupted electron transport chain and alpha-toxin production (6, 73, 74, 79, 106). A review of the literature shows that *S. aureus*, as well as other SCVs from other genera (e.g., *E. coli*, *S. epidermidis*, *Salmonella*, and *P. aeruginosa*), demonstrates reduced electron transport activity as assessed by decreased respiration, decreased methylene blue dye reduction, decreased oxidative metabolism of sugars, reduced ATP production, or decreased tetrazolium dye reduction (11, 15, 16, 20, 21, 28, 51, 54, 78, 81, 82, 94, 104, 106, 108, 115). In *S. aureus* SCVs, reversal of the SCV phenotype by adding compounds that can be utilized to repair the defect in electron transport simultaneously (6, 55, 73, 74, 78, 104–106) or by complementing the genetic defect in *trans* enhances alpha-toxin production (106). Compounds that have been tested are menadione and hemin. Hemin is used in cytochrome biosynthesis, and menadione is isoprenylated to form menaquinone (9, 47, 101) (Fig. 1). Both menaquinone and cytochromes are components of the electron transport system in *S. aureus*. Well-characterized *S. aureus* hemin biosynthetic mutants demonstrate an SCV phenotype, including decreased coagulase activity, aminoglycoside resistance, decreased pigmentation, slow growth, and reduced hemolytic activity (59, 106). Finally, anaerobic growth of five strongly hemolytic *S. aureus* clinical isolates on rabbit blood agar produced nonhemolytic colonies (P. van Langevelde and R. A. Proctor, unpublished results). Anaerobic growth downregulates menaquinone biosynthesis in *S. aureus* (9) and electron transport. Taken together, these observations show that alpha-toxin production is dependent upon electron transport.

MECHANISMS OF REDUCED ALPHA-TOXIN PRODUCTION IN *S. AUREUS* SCVs

Reduced alpha-toxin production seems to arise as a selective, rather than a generalized, response to decreased electron transport. This results in two populations of organisms: an aggressive (alpha-toxin-producing) subpopulation that causes acute destruction of host tissues and a quiescent subpopulation (i.e., SCVs) that can persist within the protective environment of host cells yet cause recurrent disease when the electron transport chain is reconstituted. Our initial thoughts were that the reduced coagulase and alpha-toxin production probably arose due to reduced availability of amino acids that are needed for protein synthesis because all amino acids in *S. aureus* SCVs are imported via ATP-dependent mechanisms (74, 91). This seems unlikely in view of the ability of *S. aureus* SCVs to grow over 7 orders of magnitude (106) and produce increased quantities of proteases (Jonsson et al., *9th Int. Symp. Staphylococci Staphylococcal Infect.*). An alternative mechanism could be reduced transport of alpha-toxin (59), but this suggestion is controversial as other workers find that alpha-toxin is not actively exported from *S. aureus* (S. Bhakdi [University of Giessen, Giessen, Germany], personal communication). While *S. aureus* SCVs make small amounts of coagulase, they show increased protease production, showing that exoproteins are released. Because toxin production in *S. aureus* is highly regulated, another scenario to link decreased alpha-toxin with interruption of electron transport is via interactions between metabolic products (e.g., decreased ATP) that change with blocked electron transport and regulatory molecules.

In an effort to define the mechanisms for decreased alpha-toxin production, we have begun a search for regulatory factors that might reproduce the SCV phenotype. The phenotypic control exerted by regulators of virulence factor production is shown in Table 1 (33, 34, 36–38, 61, 63, 64, 69, 98, 100, 114). Figure 2 shows how some of these regulators may interact. Regulatory factors (61, 63, 64, 98), two-component regulators (33, 34, 69), and sigma factors (56) are reasonably well-established as being involved in the production of alpha-toxin production and other exoproteins in *S. aureus*. On the other hand, a question mark is placed by the ResDE two-component regulator homologues because the exact role that this system plays in toxin production has not been studied in detail. Two groups have reported on ResDE homologue mutations in *S. aureus* (100, 114), but neither of the mutations produces typical SCVs. One mutation was in the sensor kinase and the other was in the response regulator. The mutants did not grow well anaerobically, but they grew normally aerobically, which is distinctly different from SCVs.

Table 1. Regulation of staphylococcal toxin production

Positive regulation	Negative regulation
SarA, Rlp	SarR, Rot, SarH1
σ^A	σ^B
Agr, SaeRS	ArlRS, ResDE homologues?[a]

[a]ResDE acts as a negative regulator for *spa* but a positive regulator for toxic shock syndrome toxin 1.

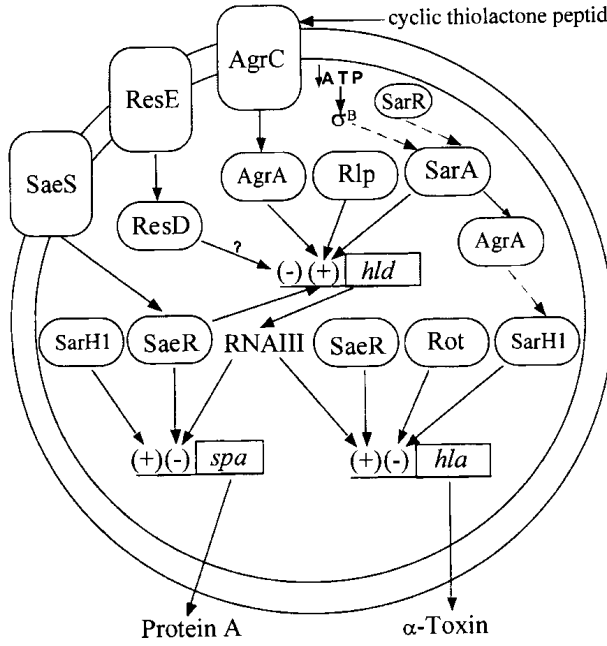

Figure 2. Hypothetical model for regulation of virulence factors in *S. aureus*. This is a schematic representation of several signaling factors that have been proposed to regulate staphylococcal virulence factor production. The sensor portions of the two-component regulators (SaeS, ResE, and AgrC) are shown as transmembrane proteins. The cognate response regulators (SaeR, ResD, and AgrA) are thought to be activated (phosphorylated) by the sensor proteins when stimulated. Solid arrows represent positive actions, whereas dashed arrows indicate negative signaling. The *agr* locus (accessory gene regulator) was the first reported global regulator of staphylococcal toxins. The *agr* operon includes a protein that is processed and released from the bacteria as an autoregulatory cyclic thiolactone peptide. RNAIII is the message from *hld*, and it has a dual role. When translated, δ-hemolysin is produced. However, when nontranscribed, RNAIII is an effector molecule that is involved in the regulation of a number of genes involved in the production of *S. aureus* virulence factors. Sar (staphylococcal accessory regulator) was subsequently identified and found to positively regulate Agr. SaeRS (*S. aureus* extracellular protein regulator) has recently been identified, and both its positive and negative actions are thought to act after RNAIII. Other protein regulators include Rot, Rlp, Sar, SarR, and SarH1. σ^B is a stress sigma factor that has negative regulatory effects on Sar. The activity of σ^B increases as ATP levels drop, thereby giving it a connection to electron transport. ResDE homologues have been identified in *S. aureus*, but their precise role in regulating exoprotein production is still undefined; therefore, a question mark is placed by this arrow.

Also, ResD has an important role in the regulation of *ctaA*, the gene for heme A which is used for catalase biosynthesis in *Bacillus subtilis*, but catalase was not tested in either report. While the idea has been put forward that menadione might be involved with ResD for SCV formation (114), this does not account for the same SCV phenotype in hemin biosynthetic mutants. Finally, neither mutation

could be phage transduced (100; P. Schlievert, personal communication). This is consistent with our findings wherein *resD* mutants were highly unstable (D. M. Bates, P. J. McNamara, and R. A. Proctor, unpublished data). Nevertheless, the SrhSR mutant showed decreased virulence in a murine pyelonephritis model, but the study did not report any measures of alpha-toxin, catalase, or other virulence factor production. Thus, while ResDE makes it an attractive candidate for the link between the expression of virulence factors and energy metabolism, more work must be performed before the question marks can be removed.

Overall, the phenotype of the SCV can be compared to the phenotypes seen when several of the known regulators are mutated, as shown in Table 2. As can be seen, increased or decreased activity of any single regulator will not produce the SCV phenotype. Hence, the SCV phenotype is likely to arise from an as yet undefined regulator or due to changes in a combination of regulators.

MECHANISMS FOR OTHER PHENOTYPIC CHANGES IN *S. AUREUS* SCVs

The nonexoprotein changes exhibited by *S. aureus* SCVs are more readily explained, as they can be linked directly to decreased electron transport activity (Fig. 1). Pigment formation (7, 20, 83, 106, 109, 113, 116) and aminoglycoside transport (15, 16, 52) require ATP and an intact electron transport system. Carotenoid pigments that give *S. aureus* SCV colonies their characteristic yellow color require ATP for their biosynthesis (56; Jonsson et al., *9th Int. Symp. Staphylococci Staphylococcal Infect.*). Interestingly, reduced levels of ATP activate σ^B, which is required for the bacteria to produce the orange pigment seen in older *S. aureus* colonies (56), but this only develops after the colonies initially turn yellow. Aminoglycoside uptake by *S. aureus* is an energy-dependent process that requires ATP and an electrochemical gradient, which is produced via the electron transport system (54, 56, 57). Decreased quantities of ATP result in slow growth, hence small

Table 2. SCV phenotype and exoprotein regulation in *S. aureus*

Extracellular protein[a]	Regulation of staphylococcal protein expression[b]								
	SCV	Agr	SaeRS	ArlSR	Sar	Rlp	Rot	SarH1	σ^B
Ser protease	↑	↑	↑	↑	↓	↑	↓	No Δ	↑
TSST-1	+	↑	↑		↑				↓
SEB–SEF	+	↑			↑				
Alpha-toxin	↓	↑	↑		↑	↑	↓	↓	↓
Beta-toxin			↑						
Protein A	↓	↓	±		↓			↑	↑
Coagulase	↓	↓	↑		↑	↓	↑		↓
FnBP	↓	↓			↑				↑
Lipase		↑	±						
Autolysis				↓					

[a]Abbreviations: TSST-1, toxic shock syndrome toxin 1; SEB-SEF, staphylococcal exotoxins B through F; FnBP, fibronectin-binding protein.
[b]Symbols: ↑, up-regulation; ↓, down-regulation; No Δ, no change; +, positive; ±, indeterminate.

colonies, because of the large number of ATP molecules used in cell wall biosynthesis (51; W. Fischer, personal communication). Finally, a block in electron transport would inhibit the mannitol-fermentative pathway of *S. aureus*, making the organisms mannitol negative (7, 20, 47, 54, 68, 92).

WHY ARE ELECTRON TRANSPORT VARIANTS FAVORED AS EMERGING PATHOGENS?

The *S. aureus* SCVs recovered from clinical isolates and from animal models are defective in electron transport (1, 11, 18, 29, 72, 78, 93). Why are electron transport variants found when there are many other auxotrophs that could produce slow growth and reduce alpha-toxin production? The prevalence of electron transport variants may be favored for several reasons. First, the pathway that is interrupted must be nonessential for bacterial survival. *S. aureus* SCVs and other bacterial species routinely survive without electron transport when grown anaerobically as long as glucose or fructose is available. Of interest, menaquinone biosynthesis is the component that is down-regulated under anaerobic conditions in *S. aureus* (9). In contrast, defects in amino acid metabolism are often lethal without an exogenous supply of the missing amino acid. Second, the defect must not be repaired by a substance readily available from the host. For example, vitamins (thiamine or menadione) and hemin are in limited supply within host cells (12, 71, 89), thus allowing the organism to maintain an SCV phenotype. While *S. aureus* tryptophan auxotrophs grew slowly on artificial medium (thus producing small colonies), they grew rapidly within cultured endothelial cells and readily lysed the monolayer (5). Third, the ability to revert to the parental phenotype would permit reversion to a more aggressive (therefore, clinically apparent) form. As menaquinone and hemin are not constitutively expressed, this allows variation in the electron transport chain activity. Thus, a nonessential pathway that can be regulated by the microorganism and that cannot be supplemented by substances within the intracellular milieu of the host cells may account for the predominance of electron transport-deficient strains in clinical SCV isolates.

SUMMARY

In summary, SCVs are found in clinical specimens. While their overall prevalence has not been firmly established, problems in discovering and identifying these organisms may cause an underestimation of their prevalence. A hypothetical model that accounts for most of the known characteristics of *S. aureus* SCVs is shown in Fig. 1. From the previous literature and our recent observations, clinical *S. aureus* SCV isolates have been shown to be auxotrophic for compounds that are biosynthesized into components of the electron transport system in almost all cases when biochemical characterization was performed. Menadione and hemin are the two most frequent substances that reverse the *S. aureus* SCV phenotype. In addition, thiamine is a cofactor used in menadione biosynthesis (9), and CO_2 may be involved in the synthesis of the porphyrin ring found in hemin (some *S. aureus* SCVs can be stimulated to grow by supplementing with either CO_2 or hemin [78]). Re-

duced activity of the electron transport system can account for most of the features typically seen in *S. aureus* SCVs; e.g., a reduction in available ATP would slow growth (cell wall biosynthesis uses large quantities of ATP), reduce pigment formation (ATP is required for carotenoid biosynthesis), and decrease aminoglycoside transport. Therefore, by down-regulating the biosynthesis of a nonessential pathway, *S. aureus* SCVs can produce multiple phenotypic changes which allow survival within the host cells, and reversal of the SCV phenotype may form the basis for recrudescent infections. This model is strongly supported by the observations with a genetically defined *hemB* mutant (106). Whether this is found with other bacterial genera is yet to be determined. An understanding of the signaling pathway(s) that results in SCVs may provide targets for drugs aimed at blocking the pathway (to prevent toxin production during sepsis) or stimulating the pathway (so that the SCV would revert to a wild-type phenotype and leave its persistent state within the host cell).

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Emerging Infections 5
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Chapter 7

Clindamycin-Resistant *Clostridium difficile*

Dale N. Gerding and Stuart Johnson

Clindamycin was the first antibiotic associated with the occurrence of pseudomembranous colitis (PMC) not attributable to *Staphylococcus aureus*. Initially, the disease was so strongly associated with use of the drug that it carried the name clindamycin-associated colitis until the occurrence of PMC in association with virtually every antibiotic was eventually described (8, 27). In the earliest endoscopic description of the disease in association with clindamycin by Tedesco et al., markedly high rates of the disease (20% diarrhea and 10% PMC) in association with use of the drug were described (27). These rates were far higher than previously or subsequently observed and raise speculation that a particularly virulent strain of *Clostridium difficile*, which was subsequently identified as the cause of PMC, was causing an outbreak at Barnes Hospital in St. Louis at the time. In light of subsequent data regarding clindamycin use as a risk factor for disease caused by clindamycin-resistant strains of *C. difficile*, it is also tempting to speculate that this outbreak was caused by clindamycin-resistant *C. difficile*.

CLINDAMYCIN RESISTANCE AMONG *C. DIFFICILE* ISOLATES IS NOT NEW

Early studies of the transmissible agent of antibiotic-associated enterocolitis identified a clostridial species that was resistant to clindamycin and was responsible for the transmission of disease from hamster to hamster (2). When this clostridial species, eventually identified as *C. difficile*, was isolated in large numbers from patients with *C. difficile*-associated diarrhea (CDAD), it was shown to have a variable susceptibility to clindamycin. Of 84 isolates of *C. difficile* from the earliest documented patients with antibiotic-associated diarrhea or colitis, 14% were resistant to clindamycin at >8.0 μg/ml and 9% were resistant at 128 μg/ml (10).

In this early study, it is remarkable that of isolates from 26 patients who were treated with clindamycin prior to becoming ill with CDAD, 11 isolates were sus-

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ceptible to clindamycin at 1 μg/ml but 6 isolates were resistant to clindamycin at 64 μg/ml. Furthermore, it was found that 6 of the 11 patients who harbored highly susceptible organisms noted the onset of diarrheal illness while they were still taking clindamycin. For four of the six patients, clindamycin was administered intravenously, but for two patients it was given orally. In contrast, all isolates of *C. difficile* were found to be susceptible to ampicillin, and 23 patients were treated with ampicillin as the causative agent for CDAD. This included 15 patients who were actively receiving ampicillin at the time of diarrhea onset. The authors analyzed the isolates of *C. difficile* that were obtained from patients who had received clindamycin and found that these isolates were significantly more resistant to clindamycin than were the isolates from patients who had been treated with other antimicrobials prior to the onset of CDAD. This established the first possible link between clindamycin resistance in *C. difficile* and the development of CDAD, as well as the association of clindamycin as an antibiotic use risk factor for the disease (10).

In 1985, Chow and colleagues published susceptibility data for 37 *C. difficile* isolates from Vancouver General Hospital in Vancouver, British Columbia, Canada, that revealed clindamycin MIC at which 50% of strains are inhibited (MIC_{50}) of 8 μg/ml and MIC_{90} of 32 μg/ml, with 81% of the isolates resistant at a clindamycin MIC of 4 μg/ml and 16% of the isolates resistant at a clindamycin MIC of 8 μg/ml (4).

In 1987, Clabots et al. reported the susceptibilities of 98 strains of *C. difficile*, 69 isolates from the Minneapolis Department of Veterans Affairs (VA) Medical Center and the others from five other U.S. hospitals (5). They found that the MIC_{50} of clindamycin was 4 μg/ml, whereas the MIC_{90} was >128 μg/ml, suggesting high-level resistance in a bimodal distribution. Subsequently, these authors published an account of a nosocomial *C. difficile* outbreak at the Minneapolis VA Medical Center in 1985 that was characterized by a unique plasmid pattern and high-level clindamycin resistance in the outbreak organisms (6). In that study, 171 unique isolates of *C. difficile* were tested, and 90 (53%) were found to be resistant to clindamycin. Typing of these isolates by a variety of methods indicated that a single epidemic clindamycin-resistant strain was responsible for the outbreak. This serves to illustrate the difficulty in interpreting susceptibility data if an epidemic strain is prevalent in a hospital and constitutes a large portion of the population being tested. It also illustrates the potential for a clindamycin-resistant strain or clone of *C. difficile* to produce an outbreak in a clinical hospital setting.

In 1988, Delmee and Avesani in Belgium separated *C. difficile* isolates into serogroups and determined their susceptibilities to clindamycin (9). They found no resistance to clindamycin in serogroups A, B, F, G, H, and X, whereas clindamycin resistance in serogroup C organisms was found in 30 of 32 isolates (94%), and resistance in the other serogroups (D, I, and K) ranged from 10 to 55%. Furthermore, of 64 patients reviewed for antibiotics associated with CDAD, all 7 patients who had taken clindamycin were found to have disease caused by clindamycin-resistant serogroup C *C. difficile*.

Overall, 21% of the 308 *C. difficile* isolates were resistant to clindamycin, yet by serogrouping, the authors were able to show that clindamycin resistance was

concentrated in specific groups of strains of *C. difficile*. Serogrouping is not sufficiently discriminating to differentiate a single clone of *C. difficile*, as evidenced by the inclusion of both toxin-positive and toxin-negative strains within the same serogroup. Thus, the concentration of resistance within specific serogroups could represent one or several clones of *C. difficile* bearing clindamycin resistance. Similarly, Mullany et al. reported that 41% of a collection of 121 largely European strains of *C. difficile* were resistant to clindamycin (breakpoint, 4 μg/ml), with resistance concentrated in specific radio-polyacrylamide gel electrophoresis (PAGE) types (18). Resistance of the macrolide-lincosamide-streptogramin (MLS) type was 100% in radio-PAGE type W, 67% in type Y, and 25 to 33% in types B, E, H, and X, with clindamycin and erythromycin MICs of ≥500 μg/ml. Radio-PAGE type W corresponds to serogroup C of Delmee and Avesani.

Taken together, the above-cited studies from the United States, Canada, and Europe confirm the widespread presence of clindamycin resistance among *C. difficile* isolates during the first 10 years following the discovery that the organism was the cause of PMC. In addition, at least two of the studies suggest that resistance varies with the serotype or molecular type of *C. difficile.*

CLINDAMYCIN SUSCEPTIBILITY IN THE 1990s

In 1993, the susceptibilities of 39 isolates of *C. difficile* from seven centers in North America were determined by Sheikh and colleagues (26). They found a clindamycin MIC_{50} of 4 μg/ml, and a MIC_{90} of 128 μg/ml. Using 50 isolates of *C. difficile* obtained in Sweden in 1995, Nord found a clindamycin MIC_{50} of 4 μg/ml and MIC_{90} of 8 μg/ml, with 50% of the isolates resistant to clindamycin at a MIC breakpoint of 4 μg/ml (20).

In 1999, Barbut and colleagues reported the comparative clindamycin susceptibilities of 198 strains of *C. difficile* from France, 100 isolated in 1991 and 98 isolated in 1997 (1). The data are reported as "reduced susceptibility" rather than resistance, and a clindamycin MIC of ≥2.0 μg/ml was used. Under these less stringent criteria, 86% of the *C. difficile* isolates in 1991 and 74.5% of the isolates in 1997 demonstrated reduced susceptibility to clindamycin. The isolates from 1997 were more susceptible to clindamycin than those from 1991, but there was a major shift in the serogroups of *C. difficile* isolated in the two periods. In 1991, serogroup C was the predominant serogroup, with 37% of all isolates. Serogroup C had previously been shown to carry resistance to clindamycin in 94% of strains. In contrast, in 1997 serogroup C constituted only 6% of the isolates. This shift in serogroups may have largely accounted for the increase in clindamycin susceptibility found in 1997.

CLINDAMYCIN RESISTANCE IS TRANSFERABLE AND LINKED

Studies by Dzink and Bartlett in 1980 and Delmee and Avesani in 1988 indicated a nearly perfect correlation of resistance to clindamycin and erythromycin, suggesting that the majority of these resistances may be linked (9, 10). A 1980 paper by Ionesco indicated that clindamycin and erythromycin resistances in *C. difficile*

were not transferable but were linked and curable, in contrast to tetracycline resistance, which was transferable but not curable (13). Subsequent studies by Wust and Hardegger found that resistances to clindamycin, erythromycin, and streptogramins were linked and were transferable to a susceptible strain of *C. difficile* (29, 30). Transfer was achieved in only one donor-recipient pair out of many combinations tested (possibly explaining the previous failure by Ionesco to document transfer), and the transfer frequency was low for erythromycin-clindamycin-streptogramins at frequencies of 1×10^{-8} to 4×10^{-8}, at the very limits of detection. Tetracycline transfers, in contrast, occurred at frequencies of 3×10^{-7} to 5×10^{-7}. Efforts to cure or eliminate resistance were unsuccessful, and the authors concluded that plasmid DNA was not involved in the transfer of resistance determinants. They also concluded that transfer of MLS resistance, at least in vitro, was a rare event (29).

Subsequently, Hachler et al. showed that erythromycin-clindamycin resistance could be transferred to another species, *S. aureus* (12). The resistance gene was designated *ermZ,* and was shown to have homology with *ermB*, which is associated with the *S. aureus* transposon Tn*551*. No association with plasmid transfer could be proven, and the genetic resistance location was presumed to be chromosomal, with transfer by means of a possible conjugative transposon.

Mullany et al. also demonstrated that MLS resistance (*ermBZ*) from *C. difficile* strain 630 (29) was transferable to *C. difficile* and transferable to and from *Bacillus subtilis* (18). Transfer frequencies were low at 10^{-9}. They showed that transfer into the recipient chromosome occurred in the absence of detectable plasmid DNA via a putative conjugative transposon designated Tn*5398*, which was found in six *C. difficile* strains. Farrow et al. further examined *C. difficile* strain 630 and found that it contains two copies of the *erm*(B) gene, the new nomenclature designation for *ermZ* (11). *erm*(B) was also the resistance gene subsequently discovered to be present in the epidemic *C. difficile* strain found in four U.S. hospitals in the 1990s (15). More than one *erm* gene has been found in *C. difficile* by using dot blot hybridization techniques, suggesting that MLS resistance is likely polygenic (23).

WHAT FECAL CLINDAMYCIN CONCENTRATION CONSTITUTES RESISTANCE?

The accepted breakpoint for clindamycin resistance based on drug concentrations in serum is an MIC of >4 μg/ml. A clinical breakpoint definition for concentrations of clindamycin in stools has not been established. Fecal clindamycin concentrations in the hamster model following a 3-mg oral dose (~30 mg/kg of body weight) were found to be 60 to 90 μg/g 1 day after administration and have been found to remain in the 4- to 6-μg/g range for up to 11 days following oral clindamycin administration (17). This long persistence of clindamycin in feces may be responsible for the observed high risk of CDAD associated with use of the drug. It also may account for an increased risk of clindamycin-resistant *C. difficile* infection in patients who have taken clindamycin (7, 15, 21).

These observations suggest that high-level clindamycin resistance (MIC, ≥128 μg/ml) would favor successful vegetative growth of *C. difficile*. Lower levels of

resistance (MIC, 8 to 64 μg/ml) appear to be less advantageous but still more likely to support successful growth in the presence of low fecal clindamycin concentrations than those of fully susceptible strains of *C. difficile*. Interestingly, essentially all strains of *C. difficile* are susceptible to ampicillin, yet CDAD has been reported to occur commonly while patients are taking ampicillin (5, 10). The speculation regarding the mechanism by which *C. difficile* is able to infect patients taking ampicillin has been that beta-lactamases in stool inactivate ampicillin, allowing *C. difficile* growth. Larson and Borriello found levels of ampicillin in the stools of hamsters on days 1, 2, and 3 following an oral dose of 3 mg to be <0.4 μg/g in seven of nine determinations (17). In only one of these stool specimens was beta-lactamase activity detected.

THE ROLE OF ANTIBIOTICS IN *C. DIFFICILE*-ASSOCIATED DIARRHEA

Any antibiotic use may be associated with subsequent CDAD, including metronidazole and vancomycin, agents used to treat the disease. The current best explanation of this is that the disruption of normal intestinal flora is likely the determining antibiotic event that makes patients susceptible to infection with *C. difficile*. CDAD has been postulated to be at least a "three-hit" disease (14). The initial event is antimicrobial exposure, followed by acquisition of *C. difficile*, which is then followed by a third hit that may include various host and organism factors that combine to determine if the patient will become ill with CDAD or become an asymptomatic carrier of the organism. One of the factors that determines if CDAD will occur has been shown to be the anamnestic serum immunoglobulin G antibody response of the host to *C. difficile* toxin A (16). Patients who are able to quickly mount a high antibody response to toxin A are not as likely to develop CDAD as those that have a slow or no antibody response.

Although clindamycin was strongly associated with CDAD in the 1970s, since the mid-1980s the second- and third-generation cephalosporins have accounted for most of the attributable risk of CDAD. The critical question regarding which agents have the highest relative risk of CDAD remains unanswered at this time. In general, clindamycin and the later-generation cephalosporins continue to be considered in the high-risk category, but because cephalosporin use is so much more frequent than clindamycin use, most reports attribute CDAD to cephalosporins as the most frequently used antibiotic group (3, 28, 31).

THE CLINICAL IMPORTANCE OF CLINDAMYCIN USE AND CLINDAMYCIN RESISTANCE IN *C. DIFFICILE*

In 1980, Dzink and Bartlett analyzed the clindamycin susceptibilities of isolates of *C. difficile* that were obtained from patients and correlated resistance with clindamycin treatment prior to the onset of CDAD (10). They found that the isolates from patients treated with clindamycin were significantly more resistant to clindamycin than were the isolates from patients who had been treated with other antimicrobials prior to the onset of CDAD. Delmee and Avesani reported that all

the cases (seven of seven) of CDAD that occurred in association with the use of clindamycin were caused by serogroup C toxin-positive *C. difficile* strains that were clindamycin resistant (9). This study was important in showing not only the relationship of clindamycin use and clindamycin resistance but also the possible clonal nature of clindamycin-resistant strains.

Clabots et al. in 1988 reported an outbreak of *C. difficile* diarrhea caused by a clindamycin-resistant strain bearing a cryptic plasmid (6). During a 3-month outbreak, new cases of CDAD more than doubled, and 67% of the isolates recovered were resistant to clindamycin, a significant increase from the 42% resistance in the previous 5 months and 43% resistance in the 4 months after the outbreak. Although the authors did not correlate risk of infection with this strain with the use of clindamycin, they did place clindamycin use under restriction and observed in retrospect an associated decline in CDAD rates, suggesting that clindamycin resistance and clindamycin use were important risk factors in this outbreak.

Perhaps the most convincing evidence of the clinical importance of clindamycin resistance and clindamycin use was first reported by Pear et al. in 1994 (21). An Arizona VA Medical Center observed a 13-month outbreak of CDAD at a rate five times the baseline that continued despite a major infection control campaign. Molecular typing revealed a single restriction endonuclease analysis (REA) type of *C. difficile*, J7, that was clindamycin resistant (MIC, >256 μg/ml) and constituted 59% of the isolates. Most compelling was the evidence for clindamycin as a risk factor for CDAD compared to other antimicrobials, as well as the high hospitalwide use of clindamycin that placed the hospital in the 99th percentile for clindamycin use among comparable institutions. The outbreak ended abruptly and dramatically with the institution of clindamycin use restrictions. In addition, the clindamycin-resistant epidemic REA type J7 disappeared with the cessation of the outbreak (21).

In an experience similar to that of Pear et al., Climo and colleagues reported an outbreak of CDAD that was caused by a clindamycin-resistant clone of *C. difficile* (7). Only 9% of *C. difficile* isolates in this outbreak were susceptible to clindamycin (MIC, $\leq$4 μg/ml). Disease caused by the resistant clone was associated with the use of clindamycin. A successful hospitalwide program of clindamycin restriction reduced clindamycin usage with an associated decrease in CDAD rates from 11.5 to 3.3 cases/month ($P < 0.001$). The clindamycin susceptibility of *C. difficile* isolates also increased from 9 to 61% ($P < 0.001$). As was the case with the study by Pear et al., and probably the study by Delmee and Avesani, these were clonal clindamycin-resistant *C. difficile* organisms causing outbreaks related to clindamycin use (9, 21). Quale et al. also reported a significant decline in CDAD rates in association with control of clindamycin use ($P = 0.006$), but measures included control of cefotaxime and vancomycin as well as clindamycin (22).

FOUR HOSPITAL OUTBREAKS OF RELATED CLINDAMYCIN-RESISTANT *C. DIFFICILE* INFECTION

A collaborative typing study involving epidemic and nonepidemic *C. difficile* isolates revealed that the epidemic strain from the Arizona CDAD outbreak men-

tioned above was also responsible for large hospital outbreaks in three other states (24). This epidemic strain was identified as J7/J9 by REA. Two of these hospital outbreaks were previously reported as isolated events, and the role of clindamycin was not apparent or had not been assessed (19, 25). Because of the strong association of the Arizona outbreak with clindamycin use, this association was examined retrospectively in the other hospital outbreaks. Clindamycin susceptibility testing was performed on all available isolates by the E-test, and representative isolates from each outbreak were examined for the presence of a previously described erythromycin resistance determinant, *erm*(B), by DNA hybridization and PCR (15). In addition, a case-control study was performed in three of the outbreaks to determine specific antimicrobial use within 60 days of the onset of CDAD due to the epidemic strain and CDAD due to nonepidemic strains.

High-level resistance to clindamycin (MIC, >256 μg/ml) was present in all 85 epidemic-strain isolates (100%) but in only 7 of 46 nonepidemic strain isolates (15%). The MICs for the 39 clindamycin-susceptible isolates were <4 μg/ml in 34 isolates and 6 μg/ml in 5 isolates. The epidemic strain was also highly resistant to erythromycin (MIC, >256 μg/ml) and resistant to ciprofloxacin (MIC, >32 μg/ml) but susceptible to ampicillin (MIC, 0.75 μg/ml) and tetracycline (MIC, 0.06 μg/ml). Representative epidemic-strain isolates from each outbreak were shown to contain an *erm*(B) gene identical or closely related to the *erm*(B) gene from *C. difficile* strain 630 (29). The ErmB determinant in strain 630 has recently been shown to contain two copies of the *erm*(B) gene, a novel genetic arrangement for an MLS resistance determinant (11).

The case control study showed that prior clindamycin use was more common for CDAD cases associated with the epidemic strain than for CDAD cases associated with nonepidemic strains (Table 1). This relationship was the same in each of the three outbreaks, despite different patterns of clindamycin use in each of the three hospitals, as reflected by the frequency of exposure to clindamycin among the nonepidemic cases, which ranged from 7% in Massachusetts to 23% in Arizona. The pooled odds ratio for clindamycin use among epidemic-associated CDAD cases was 4.35 (95% confidence interval [CI], 2.0 to 9.38; $P < 0.001$), whereas there was no significant association between usage of other antimicrobials and the epidemic-associated CDAD cases (Table 2).

Table 1. Clindamycin use prior to CDAD episodes due to the epidemic J7/J9 strain and CDAD due to nonepidemic strains in three large hospital outbreaks (15)

Hospital site	Clindamycin use[a]	
	Epidemic-strain-associated CDAD	Nonepidemic-strain-associated CDAD
Arizona	15/33 (45)	7/30 (23)
Massachusetts	9/30 (30)	4/60 (7)
New York	11/20 (55)	1/9 (11)
Total	35/83 (42)[b]	12/99 (12)

[a] Number of patients with prior exposure to clindamycin/number of cases of CDAD (percentage).
[b] Pooled odds ratio for clindamycin use associated with CDAD due to the epidemic strain, 4.35 (95% CI, 2.02 to 9.38), $P < 0.001$.

Table 2. Pooled odds ratios for antibiotic use by patients with CDAD due to the epidemic J7/J9 strain and those with CDAD due to nonepidemic strains (15)

Drug	OR[a]	95% CI	*P* value
Clindamycin	4.35	2.0–9.38	<0.001
Cefazolin	1.13	0.53–2.41	0.74
Cephalosporins[b]	1.02	0.45–2.32	0.95
Ampicillin	0.43	0.16–1.20	0.10
Vancomycin	1.09	0.49–2.45	0.83
Aminoglycosides	1.10	0.45–2.71	0.83

[a] OR, odds ratio.
[b] Third-generation cephalosporins, ceftazidime, ceftriaxone, and ceftizoxime.

This study demonstrated that large outbreaks of CDAD in geographically diverse regions within the United States were caused by a specific, highly clindamycin-resistant strain or clone of *C. difficile* and that clindamycin use was a specific risk factor. The unique association of clindamycin with CDAD has been recognized since the early 1970s, and this study may partially explain that association. In general, the role of antimicrobial agents in the predisposition to CDAD is likely to lie in the disruption of the normal intestinal flora. Prior to the studies of clindamycin resistance, no association had been made between the susceptibility of the infecting *C. difficile* strain and the precipitating antimicrobial agent. For example, as mentioned above, *C. difficile* is almost universally susceptible to ampicillin, yet this agent is frequently associated with CDAD. The presence of *erm*(B) in *C. difficile* appears to further increase the risk of CDAD, an established complication of antimicrobial therapy. This gene, which has been postulated to reside on a transposon in *C. difficile* strain 630, is already present in at least some clinical *C. difficile* strains in the United States and potentially worldwide (18).

CONCLUSIONS

Resistance to clindamycin in *C. difficile* has been present since its discovery as the cause of CDAD. Evidence from selected carefully studied outbreaks indicates that the incidence of CDAD associated with the use of clindamycin is increased when *C. difficile* is resistant to clindamycin. These outbreaks appear to be a result of clonal dissemination of clindamycin-resistant strains in selected hospital environments. It is not clear if overall resistance to clindamycin in *C. difficile* is increasing or remains stable. The only study to address this question showed a decrease in resistance but also a change in the *C. difficile* serogroup population from the first sample period to the second.

The linkage and transferability of clindamycin, erythromycin, and streptogramin B resistance has been documented in selected strains of *C. difficile.* The mechanism of MLS resistance via the *erm*(B) gene is well documented in one closely related group of *C. difficile* isolates responsible for major outbreaks in four U.S. hospitals. Restriction of clindamycin use in clonal outbreaks of clindamycin-resistant *C. difficile* infection has been highly successful not only in stopping the outbreak, but in producing marked reduction in isolation of the epidemic strain. *C. difficile* joins

the ranks of nosocomial pathogens for which antibiotic resistance contributes to pathogenicity. Fortunately, *C. difficile* clindamycin resistance also illustrates well the effectiveness of antimicrobial restriction as a strategy to reduce infection rates due to antibiotic-resistant hospital pathogens, a model that may also be profitably employed to control nosocomial outbreaks caused by other resistant species (7, 21).

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Chapter 8

Water-Transmissible Diseases and Hemodialysis

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End-stage renal disease (ESRD) is a condition that affects more than 1 million people around the world (38). Approximately 200,000 new patients start on renal replacement therapy every year.

Among the renal replacement therapy modalities, hemodialysis represents 80% of the first treatment option and 60% of the final treatment of choice (7). In hemodialysis patients, infections are associated with considerable morbidity and mortality, ranking second as a cause of death, after cardiovascular diseases. Among infections not directly related to the dialysis setting, pneumonia, urinary tract infections, and cutaneous infections are the most frequent (35). Among infections acquired during hemodialysis sessions or associated with the dialysis devices, vascular access infections are responsible for almost 50% of all infections and *Staphylococcus aureus* is the most frequent agent (21). Hepatitis B and C virus infections have been largely documented in hemodialysis units and are in part due to environmental transmission (10). Finally, water used during dialysis sessions may be responsible for the transmission of an increasing number of infections, with large and severe outbreaks being recently reported.

Hemodialysis is a complex process for depuration of undesirable solutes that accumulate in advanced chronic renal failure. This is accomplished by the use of dialyzers, which in turn are composed of semipermeable membranes. These membranes act as filters for the passage of the undesired products (e.g., urea) through diffusion and convection from the blood compartment within the dialyzer to the dialysis fluid compartment. This dialysis solution is a mixture of salts and water; the water is properly treated in each dialysis center. Under normal circumstances, fluid moves only from the blood compartment to the dialysis fluid compartment. In contrast, passage of fluid in the inverse direction, i.e., from the dialysate compartment to the blood side (backfiltration), may eventually occur. Backfiltration

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could theoretically increase the risk for the passage of bacteria or bacterial products from the dialysate to the blood compartment (and thus to the bloodstream), leading to pyrogenic reactions and other diseases. This has been a major concern, particularly since more-permeable hemodialysis membranes (high-flux membranes) have been developed and employed. Based on the molecular mass of lipopolysaccharide (LPS) (on the order of 10^6 Da), it is not expected that LPS could cross the dialysis membrane. However, bacterial products with lower molecular weights, such as LPS fragments, lipid A, and muramyl peptides, may also be present in the dialysate and may cross the hemodialysis membrane. These products possess monocyte-stimulating properties that lead to cytokine production, which has been used as an index of their passage from the dialysate.

Different in vitro studies have demonstrated that the passage of bacterial products is more likely to occur when cellulosic membranes (such as cuprofane and cellulose acetate), as opposed to the synthetic ones (such as polysulfone and polyacrilonitrile) (30, 33), are used. In part, this is due to a higher degree of adsorption of these substances to the synthetic dialysis membranes on the dialysate site (41). It has also been demonstrated that the passage of these bacterial products is not exclusively due to backfiltration but may also occur through diffusion of these products across the dialysis membrane (33). Furthermore, the practice of reutilization of the dialysis filters has also been associated with an increased risk of exposure of blood to these bacterial products. Reuse of dialyzers is a common practice in dialysis centers and is performed in 82% of American centers (40). Although it is considered a safe process, the use of disinfectants and high-pressure water may alter and increase the dialysis membrane permeability, favoring backtransport. In one study, it was demonstrated that the reprocessing of high-flux polysulfone dialyzers with bleach increased the risk of reverse transfer of bacterial products from contaminated dialysate, and this risk appeared to increase with the number of reuses (39).

WATER AS A VEHICLE FOR DISEASE TRANSMISSION IN HEMODIALYSIS

A single patient may be exposed to 150 liters of water during each dialysis session (14). Water used in dialysis sessions is the major source of bacteria, bacterial products, and electrolytes that may eventually reach the patients' bloodstream and cause different diseases. Because of the close contact with the patients' blood inside the dialyzer, water used in hemodialysis sessions must be properly treated to minimize the effects of occasional episodes of backtransport of bacteria and chemical contaminants. Very small amounts of chlorine, fluoride, aluminum, potassium, sodium, copper, zinc, calcium, cadmium, and other common constituents of source water are expected to remain after water treatment. With respect to microorganisms, the major concern is the growth of bacteria adapted to the water environment and capable of proliferating under restrictive conditions, such as those in the low-nutrient medium represented by treated water. These bacteria are mainly strains of nonfermenter gram-negative bacteria (*Pseudomonas* spp., *Acinetobacter* spp., *Stenotrophomonas maltophilia, Burkholderia cepacia, Alcaligenes* spp., *Fla-*

vobacterium spp., *Achromobacter* spp., and others) and also strains of nontuberculous mycobacteria (*M. chelonae, M. fortuitum, M. gordonae, M. scrofulaceum, M. kansasii, M. avium,* and *M. intracellulare*) (14). Not surprisingly, these agents are responsible for the majority of the outbreaks related to water contamination in dialysis facilities.

More important than the bacteria themselves are their products or constituents—mainly lipopolysaccharide or exotoxins, but also other components of bacterial wall or membranes, such as peptidoglycan and muramylpeptides. Smaller in size, these particles may easily reach the bloodstream through the pores of the dialysis filters if they are present in considerable amounts in water or dialysis fluid (28, 30) (Table 1).

Purified water for proper use in hemodialysis sessions may be obtained with the employment of water treatment stations (9, 14). These water treatment stations in turn comprise a pretreatment stage where water is cleared of large particles and chemical constituents. Large particles (diameter, 5 to 500 μm) are removed by sand or sediment filters. Softeners or deionizers are sequentially employed for electrolyte

Table 1. Water contaminants frequently related to pyrogenic reactions during dialysis sessions

Contaminant
Microorganism products
LPS
Muramyl peptides
Pseudomonas aeruginosa exotoxin
Peptidoglycan
Microcystin (algae)
Mycobacteria
M. chelonae
M. fortuitum
M. gordonae
M. scrofulaceum
M. kansasii
M. avium
M. intracellulare
Bacteria
Enterobacteriaceae
Serratia spp.
Enterobacter spp.
Nonfermenter gram-negative bacteria
Pseudomonas spp.
Acinetobacter spp.
S. maltophilia
B. cepacia
Alcaligenes spp.
Flavobacterium spp.
Achromobacter spp.

removal. Activated carbon filters are finally added to the pretreatment phase to remove chlorine, chloramine, and other organic components. Removal is achieved through adsorption by the microporous structure of the activated carbon.

This pretreatment stage is generally effective in lowering the total amount of cations and anions usually present in different water sources. On the other hand, the total load of bacteria and bacterial products rises sharply during the pretreatment. This is due to the porous nature of the devices, which allows bacteria to be retained and proliferate within the pores, and also because of chloride removal by the carbon filters.

The most commonly used device to remove bacteria and bacterial products employed after the above-mentioned pretreatment stage is the reverse-osmosis membrane. A membrane separation process based on molecular sieving and ionic exclusion (27) achieves elimination of bacterial components and also of the remaining amounts of chemical components. These membranes are usually able to retain and remove up to 99% of bacteria and bacterial products such as endotoxin. These devices must, however, be routinely disinfected, because bacterial multiplication within the membranes' pores has already been documented. Although more than 99% of U.S. dialysis centers employ reverse osmosis in their treatment stations, this is not the rule for developing countries.

After the water treatment stage, some other methods can be applied to achieve even lower bacterial counts. UV radiation has been proposed but is rarely used because some gram-negative strains may be resistant and overgrow. In addition, there is no effect on endotoxins, and in fact increased levels can be found due to bacterial wall destruction (15). Storage tanks, if necessary, must meet some specific standards to avoid slime formation, which is particularly common with nonfermenter gram-negative bacteria. These storage tanks must permit complete and adequate water drainage after regular disinfections and must have a preferential oval-shaped bottom, which minimizes the risk of slime formation. The pipes of the water distribution and delivery system to the dialysis machines must also meet some standards to avoid slime formation. They must be as short as possible, and the system must be free from rough joints, dead-end pipes, and unused branches and taps (3, 14, 34). The water distribution system must be periodically disinfected to avoid contamination and slime formation. Chlorine disinfectants as well as peracetic acid and even hot water (80°C) are usually employed to achieve effective disinfection.

Some authors advocate the use of ultrafilters with a pore size of less than 0.1 μm after the treated water is mixed with the dialysis fluid concentrate and before it reaches the dialysis filter (17, 32). Ultrafilters are effective for bacterium and endotoxin removal and are associated with lower rates of pyrogenic reactions. This may be particularly useful when a bicarbonate-based dialysis solution is employed, as bicarbonate favors bacterial growth. Table 2 summarizes water treatment devices employed in dialysis centers and their possible related problems. Figure 1 exemplifies a water treatment station in Brazil.

After adequate water treatment, specific standards must be met for the use in hemodialysis sessions. These standards have been developed by the observation of pyrogenic episodes as well as intoxication with chemical compounds and the cor-

Table 2. Basic components of water treatment stations for hemodialysis and potential drawbacks related to microorganism contamination

Step and component	Function	Drawback(s)
Pretreatment		
Prefilter	Elimination of large particles	Bacterial growth
Activated-carbon filter	Removal of organic compounds	Bacterial growth and difficult to sanitize
Softener	Removal of water hardness compounds	Bacterial growth and difficult to sanitize
Primary treatment		
Reverse osmosis	Effective removal of ions and bacterial products	Wears off
Deionizer	Final removal of ions ("polishment")	Noneffective for bacterial products
Ultrafilter	Removal of bacteria and their products	Bacterial growth on the membrane
Posttreatment		
Storage tank	Reservoir for treated water	Bacterial growth
UV radiation	Killing of bacteria	Increase in bacterial products

Figure 1. Water treatment system in a Brazilian private hospital. From right to left are seen one sand filter, two softeners (ion-exchange filters), two charcoal (carbon) filters, and a set of reverse-osmosis membranes.

Table 3. AAMI standards for treated water for hemodialysis sessions: maximum allowable chemical contaminant levels[a]

Chemical component of water	AAMI standard for treated water (mg/liter)
Calcium	2
Magnesium	4
Sodium	70
Potassium	8
Fluoride	0.2
Chlorine	0.5

[a]Data from reference 6.

relation of these observed conditions with levels of bacteria, endotoxin, and ions present in both treated water and dialysate. In the United States, the Association for the Advancement of Medical Instrumentation (AAMI) recommends that treated water in different points of the treatment system must contain less than 200 CFU of viable bacteria per ml and less than 1 ng of endotoxin per ml (as shown by the *Limulus* lysate assay). For the dialysate, as higher bacterial counts are expected, particularly when bicarbonate-based solutions are used, no more than 2,000 CFU/ml is allowed (6). Surveillance should be conducted on a regular basis, with at least a monthly evaluation. In Brazil and in different South American countries, the standards to be met are very similar to the American ones, but the values may differ for European countries and Japan (Tables 3 and 4).

Some specific recommendations related to the disinfection procedures of filters, reservoirs, and water distribution systems must be followed to maintain water quality with respect to microbiological standards. However, despite strict adherence to these practices, it may be difficult to achieve the proposed standards, and compliance rates as low as 70% have been reported even in developed countries (5, 29).

CLINICAL CONSEQUENCES OF USING POOR-QUALITY WATER AND DIALYSIS FLUID IN HEMODIALYSIS PATIENTS

Clinical Consequences of Chemical Contamination

Inorganic water contaminants such as trace minerals, heavy metals, and chemicals are generally not toxic to the general population. This may not be the same for dialysis patients, as they are exposed to a large volume of water per week

Table 4. Microbiologic standards for treated water and dialysis solution in different countries[a]

Country (yr)	Water standard (CFU/ml)	Dialysis fluid standard (CFU/ml)
Europe (1997)	<100	NS[b]
Japan (1995)	NS	<100
Canada (1986)	<200	NS
United States (1996)	<200	<2,000
Brazil	<200	<2,000

[a]Data from reference 9.
[b]NS, not specified.

across a relatively nonselective membrane that is permeable to a variety of substances, including some contaminants.

The water composition used in dialysis depends on its source, seasonal variations, and the municipal water treatment processes (2, 26). Water sources may be broadly classified into two major categories: surface water and groundwater. In surface water (rivers, streams, reservoirs, lakes, artificial water basins, and dams), the contaminants include organic materials and chemicals coming from agriculture and industry. In underground water (bore holes and wells) the contaminants depend on the geology of the area and are predominantly inorganic, including water hardness compounds, sulfates, chlorides, iron, and others.

For public health reasons, municipal water treatment authorities deliberately add substances to source water that may be hazardous to dialysis patients. As an example, fluoride, added by water companies in order to reduce tooth decay, and aluminum, added to clarify the water, are substances associated with bone disease in dialysis patients (2, 13, 26). Finally, water contaminants can also be added in dialysis units due to inadequate use of disinfectant products, due to copper or galvanized piping, or even as a result of an inappropriate water treatment system.

The presence of inorganic contaminants is associated with adverse outcomes in hemodialysis patients, including anemia, bone disease, and intradialytic nausea and vomiting (2, 13, 16, 18, 26, 42, 43). Clinical consequences of exposure to water contaminants are summarized in Table 5.

Recently, a high chloramine exposure due to an increased amount of this contaminant in source water by the water company in the United Kingdom was associated with the occurrence of erythropoietin resistance in hemodialysis patients

Table 5. Water contaminants and clinical consequences

Contaminant(s)	Source	Adverse event(s)
Aluminum	Municipal water	Encephalopathy, bone disease, anemia, abnormal myocardial function
Arsenic	Source water	Neurological damage
Cadmium	Source water	Anemia
Calcium, magnesium	Source water, municipal water	Nausea, vomiting, seizures, hypertension, muscle weakness
Chloramines	Municipal water	Anemia, hemolysis, methemoglobinemia, erythropoietin resistance, nausea, vomiting, hypotension
Copper	Dialysis unit	Methemoglobinemia, hemolysis, anemia, nausea, vomiting, diarrhea, hypotension
Cyanotoxin	Source water	Liver failure
Fluoride	Municipal water	Nausea, vomiting, diarrhea, hypotension, chest pain, fatal arrhythmia, bone disease
Iron	Source water, dialysis unit	Hemolysis, liver damage
Nitrates	Source water	Anemia, hemolysis, hypotension, nausea, vomiting
Sulfate	Source water	Nausea, vomiting, metabolic acidosis
Zinc	Dialysis unit	Hemolysis, anemia, nausea, vomiting, fever

(16). Chloramines may also cause hemolysis, hemolytic anemia, and methemoglobinemia, which decreases red blood cell half-life (2, 13, 26, 43).

Aluminum intoxication is still frequent in many developing countries with insufficient dialysis water purification systems (2, 13, 18, 26, 42). Moderately high aluminum concentration decreases hemoglobin synthesis and interferes with iron metabolism, leading to depletion of body iron stores (18, 42). Exposure of hemodialysis patients to high levels of aluminum has also been associated with osteomalacia and progressive neurological deterioration (2, 16, 26).

Prevention of water contamination requires not only the correct use of an appropriate water treatment system at the hemodialysis unit but also a continuous attention to water quality.

Long-Term Consequences of Microbial Contamination

The repetitive stimulation of monocytes in the blood side of the dialysis membrane, which produces cytokines and other proinflammatory mediators, may lead to a chronic state of systemic inflammation. Water and/or dialysis fluid with varying amounts of bacteria and bacterial products may be the vehicle of such chronic stimulation. The clinical consequences of this inflammatory state include β_2-microglobulin amyloidosis, susceptibility to infections, and an acute-phase response syndrome, the last of which has been associated with malnutrition and an increased incidence of cardiovascular diseases. In the acute-phase response, proinflammatory cytokines such as tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-6 lead to a steep increase in the hepatic synthesis of C-reactive protein (CRP) and serum amyloid A. Other proteins are decreased, such as albumin and transferrin, and hence are called negative acute-phase proteins. Several studies reported that hypoalbuminemia is a powerful predictor of mortality in dialysis patients (31). More recent studies have revealed that low levels of albumin in serum are often associated with inflammation in ESRD patients. High levels of CRP are associated with hypoalbuminemia in dialysis patients (25).

Cardiac disease has a major impact on mortality and morbidity in ESRD patients (1). CRP has been identified as a predictor of death from cardiovascular disease in hemodialysis patients (44). Therefore, it is conceivable that the long-term consequences of the chronic stimulation of monocytes by bacterial products that cross the dialysis membrane include malnutrition and increased cardiovascular mortality.

Short-Term Consequences of Microbial Contamination

Both microorganisms and their products may cause acute disease in hemodialysis patients if they accumulate in excessive amounts in treated water or dialysis fluid.

The most harmful outbreak related to contamination of treated water with microorganism products occurred in Brazil in 1996. This outbreak was due to water contamination with microcystins, which are cyclic peptides produced by different genera of blue-green algae (cyanobacteria). Microcystins are major hepatotoxic agents and may also affect the central nervous system. Pathogenesis is related to direct DNA damage by activation of celular endonucleases. Occasional ingestion of excessively contaminated water may cause only diarrhea, but chronic ingestion

has been proposed to be related to hepatocarcinoma. On the other hand, direct inoculation into the bloodstream may frequently be fatal.

In Brazil, in February 1996, 116 out of 130 patients (89%) in a dialysis clinic in Caruaru, Pernambuco (northeastern Brazil), began to complain mainly of visual disturbances, nausea, vomiting, headache, and muscle weakness. In March of the same year, 101 patients developed clinical and laboratory evidence of acute hepatic damage, and 50 patients died as a direct consequence of liver failure (23). Pathological findings showed on autopsy a uniform liver damage, with extensive necrosis, severe cholestasis, mitochondrial changes, and apoptosis.

Further investigation showed microcystin intoxication to be the cause of the outbreak. Considerable amounts of microcystin were recovered from water used in hemodialysis sessions as well as from the patients' serum and hepatic tissue. Epidemiological investigation revealed the water reservoir used as source water to have excessive growth of cyanobacteria and high levels of microcystin. Also, in the water treatment station at the dialysis center, routine disinfection of filters was not being done and reverse-osmosis membranes were not part of the treatment system. Although no similar outbreaks have been recorded since, water intoxication with microcystins is still a threat, mainly in countries whose water harbors cyanobacteria, such as Brazil. Routine testing for microcystin is now part of the regular screening for treated water in dialysis clinics.

Much more common than microcystin intoxication are episodes and outbreaks of pyrogenic reactions, caused mainly by gram-negative bacteria and their products and less frequently by strains of mycobacteria.

Pyrogenic reactions are episodes of rigors, shaking chills, fever, nausea, hypotension, and myalgia that occur after the start of the hemodialysis session. A great proportion of these reactions is due to bacteremia related to the vascular access, with central catheters associated with an odds ratio of 7.6 in comparison to native fistulas (21) and *S. aureus* being the major pathogen. Some episodes may be associated with a distant focus of infection, with urinary tract infections, skin infections, and pneumonia being the major clinical syndromes (35). Fever, however, is frequently present before the start of the dialysis sessions.

Excluding the above-mentioned situations, pyrogenic episodes may be associated with water contamination and less frequently with equipment contamination. In contrast, fever is not present before the beginning of the dialysis session.

There are only a few reports of the endemic levels of pyrogenic reactions, mostly from local experiences rather than national statistics. In the United States, the 1997 National Surveillance of Dialysis-Associated Diseases (40) showed that 21% of the dialysis centers (648 centers) reported at least one pyrogenic reaction, contrasting with only 13% in 1976. In this report, 1.7% of the dialysis centers (53 centers) reported clusters of pyrogenic reactions.

Water contamination is associated with pyrogenic reactions more frequently (but not exclusively) when bacterial counts exceed specific levels (see above). Contamination may occur at any point in the water pathway. Source water contamination has been recorded not only with microcystin but also with gram-negative bacteria, mycobacteria, and endotoxin. Inadequate treatment of source water may in turn be associated with pyrogenic reactions by contamination of the water used to prepare

disinfectants for the reuse of dialyzers, the water distribution system, and the dialysis solution. Contamination of the dialysis machine, though less frequent, may also occur. Contamination of the water distribution system and reservoir tanks has been reported and may be associated with prolonged outbreaks due to biofilm formation. Slime formation, frequently associated with nonfermenter gram-negative bacteria, occurs mainly in large-distribution polyvinyl chloride systems with rough joints and dead ends and with stagnant (noncirculating) water. Once formed, this glycocalyx-like extracellular material is very difficult to remove, and only repeated disinfections may achieve this goal. Glutaraldehyde (2%) and formaldehyde (2%) are more effective than the widely used chlorine-based solutions. Outbreaks due to biofilm formation may occur even in the presence of low bacterial counts in the freely flowing water and may sometimes be difficult to recognize (9).

Endotoxin may cause pyrogenic reactions by direct contact with the patients' bloodstreams, after passing through the dialyzer pores and stimulating the production of cytokines (mainly IL-1). Alternatively, endotoxin may stimulate monocytes on the dialyzer surface to produce cytokines, without actually traversing the membrane. Other components of the dialysis membrane, however, may also be able to activate monocytes and induce cytokine release, leading to pyrogenic reactions as well.

There are several reports of pyrogenic reactions related to water contamination in medical literature. Between January 1980 and June 1999, the Centers for Disease Control and Prevention investigated 16 outbreaks of bacteremia or pyrogenic reactions in hemodialysis patients in the United States. These outbreaks were related to dialyzer reuse in eight instances, inadequate water treatment in five, contaminated dialysis fluid in three, contaminated dialysis machines in two, and central catheter use in one (36). Some of these outbreaks illustrate both clinical features and specific mechanisms of disease transmission.

The dialyzer reuse process may be responsible for pyrogenic reactions. In 1995, Rudnick et al. (37) reported 22 pyrogenic reactions that were related to high concentrations of endotoxin in the water used to prepare the disinfectant employed in the dialyzer reuse process. Endotoxin and bacteria, if present in the disinfectant solution, may be retained by the dialyzer micropores and cause a pyrogenic reaction in the following dialysis session. There are several other examples of contaminated water for dialyzer reuse that resulted in such reactions. In 1985, Bolan et al. (8) described an outbreak of pyrogenic reactions due to strains of *M. chelonae* that affected 27 out of 140 patients attending a dialysis clinic in Louisiana. Fourteen patients had bacteremia alone, three had soft tissue infections, one had an access graft infection, and nine had disseminated disease. Fever (88%), malaise (83%), and anorexia (83%) were the main complaints. Within a 1-year follow-up, 50% of the patients had died. Further investigation showed that the source water was heavily contaminated with different species of nontuberculous mycobacteria. Also, the water treatment system was inadequate and the disinfectant employed for dialyzer reuse was 2% formaldehyde, which is inadequate to eradicate the mycobacterial strains. These strains persisted within the dialyzer structure and subsequently were transferred to the patients' bloodstreams. Although a 2% solution is effective for the eradication of gram-negative bacteria, a 4% solution is needed to eliminate

mycobacteria. Inadequate disinfectant concentration (36) or disinfectants that have been shown to destroy the membrane integrity have also been responsible for outbreaks of gram-negative-organism bacteremia and endotoxemia (11).

Inadequate water treatment may be frequently implicated in pyrogenic reactions. This may be caused by the lack of adequate water treatment stations and is frequently due to the absence of reverse osmosis membranes (20). On the other hand, even with adequate treatment systems, water quality standards may be difficult to achieve, leading to epidemics of pyrogenic reactions (19, 22). We have had our own experience in São Paulo, in southeastern Brazil, at the Kidney and Hypertension Hospital (unpublished data). During June 2000, our outpatient dialysis clinic registered 38 pyrogenic reactions in 38 patients. *S. maltophilia* (32%) and *B. cepacia* (18%) were the main pathogens recovered from blood cultures. In 32% of the pyrogenic reactions, blood cultures were negative, suggesting that endotoxin or other bacterial products were responsible for the reactions. Culture-positive and culture-negative pyrogenic reactions were clinically indistinguishable. The assistant nephrologist was accustomed to administering antibiotics based on the severity of clinical symptoms. Correlating the prescription of antibiotics with further blood culture results, the rate of empiric antibiotic administration was the same (30%) for either *S. maltophilia, B. cepacia,* or culture-negative pyrogenic reactions. Host factors as well as the inoculum size may be the main factors associated with the severity of the reactions. Another characteristic of this outbreak was that, although no deaths occurred and more than 95% of the patients were managed without hospitalization, only those patients whose dialysis was performed with a central line had recurring pyrogenic episodes with the same strain, even with proper antibiotic treatment. Only with catheter removal was complete resolution of the infection achieved. In this particular outbreak, water became contaminated inside a reservoir tank (after reverse-osmosis treatment) and further contaminated the entire water distribution system. Pulsed-field gel electrophoresis analysis showed identical molecular profiles for a strain of *S. maltophilia* recovered from water and strains recovered from the blood of symptomatic patients. Only after repeated disinfections of the storage tank was the outbreak finally controlled.

Although less frequent than water contamination as a cause of pyrogenic reaction during dialysis, dialysis machines and the dialysis setup process are sometimes implicated. Contamination of dialysis machines and lines due to backflow of bacteria from waste handling options (WHO) (a waste port designed to dispose of the saline used to flush a dialyzer before the machine is used for a patient) has been described. Three outbreaks with gram-negative-organism bacteremia due to this mechanism have been described in Canada, the United States, and Israel (12). In a single report in Chicago, 29 episodes of bloodstream infections with 16 different pathogens were caused by WHO contamination. Although no deaths related to infection occurred, 21 patients required hospital admission and intravenous antibiotic administration (4). The WHO, however, are disposable elements in the dialysis process. Direct contamination of the internal parts of the dialysis machines is seldom reported. It is recommended that during the disinfection process, the disinfectant must reach the entire water pathway.

There are a few examples of pyrogenic reactions due to contamination during the dialysis setup. In a report from Thailand (24), nine patients were reported to have bloodstream infections due to *B. cepacia*. A molecular analysis showed that the blood isolates were identical to *B. cepacia* strains isolated in the chlorexidine-based solution used in the catheter site disinfection process. One patient died as a direct consequence of infection, and the remainder of the patients failed to respond to antibiotic therapy until the catheter was removed.

These epidemiological mechanisms of water-transmissible infections in hemodialysis are summarized in Table 6.

CONCLUSIONS

ESRD patients are an expanding population highly susceptible to infectious diseases. In hemodialysis patients, which represent the great majority of patients on renal substitutive therapy, infections are second only to cardiovascular diseases as the cause of death. In this set of patients, water has been shown to be a vehicle for disease transmission, particularly of infectious diseases. Because of the close contact with the patients' bloodstreams, water must be properly treated before use in hemodialysis sessions and must meet specific microbiological and chemical standards. Reuse of dialysis filters is a known risk factor for backtransport of water contaminants to the patient's bloodstream. In what refers to microbiological contamination, nonfermenter gram-negative bacteria, strains of mycobacteria, and mainly bacterial products are the major concerns. Excessive accumulation in water or dialysis fluid, along with increased permeability of the dialyzer membrane, is related to outbreaks of pyrogenic reactions, disseminated mycobacterial diseases, endotoxemia, and large and fatal outbreaks such as an epidemic of microcystin intoxication in Brazil, where 50 patients attending a dialysis clinic died due to liver failure.

Since the great majority of these outbreaks arise from source water contamination or improper water treatment, optimal water treatment stations coupled with

Table 6. Epidemiology of water-transmissible diseases in hemodialysis[a]

Origin(s) of microbial contamination	Agent	Clinical consequences	Reference
Source water	Cyanobacterium (microcystin)	50 deaths due to hepatic failure	23
Water for dialyzer reprocessing	Endotoxin	22 pyrogenic reactions	37
Source water and incorrect disinfectant concentration	*M. chelonae*	27 mycobacterial infections (13 deaths)	8
Water reservoir after reverse osmosis	Nonfermenter gram-negative bacterium	38 pyrogenic reactions and bacteremia	Unpublished
Dialysis machine (WHO)	Gram-negative bacterium	29 bloodstream infections	4
Chlorhexidine used for catheter disinfection	*B. cepacia*	9 bloodstream infections (1 death)	24

[a]Examples of outbreaks and the underlying mechanism of microbial contamination are shown.

surveillance of water quality and pyrogenic reactions are the cornerstone measures for prevention. National databases should be built to define endemic acceptable levels of pyrogenic reactions, and the development of a consensus concerning disinfection modalities and water treatment options should also be encouraged.

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Emerging Infections 5
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Chapter 9

Mycobacterium ulcerans Infection and Buruli Ulcer Disease: Emergence of a Public Health Dilemma

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After tuberculosis and leprosy, Buruli ulcer disease (BUD) is the third most common mycobacterial disease among immunocompetent people in the tropical world. BUD (also known as Bairnsdale ulcer or Searles' ulcer), caused by *Mycobacterium ulcerans*, is characterized by indolent, necrotizing ulcerations of the skin. The skin lesions progress over weeks to months from typically painless, subcutaneous nodules or plaques to large, undermined ulcers, usually in the absence of systemic signs of illness. Adverse sequelae are common and include extensive scarring, flexion contractures, osteomyelitis, loss of a limb(s), and blindness. In 1897, BUD was first described by Sir Albert Cook in Uganda (31), and in 1948, the mycobacterial etiology was defined (35). Since then, the disease has been identified in many tropical and temperate parts of the world including West and Central Africa, Australia, Papua New Guinea, Malaysia, the South Pacific, and Central and South America (6). Over the past decade, there has been a noted increase in BUD in several West African countries (33, 36, 38). In areas where it is endemic, BUD has replaced tuberculosis and leprosy as the most prevalent mycobacterial disease, affecting up to 22% of the population in some communities (6). Because the current standard of treatment is surgical removal of the affected tissue and possible skin grafting, the economic burden on health care systems is substantial (5).

Despite the long history of study, the wide geographic distribution, and the severity of BUD, basic questions remain regarding the route and reservoir of in-

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fection, diagnosis, treatment, and public health control strategies. Currently, control strategies for BUD are limited to early treatment to reduce hospitalization, extent of surgery, sequelae, severity of morbidity, mortality, and costs. Surgical intervention at the preulcerative stage of disease is believed to prevent the development of the ulcerative stage of disease; however, confirmation of the diagnosis at the preulcerative stages of disease is difficult, because no rapid direct or indirect assays for laboratory confirmation are currently available. In addition, alternative treatment strategies to surgery have not been rigorously evaluated, because of the inability to rapidly confirm suspected cases, which is necessary for valid clinical trials.

In response to an emergence of this poorly understood disease, the World Health Organization (WHO) launched the Global Buruli Ulcer Initiative in 1998 (4, 67). The primary goals of this initiative are fourfold: first, to strengthen efforts to accurately estimate the burden of BUD and provide primary health care services in high-prevalence countries; second, to identify modifiable risk factors for *M. ulcerans* infection and refer BUD patients for medical therapy earlier in the course of the infectious process; third, to develop new diagnostic tests to identify patients with *M. ulcerans* infection early in the course of the infectious process so that antimicrobial chemotherapy can be evaluated; and fourth, to characterize *M. ulcerans* toxins to foster the development of a preventive vaccine or therapeutic antitoxin directed at one or more toxic antigens.

EPIDEMIOLOGY

BUD occurs in tropical countries (Fig. 1), with prominent foci in West Africa (1, 15, 38, 40, 41), Central Africa (7, 11, 59; E. Delaporte, S. Alfandari, and F.

Figure 1. Global distribution of BUD.

Piette, Letter, *Clin. Infect. Dis.* **18:**839, 1994) and the western Pacific (11, 26, 53); cases have also been reported from Asia and the Americas (6, 19, 66). Shaded areas in Fig. 1 do not represent the extent of the problem but indicate only those countries where the disease has been reported. The incidence and prevalence of the disease worldwide are not precisely defined, due to inadequate surveillance data at national and international levels. Moreover, the lack of routine case confirmation and the likely underreporting from passive surveillance systems make estimating the global burden of BUD difficult.

Incidence and prevalence rates for BUD have been reported in only a few locations under special circumstances. In Uganda, Buruli ulcer cases were observed for the first time in a group of 2,500 Rwandan refugees who had moved to a camp near the Nile River in 1964; this cohort was monitored intensively through 1970, when they moved away from the area of endemicity (3, 8). The highest incidence rate recorded was 7 per 100 people per year in the 10- to 15-year-old age group in 1966 and 1967 (3, 8). In the course of conducting a case-control study in the Daloa region of Ivory Coast, overall prevalence of past or present BUD was estimated to be 1.3 to 1.6%, with specific villages reaching 16% (6). More recently, in villages of the Amansie West district of Ghana where BUD is endemic, prevalence has been reported to range from 4 to 22% (6). To date, this increase has not been associated with underlying human immunodeficiency virus (HIV) infection (36).

Populations at risk appear to be residents of tropical climates who are exposed to stagnant, slowly moving water sources (13, 36). In one study, the incidence of BUD in a population located adjacent to swampy regions of the Nile River fell significantly when the population was relocated elsewhere (10). In West Africa, the majority of those afflicted are poor, rural farming communities that live adjacent to swampy environments (36, 67). In almost all of the case series analyzed, the sexes are equally represented, but children between the ages of 2 and 15 are disproportionately affected. The reason for this age distribution is unknown, but some aspect of behaviors among children or the absence of acquired immunity is presumed to put them at higher risk.

In 1991, in response to an increase in BUD patients reported by health care providers in the Daloa region of Ivory Coast, the Centers for Disease Control and Prevention conducted a case-control study in collaboration with the Ministry of Health of Ivory Coast (36). Since little was known about the suspected mode of transmission, a wide range of potential exposures were explored. Participation in farming activities near the Lobo river in the Daloa region was identified as a risk factor for disease (odds ratio for each 10-min decrease in walking distance between fields and the river, 1.52; 95% confidence interval, 1.01 to 2.28; $P = 0.0467$). In addition, wearing long pants was found to be protective (odds ratio, 0.20; 95% confidence interval, 0.06 to 0.62; $P < 0.005$) (36). Yet this disease does not appear to discriminate by race or socioeconomic status. In Australia, BUD appeared in a relatively wealthy Caucasian community near a golf course (55). The golf course water irrigation system, which relied on water pumped from an adjacent swamp, was implicated (55). The outbreak abated following the cessation of irrigation with

swamp water. Subsequent environmental investigations identified the presence of *M. ulcerans* DNA in rotting swamp vegetation (55).

Partly due to a long-standing inability to culture *M. ulcerans* from environmental specimens (47), the precise source of infection for humans is unknown. However, *M. ulcerans* is clearly associated with swamp or riverine environments, and outbreaks appear to be related to environmental changes involving surface water. Damming of streams to create artificial lakes in Nigeria and Australia (43, 55) and flooding in Uganda were associated with outbreaks of BUD (3). Several other man-made modifications to the environment are hypothesized to be linked with the current emergence of this disease in West Africa.

Based on PCR amplification of specific genomic sequences of *M. ulcerans*, it has been hypothesized that predatory insects of the genera *Naucoris* and *Diplonychus*, which feed on other water-filtering organisms, could passively concentrate *M. ulcerans* and possibly transmit *M. ulcerans* to humans (47; F. Portaels, P. Elsen, A. Guimaraes-Peres, P. A. Fonteyne, and W. M. Meyers, Letter, *Lancet* **353:**986, 1999). Koalas, rats, opossums, and a captive alpaca have been shown to be naturally infected in areas of endemicity in Australia (29, 39), and armadillos, rats, mice, guinea pigs, lizards, and cattle can be infected experimentally (47, 68).

The route of infection is unknown. The organism is postulated to enter at sites of trauma to the skin (15, 37), following contact with contaminated water or vegetation, or possibly an insect vector (J. Hayman, Letter, *Lancet* **337:**124, 1991; Portaels et al., letter). There is no confirmatory evidence for human-to-human transmission (9), although prolonged skin-to-skin contact leading to ulcer has been suggested (6).

DISEASE SURVEILLANCE

Three factors mitigate against the effectiveness of a passive disease surveillance system for BUD: (i) the disease typically occurs in very remote areas where villagers have little contact with the health care system, (ii) the disease is often considered by villagers to have no effective treatment, and (iii) treatment is often sought from traditional healers. Fortunately, there is an existing active surveillance system in West Africa that serves as an ideal model for active BUD surveillance. Like BUD, guinea worm presents as a cutaneous disease and occurs in areas adjacent to open freshwater sources. The Guinea Worm Eradication Program derives its success from an ongoing, active surveillance system relying on village health workers trained in the accurate recognition of a cutaneous guinea worm infection. Guinea worm patients are identified by village-based health workers who report their cases to a district coordinator. These surveillance data are forwarded to the regional and national levels, where the information is used to monitor the effectiveness of the program.

In Ghana, the disease is currently monitored through a passive surveillance system, using data collection forms developed by the WHO. A probable case of BUD is defined as a painless or minimally painful cutaneous ulcer with induration and undermined borders, a nonulcerative edematous plaque, or a subcutaneous nodule (active disease) or a depressed stellate scar without a history of previous burn at

the site of the scar (inactive disease) (6). A confirmed case is defined as a probable case with characteristic histopathology on skin biopsy, a positive acid-fast bacillus (AFB) smear taken from the ulcer, or a positive culture or PCR-based test for *M. ulcerans* (25).

PATHOGENESIS

M. ulcerans, in contrast to other mycobacteria, primarily grows as extracellular microcolonies within host tissues (28). The organism disrupts macrophages and adipose cell monolayers in vitro, in lieu of growing within these cells (45; K. Dobos, M. Deslauriers, P. Small, F. Quinn, and C. King, unpublished data). Rastogi and colleagues (50) have shown that although *M. ulcerans* infects and persists in murine macrophages after 4 days, no intracellular growth occurs. Others have demonstrated that culture filtrates (CF) from *M. ulcerans* suppressed phagocytosis of the bacilli, and they speculated that this in vitro suppression of phagocytosis might explain why the bacilli are only rarely observed within host cells during human disease (45). The necrosis observed in the skin lesions of *M. ulcerans* infections and absence of intracellular growth led to speculation that the bacteria produce a cytotoxin or other necrotic bacterial component. The CF from *M. ulcerans* has been shown to produce a cytotoxic effect on cultured fibroblasts (51). This material also simulated clinical and histopathologic changes when injected into guinea pigs, similar to those seen in BUD (34). An initial analysis of the CF resulted in the identification of a high-molecular-weight phospholipoprotein-polysaccharide complex that retained the ability to produce a cytotoxic effect on cell monolayers (30) and that may also be immunosuppressive (45).

Recent studies by George and colleagues have led to the identification and characterization of a unique polyketide in *M. ulcerans*, named mycolactone based on its origin and chemical structure (21). Mycolactone has been shown to induce rounding and detachment of murine fibroblasts due to the arresting of cell growth (20). Further studies have shown that intradermal injection of mycolactone produces lesions in guinea pigs similar to those observed in human disease (21) and that mycolactone induces apoptosis secondary to cell arrest (21, 22). Studies using protein-deficient media for the characterization of cytotoxic factors secreted from *M. ulcerans* have identified phospholipase activities (23, 42) and necrotic and apoptotic activities in cell models (Dobos et al., unpublished data) and guinea pigs (M. Deslauriers, J. Guarner, K. M. Dobos, J. Bartlett, S. R. Abner, C. H. King, and F. D. Quinn, unpublished data) that may augment the toxicity of *M. ulcerans*. Collectively, these studies demonstrate that the cytotoxic effects demonstrated by *M. ulcerans* in human disease may result from multiple factors.

CLINICAL PRESENTATION

The clinical spectrum of BUD ranges from a typically painless, subcutaneous nodule to large, undermined ulcers in the absence of systemic signs of illness (Color Plates 3 and 4 [see color insert]) (31). Adverse sequelae are common and include extensive scarring, flexion contractures, osteomyelitis, loss of a limb(s), and blind-

ness (Color Plate 5 [see color insert]). Death, when it occurs, is usually attributed to secondary bacterial infection, followed by sepsis, gangrene, or tetanus.

The disease is believed to begin with the entrance of the organism into the dermis or subcutis, where after a latent period, the bacteria proliferate and secrete toxin (29, 31). As with other mycobacterial infections, it seems likely that only a fraction of persons infected develop active cutaneous disease. The infectious dose for *M. ulcerans* is unknown. Animal models for *M. ulcerans* infection include mice, guinea pigs, and armadillos, but it is difficult to extrapolate from these to humans. Inoculation of 10^3 to 10^4 CFU is sufficient to induce swelling in the mouse footpad model (16). The time from infection to active disease is also unknown, but estimates range from 1 to 4 weeks to 3 to 4 months (3, 58). As noted previously, the ulcerative process is caused by the production of one or more *M. ulcerans* toxins (unique among mycobacteria) (22; Dobos et al., unpublished data), leading to coagulative necrosis of subcutaneous adipose tissue. The toxin appears to cause preferential necrosis of adipocytes, without provoking a local inflammatory response. At this stage, a preulcerative lesion may become clinically apparent in the form of a painless, palpable subcutaneous nodule. Alternatively, active BUD can first manifest as a raised papule; diffuse, nonpitting edema; or well-defined, elevated, indurated plaque (6). Left untreated, a preulcerative lesion may progress to an ulcer over the course of several weeks to months (43, 58). Hematogenous dissemination of *M. ulcerans* infection has also been hypothesized, particularly among patients with multiple lesions or recurrent disease at a distant site (31, 49).

LABORATORY CONFIRMATION OF *M. ULCERANS* INFECTION

Current methods used to confirm the diagnosis of BUD include (i) Ziehl-Neelsen staining of AFB, (ii) microbiological culture, (iii) histopathology, (iv) dual skin testing using burulin (purified protein derivative [PPD] of *M. ulcerans*) and tuberculin (PPD of *Mycobacterium tuberculosis*) (61, 62), and (v) PCR. One of the major goals of the WHO's Global Buruli Ulcer Initiative is to develop new diagnostic tests to identify patients infected with *M. ulcerans* prior to the development of advanced ulcerative disease (4, 6). Serologic assays for detection of antibodies against the secreted proteins found in *M. ulcerans* CF (MUCF) are being investigated (17). Such tools would allow the evaluation of antimycobacterial chemotherapy for early and advanced BUD and improved case confirmation for patient management and surveillance.

Ziehl-Neelsen Staining of AFB

Ziehl-Neelsen staining of AFB is a relatively simple technique, requiring swabbing of the undermined edge of the ulcer, deposition of the exudate onto a glass microscope slide, and a 5-min staining procedure. Although inexpensive and rapid, the use of AFB staining for the diagnosis of BUD is problematic due to the lack of sensitivity and specificity for the detection of the *M. ulcerans* bacilli.

Microbiological Culture

With adequate laboratory capacity, microbiological culture of swabs or tissue biopsies should be attempted using Löwenstein-Jensen or other selective media. Despite being considered the gold standard, the sensitivity of this method may also be low; however, a high bacillary load in the tissue may increase the success of recovery of *M. ulcerans*. Because of the slow growth rate of *M. ulcerans* and the necessity to perform confirmatory high-pressure liquid chromatography or PCR analyses for species verification, diagnosis via culture is rarely useful for clinical management. Procedures for isolation of *M. ulcerans* from biopsied tissues are provided in Fig. 2. Significant efforts should be made to optimize survival of mycobacteria during patient sample collection and transport. Patient samples should be placed promptly into sterile test tubes containing P5 transport medium (modified Dubos medium supplemented with polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin [PANTA] and 0.5% agar [7, 44, 52, 57]). Because most strains of *M. ulcerans* are sensitive to temperatures of ≥37°C and prefer 32°C, patient samples and cultures must be transported at cooler temperatures, e.g., 25°C

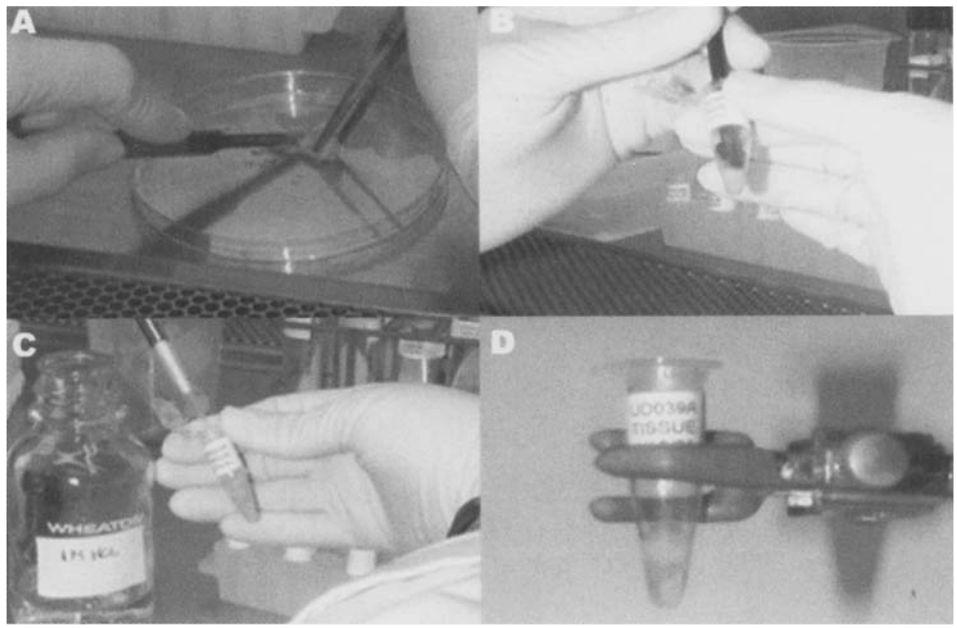

Figure 2. Culture isolation. (A) Tissue preparation. Tissue samples are removed from transport tubes and superficial layers and scar tissue are aseptically removed with a sterile scalpel. (B) Tissue homogenization. Tissue (mostly dermis and subcutaneous fascia) is homogenized using a sterile plastic pestle and phosphate-buffered saline. (C) Decontamination. Tissue homogenate is decontaminated with HCl and then neutralized with NaOH. (D) Sedimentation of bacilli. After decontamination, tissue homogenate is centrifuged; fatty tissue remains on the top layer, while bacteria settle to the bottom of the tube.

(46). As with most serious infections, culture confirmation of infection can optimize clinical management of suspected BUD patients. Because *M. ulcerans* is infrequently isolated, every well-characterized clinical isolate has the potential to add to the understanding of the molecular epidemiology of *M. ulcerans*.

Histopathology

Histopathologic examination of lesions is considered to be a time-tested, sensitive, reliable technique, but it requires significant expertise (12, 27). Compared to AFB staining and culture, the histopathologic diagnosis of BUD is more sensitive and specific (the characteristic subcutaneous necrosis observed in lesions is unique for *M. ulcerans* infection). The pathologic features of BUD vary depending on the stage of disease. In all stages, the three skin compartments (epidermis, dermis, and subcutaneous adipose tissue) may be affected to different degrees. The main histopathologic feature during the nodule stage is the necrosis of the subcutaneous adipose tissues (Color Plate 6A [see color insert]). This necrosis can be classified as coagulative since the tissue appears to maintain its architecture and shows intense eosinophilia due to loss of the normal basophilia imparted by cytoplasmic RNA. The adipose tissue septa (Color Plate 6A) demonstrate edema, fibrin deposition, mild inflammatory infiltrate mostly composed of neutrophils, and blood vessels that can show a variety of changes (thickening of the media, dilated lumen, and occasionally inflammation and thrombosis). It is usually in the subcutaneous septa that abundant clumps of AFB can be found (Color Plate 6B). In general, the subcutaneous nodule shows necrotic adipocytes (ghost cells) (Color Plate 6A), which may show calcifications. The deep dermis usually shows different degrees of elastolysis (separation of collagen fibers by basophilic material) (Color Plate 6A) and mild inflammatory neutrophilic infiltrate. The changes in the superficial dermis and epidermis seem to be reactive to the pathologic process in the subcutaneous tissue and consist of either focal or bandlike chronic inflammatory infiltrates (composed of lymphocytes, macrophages, and plasma cells) and various degrees of psoriasiform epidermal hyperplasia. Damage to the overlying epidermis ensues, leading to an ulcer with deeply undermined edges, often reaching the fascia but sparing muscle (27, 31, 67). During the ulcer stage of BUD, similar histopathological features can be seen in the subcutaneous tissue, e.g., coagulative necrosis. However, the changes can be obliterated by intense inflammation where the epidermis has been lost. The inflammatory infiltrate at this time point can be a mixture of acute cells (neutrophils), chronic cells (lymphocytes, macrophages, and plasma cells), and granulomatous inflammation (epithelioid macrophages and multinucleated giant cells). The inflammatory infiltrate is usually most intense in the dermis (Color Plate 7 [see color insert]) surrounding the ulcer. The epidermis can demonstrate various degrees of pseudoepitheliomatous hyperplasia (Color Plate 7), which is due to the need to repair the ulcerated area. During this stage, AFB can be difficult to find but may be found in the necrotic base of the ulcer or in the necrotic fat at the ulcer margin, which can extend quite far underneath the damaged skin above. Healing starts with the formation of granulation tissue at the base of the ulcer, and it may be linked to the initiation of a cell-mediated immune response (65). Some of the

difficulties with using histology for the diagnosis of *M. ulcerans* are the level of expertise required for sample preparation and interpretation, its impracticality for use in rural field conditions where BUD is endemic, and the time required to process each biopsied specimen.

Dual Skin Testing

Although in West Africa areas of endemicity for BUD and tuberculosis overlap, delayed-type hypersensitivity skin tests using burulin (PPD of *M. ulcerans*) and tuberculin (PPD of *M. tuberculosis*) can be used to screen for evidence of *M. ulcerans* infection. Dual skin testing requires the intradermal injection of burulin and *M. tuberculosis* PPD into the flexor surface of the forearm and measurement of induration at 48 or 72 h (each test is considered positive at ≥10-mm induration). While inexpensive and relatively sensitive, burulin testing is generally positive in BUD patients with active or healed disease (62). But burulin response in patients with early disease is rarely positive, suggesting that onset of cellular immunity develops late in the infectious process (17). Unfortunately, when both burulin and tuberculin tests are positive, differentiating *M. ulcerans* infection from *M. tuberculosis* infection (or identifying concomitant infections) is not possible through skin testing, as there is substantial cross-reactivity between the two skin tests (24, 61, 62).

PCR

Recent developments in PCR for the detection of *M. ulcerans* show promise as sensitive rapid assays (25, 56). PCR for the detection of *M. ulcerans* was first described for the gene coding for 16S rRNA (48) and the gene coding for the 65-kDa heat shock protein (54). Recent analyses of the *M. ulcerans* genome led to the discovery of a unique 1,109-bp insertion sequence, IS*2404* (56). A comparison of these three PCR assays demonstrated the greatest sensitivity and specificity when the IS*2404* sequence was amplified, especially using nested PCR (25). While PCR diagnosis is specific and sensitive, the cost and strict requirements for control of potential DNA contamination prohibit its utility as a routine clinical diagnostic tool. However, potential advantages of PCR in the detection of environmental reservoirs of *M. ulcerans* and in molecular epidemiological studies have been reported (63–65).

New Diagnostic Methods

The humoral immune response to *M. ulcerans* may ultimately prove to be a rapid and reliable diagnostic test for *M. ulcerans* infection and BUD, as a strong humoral immune response is observed with all other known mycobacterial infections (14, 32). To assess the potential for development of a serodiagnostic assay for BUD, the humoral immune response to *M. ulcerans* antigens among BUD patients was evaluated in a study in the Daloa region of Ivory Coast (36). The humoral response was measured and compared to the delayed-type-hypersensitivity responses of these same patients (using burulin and tuberculin) (17). The sensitivity

of the burulin skin test was found to be dependent on the stage of the disease, and sensitivity for confirmation of early disease was low (34%). However, there was no correlation between disease stage and the serum antibody response to MUCF, with a sensitivity of 71%, suggesting that the MUCF serologic response may be useful in the early diagnosis of BUD (17). Among these seroreactive patients, significant antibody reactivity was observed against two specific *M. ulcerans* proteins of 70 and 38 kDa (Fig. 3) (17). Similar studies conducted by Gooding et al. corroborate the potential for a serologic assay for the diagnosis of BUD (24). In this study, the antibody response to sodium dodecyl sulfate-soluble *M. ulcerans* antigens was evaluated by Western blot analysis in BUD patients. Antibody reactivity was found in 9 of 11 (82%) BUD patients but in none of the controls. Overall, these findings suggest that the serologic response of BUD patients to specific proteins present in MUCF can be utilized for the diagnosis of BUD.

PROTEOMICS IN THE DEVELOPMENT OF DIAGNOSTIC ASSAYS

Persons with BUD produce a specific antibody response to the MUCF proteins (17, 24; S. L. Kihlstrom, K. M. Dobos, M. R. Evans, H. S. Thangaraj, R. Phillips, D. Ohene-Adjei, M. H. Wansbrough-Jones, and C. H. King, submitted for publication). Because these proteins appear to be important as diagnostic tools as well as potential vaccine candidates, a complete analysis of the protein composition of

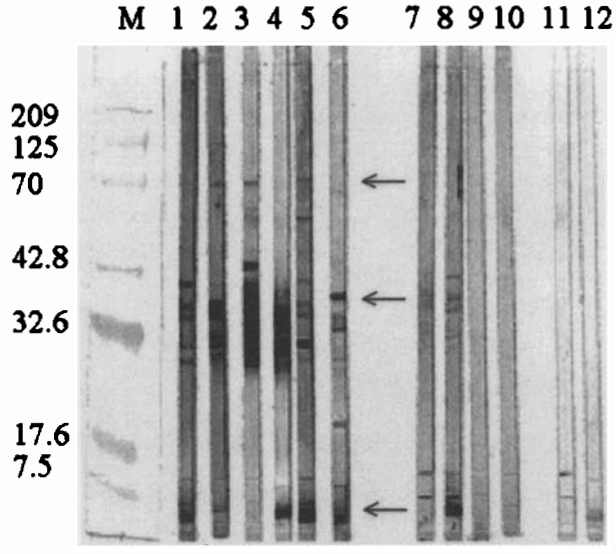

Figure 3. Western blot reactivity to MUCF. M, molecular size markers; lanes 1 to 6, representative BUD patient sera with reactivity to the MUCF; lanes 7 to 10, representative antibody reactivity in healthy persons from the area where the disease is endemic; lanes 11 and 12, serologic reactivities to the MUCF of two representative tuberculosis patients. Arrows indicate reactivity to the 70-, 38-, and 5-kDa antigens.

the MUCF is currently underway. Using two-dimensional polyacrylamide gel electrophoresis, a map of the CF proteins of *M. ulcerans* has been generated (Fig. 4A). The coupling of this electrophoretic technique with Western blot analysis allowed the identification of proteins from the MUCF recognized by human BUD patient sera (Fig. 4B). Further molecular characterization of abundant proteins within the MUCF was achieved by N-terminal amino acid sequencing. One of these proteins, with a molecular mass of 38 kDa, corresponded to a putative diagnostic antigen for BUD and contained at its N terminus a proline-rich sequence that frequently precedes mycobacterial glycosylation sites (Table 1) (17). In addition, a 28-kDa protein with a sequence identical to that of MPT 51 and a 31-kDa protein with an amino acid sequence identical to that of antigens 85A and 85B of *M. tuberculosis* were identified (Table 1). Further detailed proteomic maps of *M. ulcerans* will be

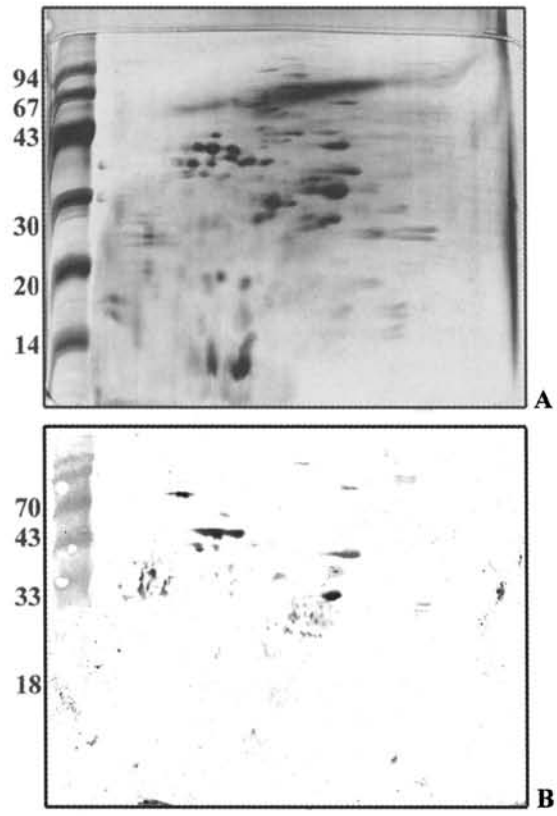

Figure 4. Two-dimensional gel electrophoresis (2D GE) mapping of the MUCF. (A) Silver-stain analysis. Fifty micrograms of MUCF protein was resolved by 2D GE using a pH gradient of 4 to 6.5 and discontinuous sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis. (B) Western blot analysis. Fifty micrograms of MUCF was resolved by 2D GE as in panel A, transferred to nitrocellulose, and probed with BUD patient sera.

Table 1. Candidate serodiagnostic proteins from *M. ulcerans* CF

Protein (kDa)	Antiserum reactivity	MTB monoclonal antibody reactivity[a]	N-terminal amino acid sequence[b]	Identity
38	Yes	No (polyclonal 45)	APAPAPAPAT	Characterization in progress
31	Yes	Yes (MT)	FSRPGLPVEY	MT
30	Yes	No (polyclonal MT)	ND	MT
28	Yes	No	APYETLMVPS	MPT 51 homolog

[a]Abbreviations: MTB, *M. tuberculosis*; MT, mycolyl transferase.
[b]ND, not determined.

useful in the identification and analysis of serodiagnostic and vaccine candidates for this emerging mycobacterial disease.

TREATMENT

Surgery is the current recommended treatment for all stages of BUD (4, 67). Ulcerative lesions require wide debridement followed by skin grafting, techniques requiring referral to specialized health care facilities. Antimicrobial therapy has not been systematically evaluated among patients presenting with early BUD, because patients only rarely present to primary health care facilities for painless nodular lesions and limited laboratory confirmatory techniques (4, 67). Combination antimicrobial therapy with rifampin and dapsone for ulcerative lesions has demonstrated modest benefit (18). However, combination antimicrobial therapy with two or more bactericidal agents has not been systematically evaluated in early or advanced disease.

PREVENTION

Due to a fundamental lack of understanding of the mechanism of disease transmission, prevention strategies have not been developed. BCG vaccination has been suggested to have a moderate, short-lived protective effect against Buruli ulcer (2, 60). Currently, control methods in countries with endemic disease are limited to early diagnosis through improved active surveillance and surgical treatment. Surgical intervention at the preulcerative stage of disease is believed to prevent the development of the ulcerative stage of disease. However, well-controlled clinical trials testing this theory have not been published. Education of the population at risk may be an important strategy to reduce the marginal increased morbidity and mortality that result from delayed treatment. Further investigation of inexpensive prevention strategies, such as wearing protective clothing when farming and the immediate cleansing of traumatic skin injuries, are warranted. Since no diagnostic test is currently available that rapidly confirms *M. ulcerans* among suspected cases, antimycobacterial chemoprophylaxis for the prevention of BUD has not been evaluated.

CONCLUSIONS

BUD, caused by *M. ulcerans*, has been identified in many parts of the world, predominantly in tropical regions (6). Over the past decade, there has been a purported dramatic increase in BUD in several West African countries (6, 67); this increase has not been associated with HIV infection (36). Reasons for the emergence remain elusive.

Although *M. ulcerans* DNA has on occasion been recovered from the environment, the reservoir for *M. ulcerans* remains undefined; observational studies suggest that *M. ulcerans* infection is transmitted through skin after contact with water, vegetation, or possibly an insect vector (63; Portaels et al., letter). Further environmental investigations should be pursued using optimized molecular and microbiological methods.

Current treatment strategies for BUD are surgical. While early lesions can be excised, estimates of recurrence range from 15 to 20% (6). Larger ulcerative lesions are typically managed by wide debridement followed by skin grafting. These techniques are costly and require referral to specialized health care facilities (5). Anecdotal information suggests that antimycobacterial therapy for preulcerative BUD may be beneficial, and combination therapy is associated with modest improvement in ulcerative disease. However, well-controlled clinical trials are needed for describing the benefit of early medical intervention. Because patients rarely present to primary health care facilities with early lesions, improved surveillance and community education must be linked with these efforts.

Currently, international efforts directed by the WHO Buruli Ulcer Initiative are targeted towards defining the worldwide burden of this disease, the source(s) and route(s) of transmission, improved diagnostic assays, the mechanisms of pathogenesis, less invasive treatment options, and improved public-health control strategies. Over the past two years there has been steady progress in establishing surveillance systems utilizing standardized WHO reporting tools and conducting epidemiologic field work in West Africa. Improved diagnostic assays that are rapid, simple, and cheap are essential to progress in understanding the epidemiology, treatment, and control of this mysterious disease.

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Chapter 10

Borrelia: a Diverse and Ubiquitous Genus of Tick-Borne Pathogens

Alan G. Barbour

The spiral bacteria that Leeuwenhoek observed in mouth scrapings with his microscope were undoubtedly spirochetes, and for a time oral spirochetes were included in the genus *Borrelia* on the basis of similar appearances. Subsequent taxonomies took into account biological and physiological characteristics, as well as morphologic features, and restricted membership in the genus *Borrelia* to certain aerobic spirochetes with dependence on arthropods, usually ticks, for transmission between vertebrate reservoirs (12). This was a coherent group of vector-borne pathogens that could be readily distinguished in their epidemiology and pathogenesis from other spirochetal disease agents such as *Treponema pallidum*, the cause of syphilis, and leptospires. There were also a variety of free-living spirochetes and commensal spirochetes of invertebrates and vertebrates to consider, but the lifestyles of these bacteria could not be confused with those of *Borrelia* species.

In the century following Obermeier's first description in 1881 of *Borrelia recurrentis* in the blood of a patient with epidemic relapsing fever, several other *Borrelia* species that cause relapsing fever were discovered. The relapsing fever species are divided into Old World and New World groups. Examples of Old World species are *B. crocidurae* of West Africa, *B. duttonii* of East and southern Africa, and *B. persica* of the Middle East. New World relapsing fever species include *B. hermsii* of the mountainous and Pacific coastal regions of the United States and Canada and *B. turicatae* of the southwestern United States, Florida, and northern Mexico. Up to 1981 the only nonrelapsing fever species of *Borrelia* were *B. anserina*, which causes avian spirochetosis, and *B. theileri*, which causes bovine borreliosis in Africa and other regions.

B. recurrentis remains the only recognized *Borrelia* species that is transmitted by lice (or any other insect). All subsequently discovered species are transmitted by ticks. The relapsing fever and avian spirochetosis agents are transmitted by argasid ticks, which have comparatively soft exoskeletons. In comparison to their

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harder-shelled counterparts, the ixodid ticks, the argasid ticks' blood feedings last only minutes rather than the hours to days of ixodid ticks. Until 20 years ago, *B. theileri* was the only species known to be transmitted by hard ticks. The principal vector of *B. theileri* in Africa and elsewhere is *Boophilus microplus*, the tropical cattle tick. Members of the genus *Boophilus*, as well as the genera *Amblyomma*, *Dermacentor*, and *Rhipicephalus*, are examples of metastriate ticks. These genera include among their species the vectors of *Rickettsia*, *Ehrlichia*, *Cowdria*, and *Anaplasma* sp. pathogens.

In 1981 spirochetes were discovered in the prostriate ticks, which is the second major group of hard ticks and is primarily made up of species of the genus *Ixodes*. Two species, *Ixodes ricinus* in Europe and *Ixodes scapularis* in the United States, had previously been associated epidemiologically with what was called Lyme disease in the United States and erythema migrans in Europe. The cause of Lyme disease and erythema migrans was unknown until the isolation of a new species, *B. burgdorferi*, first from *I. scapularis* and then from *I. ricinus* (9, 15, 19). This new species was subsequently isolated from patients with Lyme disease and related disorders in the United States and Europe, as well as from the mammalian hosts of the ticks.

All isolates from humans with Lyme disease acquired in North America have been identified as *B. burgdorferi* (reviewed in reference 5). In Europe there were other isolates of *B. burgdorferi*, but there were also strains that differed from *B. burgdorferi* in several characteristics, most notably in the molecular weights of major outer membrane proteins OspA and OspB (13). These atypical strains were first categorized as *B. burgdorferi* in the literature. But as the number of isolates from patients, ticks, and rodents grew, it became clear that there were two other species in Europe; these were named *B. afzelii* and *B. garinii* (5). The geographic range of *B. afzelii* and *B. garinii* extends into Russia, Siberia, China, Korea, and Japan. East of the Ural Mountains in Russia and in north and east Asia, the *Ixodes persulcatus* tick is the usual vector of Lyme disease. *B. garinii* has also been found among seabirds in both northern and southern arctic and subarctic regions; the vector is the tick *Ixodes uriae* (74). In the far-western United States, *Ixodes pacificus* is the vector of *B. burgdorferi* (20). *B. burgdorferi* or a related species has not been documented in sub-Saharan Africa, Australia, Central Asia, South Asia, Southeast Asia, the Middle East, or Latin America.

The recovery of *B. burgdorferi*, *B. afzelii*, and *B. garinii* from the blood, skin, and cerebrospinal fluid of human patients demonstrated the infectiousness and virulence of these species. *B. burgdorferi* has also been shown to cause arthritis, carditis, and/or neurologic involvement in experimental infections of mice, hamsters, rabbits, dogs, and nonhuman primates. As few as 10 organisms of *B. burgdorferi* injected intradermally are sufficient to cause an infection. Experimental infections have also been established using laboratory-reared ticks bearing *B. burgdorferi*. Much less work has been done on laboratory infections with *B. afzelii* or *B. garinii*.

The laboratory studies of *B. burgdorferi* and related species also revealed biological differences between this group and the relapsing fever *Borrelia* spp. (reviewed in reference 6). In contrast to a relapsing fever agent such as *B. hermsii*

that achieves as many as 10^7 spirochetes per ml of blood in a mouse, the density of *B. burgdorferi* organisms in the blood is usually not more than 10^3 per ml. Patients with relapsing fever acutely become moderately to severely ill; they suffer recurrences of the symptoms, but the usual duration of the untreated infection is a few weeks. In contrast, *B. burgdorferi* infection is less disabling during its early stage but can be persistent and lead to chronic arthritis, carditis, and meningitis. These biological differences between the Lyme disease group of *Borrelia* spp. and the relapsing fever group of species were reflected by divergence in DNA sequences between the two groups of organisms. Although the new species associated with Lyme disease were more closely related to previously known *Borrelia* species than they were to treponemes, leptospires, or other spirochetes, the new species were clearly distinct from the relapsing fever species on the basis of DNA relatedness (12).

Table 1 lists new *Borrelia* species, that is, those that have been discovered since the mid-1980s. The new species are described and discussed below; they are the focus of this chapter. In Table 1 the suspected vector and reservoir host are listed, but this information may change as further information about the epizootiology and ecology of these organisms is gained. The example of *B. garinii* given below shows that the designation of what is the critical reservoir can change. Figure 1 shows one estimate of the phylogenetic relationships of new species and selected old species, their major groupings, and their associations with different types of ticks.

NEW SPECIES IN THE LYME DISEASE GROUP OF SPIROCHETES

As investigators in North America, Europe, and Asia gathered more samples from ticks and animals in the field, isolates that could not be classified as *B. burgdorferi*, *B. afzelii*, or *B. garinii* were noted. Bissett and Hill noted that some isolates from *I. pacificus* ticks in California differed from *B. burgdorferi* in their OspA and OspB proteins (16). Anderson and coworkers identified an equally dis-

Table 1. New *Borrelia* species associated with hard ticks

Species	Common vector species	Common reservoir(s)	Geographic range
B. bissettii	*I. scapularis*	Rodents	Northeastern, north-central United States
	I. spinipalpis	Rodents	Western United States
B. andersonii	*I. dentatus*	Rabbits	Northeastern United States
B. lusitaniae	*I. ricinus*	—[a]	Mediterranean basin
B. valaisiana	*I. ricinus*	Birds	Europe, Russia
	I. nipponensis		Asia
B. japonica	*I. ovatus*	Rodents, shrews	Asia
B. tanukii	*I. tanuki*	Rodents	Asia
B. turdi	*I. turdus*	—	Asia
B. miyamotoi	*I. persulcatus*	Rodents	Asia
B. miyamotoi-like sp.	*I. scapularis*	—	Northeastern United States
B. lonestari	*A. americanum*	—	Southeastern United States

[a]—, unknown.

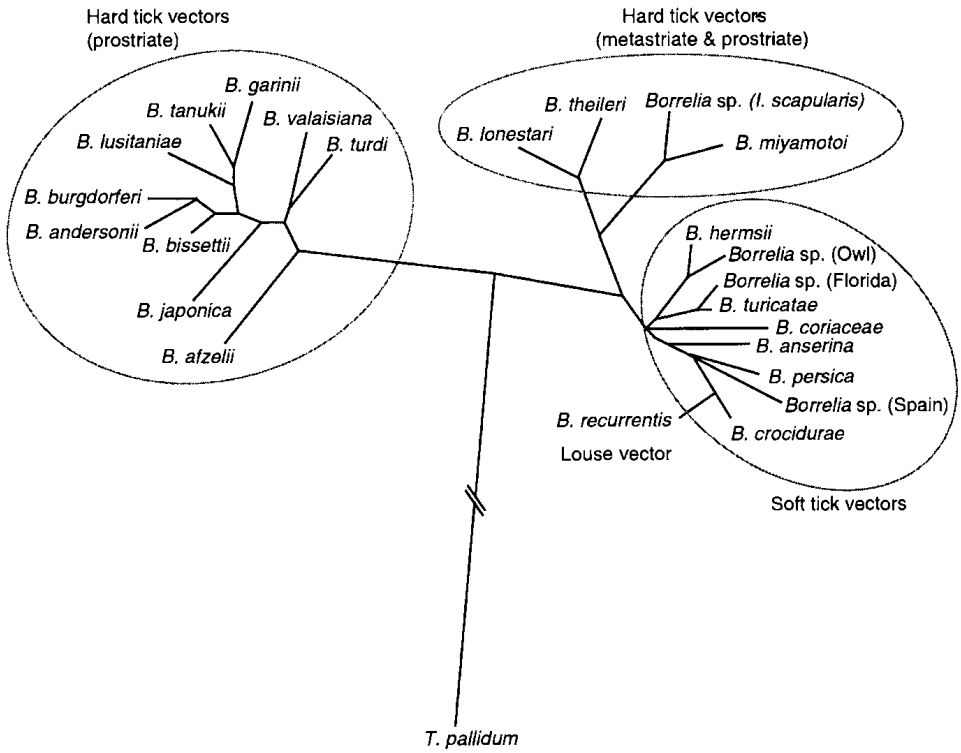

Figure 1. Unrooted neighbor-joining phylogenetic tree of selected *Borrelia* species. 16S rRNA sequences corresponding to base positions 56 to 1345 of the *B. burgdorferi* 16S rRNA gene (GenBank accession number M88329) were aligned and analyzed by using the ClustalX program package. Selected other *Borrelia* species included in the analysis (with accession numbers given in parentheses) are *B. bissettii* (AJ224141), *B. andersonii* (L46701), *B. afzelii* (X85189), *B. garinii* (AJ009749), *B. turdi* (D67024), *B. tanukii* (D67023), *B. japonica* (U42299), *B. valaisiana* (X98232), *B. lusitaniae* (X98229), *B. miyamotoi* (D45192), *B. lonestari* (U23211), *B. theileri* (U38375), *B. anserina* (U42284), *B. coriaceae* (U42286), *B. persica* (U42297), *B. crocidurae* (U42302), *B. recurrentis* (U42300), *B. turicatae* (U42299), *B. hermsii* (U42292), and *Borrelia* spp. from a patient in Spain (U28502), a dog from Florida (L37837), and an owl from Washington State (AF116903). The outgroup is the spirochete *Treponema pallidum* (M88726). Three major groupings are demarcated, and the types of ticks that are the usual vectors of members of each are indicated. *B. recurrentis* is the only known *Borrelia* species transmitted by an insect instead of a tick.

tinctive spirochete in rabbits and the rabbit tick, *I. dentatus* (3). These organisms have now been named *B. bissettii* and *B. andersonii*, respectively, for their discoverers.

In Europe Péter and colleagues (79) and Postic et al. (85) identified in *I. ricinus* two new genomic groups, which were subsequently named *B. valaisiana* (107) and *B. lusitaniae* (49), respectively. *B. valaisiana* is associated with birds in Europe (33). *B. valaisiana* was also found in east Asia in *Ixodes nipponensis* ticks (57)

and in *Ixodes columnae* ticks (28). There has been one report of the detection by PCR assay of *B. valaisiana* in skin biopsy specimens of patients with erythema migrans (91), but *B. valaisiana* has not been isolated to date from humans. *B. lusitaniae* appears to be largely restricted to the Mediterranean basin. Isolates of *B. lusitaniae* from Tunisia were the first species of the Lyme disease group to be identified in Africa (25, 49).

In Japan, *B. afzelii* and *B. garinii* were known to occur, but further studies revealed three new species that were related to *B. burgdorferi* and other Lyme disease agents: *B. japonica* (37, 86), *B. tanukii* (29, 59), and *B. turdi* (29). Two other probable species have yet to be named or further classified. One of these is represented so far by a single isolate, but this was recovered from a patient with erythema migrans in The Netherlands (106). The second newly recognized *Borrelia* sp. group comprises several diverse isolates from ticks and mammals in both California and Europe (87, 88, 109). Their pathogenic potential for humans and other animals is unknown. Of the new species discovered since 1987, *B. bissettii* and *B. japonica* are the best studied.

B. bissettii

The type strain of *B. bissettii* is DN127, which was recovered from an adult *I. pacificus* in California (16). This strain and other isolates differed from *B. burgdorferi* in the apparent sizes of their OspA and OspB proteins in polyacrylamide gels. Shortly after this report of what was at first thought to be an atypical isolate of *B. burgdorferi* came another report of an unusual isolate, this time from a larval *I. scapularis* tick collected in upstate New York (2). This was strain 25015, which was later shown to be very similar but not identical to strain DN127 of California in its *ospA* gene sequence and by other genetic criteria (40).

While the prevalence of *B. bissettii* in ticks and reservoir hosts in the northeastern United States is not known, further studies in California have yielded several more examples of *B. bissettii* (77, 94). Isolates from ticks have been taken from *I. pacificus* but also from *Ixodes spinipalpis*, a species that uncommonly bites humans because it is mainly restricted to the nests of its host rodents. The usual vertebrate host for *I. spinipalpis* is the dusky-footed wood rat (*Neotoma fuscipes*) in the area of enzooticity of California. *B. bissettii* has also been documented in Colorado in *I. spinipalpis* and in the Mexican wood rat (*Neotoma mexicana*) and the prairie vole (*Microtus ochrogaster*) (21, 63, 66). Unlike California and the northeastern United States, Colorado is not a known region of endemicity for *B. burgdorferi*. Thus, the absence of definite cases of locally acquired Lyme disease in Colorado suggests that *B. bissettii* either is incapable of causing a Lyme disease-like disorder or is only transmitted by ticks that humans rarely encounter (63).

Another area with little or no risk of Lyme disease for humans is the region of Illinois around Chicago. While northwestern Illinois and the adjoining region of Wisconsin have yielded isolates of *B. burgdorferi*, Picken and Picken identified only *B. bissettii* among 19 isolates from rodents, including the vole *Microtus pennsylvanicus*, from greater metropolitan Chicago (81). Molecular typing using pulsed-field gel electrophoresis and large restriction fragments showed that the Illinois

isolates of *B. bissettii* included some that were like the California isolate DN127 or others that were more like the New York isolate 25015. There is evidence that *B. bissettii* also occurs in the southeastern United States (62).

While *B. bissettii* can be infectious for mice, rats, hamsters, and voles in the laboratory (2, 21, 63, 77), the more susceptible animal seems to be its natural host, the wood rat. However, in comparison to *B. burgdorferi* the New York isolate (strain 25015) of *B. bissettii* did not cause detectable arthritis of joints, even though the organism could be recovered from the joints of rats (2). There have not as yet been comparable studies of the virulence of California, Colorado, or Illinois isolates of *B. bissettii*.

One possible indication of the pathogenicity of *B. bissettii* was the report of nine isolates of a *Borrelia* sp. from patients with Lyme disease or a Lyme disease-like disorder in Slovenia (80, 100). These isolates reportedly clustered more closely with *B. bissettii* than with *B. burgdorferi*, *B. garinii*, or *B. afzelii*. However, the sequences of these Slovenian strains have not been deposited for examination, and the findings have not been independently confirmed. There have been no reports of isolation of *B. bissettii* from humans in the United States.

B. japonica

B. japonica is transmitted by *Ixodes ovatus* ticks and was isolated from three species of shrews (*Sorex unguiculatus*, *Sorex caecutiens*, and *Crocidura dsinezumi*), a species of vole (*Clethrionomys rufocanus*), and two species of mice (*Apodemus argenteus* and *Apodemus speciosus*) captured in various locations in Japan (65). Comparing *B. japonica* to *B. afzelii* and *B. garinii*, the Lyme disease species that occur in Japan, Isogai and colleagues found that *B. japonica* was less pathogenic for mice by the criterion of cytokine levels in various tissues of infected animals (34). Kaneda et al. reported that while *B. japonica* infection of both immunocompetent and immunodeficient mice produced arthritis, the severity of joint inflammation was lower than what was observed with a strain of *B. burgdorferi* (36). Using a mouse model in which spirochetes were injected directly into the footpad, Masuzawa and colleagues found that *B. japonica* produced less footpad swelling than did *B. burgdorferi*, *B. afzelii*, or *B. garinii* (58).

The sole evidence of the pathogenic potential of *B. japonica* for humans is a single case report from Japan of a young girl who might have had an infection with *B. japonica* (60). The patient was bitten by a tick that was suspected on epidemiologic grounds to be *I. ovatus* and developed adenopathy near the tick bite. Using different antigen preparations, including those from *B. japonica* and from Lyme disease species, in enzyme-linked immunosorbent assays (ELISAs) and Western blot assays, the investigators concluded that the antibody response was stronger to *B. japonica* antigens than to the antigens of other species.

ERYTHEMA MIGRANS IN THE SOUTHERN UNITED STATES

For several years there have been reports of a Lyme disease-like illness from the southeastern and south-central United States. Most of these early reports were

attributable to misdiagnosis (23), but some reports of Lyme disease in the area continued after diagnostic criteria were standardized. In the reported cases the patients often had an expanding skin rash that was consistent in appearance with erythema migrans, the best single clinical indicator of Lyme disease. The problem for public health officials was that the serologic assay for antibodies to *B. burgdorferi* was usually negative in cases in which the infection supposedly was acquired in a southeastern state. Attempts to isolate *B. burgdorferi* from skin biopsy specimens of the rashes were unsuccessful.

I. scapularis occurs in the southeastern United States, but these ticks in the South are generally distinguishable from the northern variety of the species in their morphology and behavior (38). The southern variety is less likely to feed on humans. (Spielman and colleagues assert that the northern form of this tick is distinctive enough in its behavior and other characteristics to retain its own species designation, *Ixodes dammini*, the name used in the earliest studies of Lyme disease vectors in the Northeast [98].) *B. burgdorferi* occurs in the southern variety of *I. scapularis*, but these isolates have almost exclusively been from ticks collected from coastal region, including the barrier islands off North Carolina, South Carolina, Georgia, and Florida (69–71, 73, 75).

Reports of *B. burgdorferi* further inland have been rare and controversial (14, 67). In a survey of *I. scapularis* ticks from the southeastern states of South Carolina, Georgia, Florida, and Mississippi, Piesman et al. found that none of 284 collected ticks contained spirochetes (82). In the regions of endemicity for Lyme disease in the Northeast 15 to 50% of ticks collected would be expected to contain spirochetes. Even in northern California, which has a comparatively low incidence of Lyme disease, the prevalence of spirochetes in *I. pacificus* in areas of endemicity is 2 to 5%. *Borrelia*-like spirochetes were isolated from *I. dentatus* ticks from central Georgia and Missouri (68, 72), but these isolates appear to represent a different genospecies from *B. burgdorferi* (62). *I. dentatus* rarely feeds on humans (102). Several isolates from mammals in Florida and Georgia phylogenetically cluster more closely with *B. bissettii* than with *B. burgdorferi* (62). The report of isolates of *B. burgdorferi* from *I. scapularis* in Texas is in doubt, because similar organisms were allegedly also recovered from fleas and ticks that are incompetent as vectors (103). One of the isolates of *B. burgdorferi* from this study was also highly similar to a commonly used laboratory strain of *B. burgdorferi* (10, 103), and a study of five Texas isolates of *B. burgdorferi* demonstrated that the isolates were noninfectious for mice, a characteristic of serially cultivated isolates (83). These findings suggest that these Texas isolates of *B. burgdorferi* were the consequence of laboratory contamination. Furthermore, a subsequent study of *I. scapularis* ticks collected in Texas did not confirm the presence of *B. burgdorferi* in that tick (89).

The most provocative and numerous reports of a Lyme disease-like illness were from Edwin Masters, a primary care practitioner in southeastern Missouri, who noted an illness with similarities to early Lyme disease among patients in his and colleagues' practices (54, 55). The hallmark of the illnesses was a single expanding annular erythematous rash, frequently at the site of a tick bite, and often accompanied by constitutional symptoms. Color Plate 8 (see color insert) shows an ex-

ample of a rash observed by Masters and colleagues. These illnesses were treated empirically with antibiotics with known activity against *B. burgdorferi* with apparent success. Cases consistent with early Lyme disease were also reported from areas of North Carolina that do not have documented transmission or occurrence of *B. burgdorferi* in the environment (51).

These reports prompted other studies of the phenomenon. Campbell et al. carried out a retrospective case-control study of 45 Missouri outpatients with physician-observed annular rashes of ≥5 cm in diameter (22). The typical rash expanded over time and reached a mean diameter of 8 cm about 4 days after onset. More than 90% of the lesions were located on the legs or trunk, and the majority of the rashes had central clearing like erythema migrans. They were more commonly pruritic than painful or burning in sensation. Mild constitutional symptoms such as fatigue, headache, stiff neck, myalgias, and/or arthralgias were common, and fever occurred in 30% of patients. The patients ranged in age from 3 to 84 years and tended to be male, to live near the water, and to hunt. The peak in incidence of the rashes was during the late spring and early summer; no patient had onset in November through February. Twenty patients (44%) associated their rash with the bite of a tick, and five of these patients described an adult *Amblyomma americanum*, the lone star tick.

In the study by Campbell et al. (22), serologic tests did not indicate infection with the known tick-borne agents *Francisella tularensis*, *Ehrlichia chaffeensis*, *Rickettsia rickettsii*, *Rickettsia typhi*, *Coxiella burnetii*, or *B. burgdorferi*. However, more patients than expected for an area not supposed to be an area of endemicity had positive reactions against *B. burgdorferi* in the whole-cell ELISA. These sera were negative by Western blot assay by the criteria for Lyme disease diagnosis. Skin biopsy specimens from 23 patients were obtained prospectively in the years 1991 to 1993. Using procedures that would be expected to yield ≥50% positive cultures from biopsy specimens of erythema migrans during *B. burgdorferi* infection, the investigators did not recover *B. burgdorferi* or any other *Borrelia* sp. by cultivation.

Masters and his colleagues in southeastern Missouri reported on a retrospective study of a group of 17 patients, who had a physician-diagnosed erythema migrans rash, associated the rash with a bite by *A. americanum*, and who had a skin biopsy performed on the rash site (56). Nine (53%) of the patients were male, and the ages ranged from 12 to 69 years. The time of onset occurred during April to September. Nymphal, adult male, and adult female ticks of *A. americanum* were associated with the rashes. Most of the rashes were on the trunk, and the rest were on the lower extremities. The median rash diameter was 7.5 cm, and the largest was 15 cm. Fourteen of the rashes had central clearing. Only one patient had multiple lesions, and five (30%) had constitutional symptoms as well as the rash. Although spirochetes were identified by an experienced pathologist using a silver stain in 6 of 11 biopsy specimens examined histologically, cultures of the skin biopsy sampler for *B. burgdorferi* and other *Borrelia* spp. were negative. These investigators reported, however, that the patients were more likely than expected to have positive serology results in an ELISA based on *B. burgdorferi* flagella, a

subcellular structure that is cross-reactive in antigenicity between different *Borrelia* spp. (12).

A subsequent study by Masters and colleagues compared samples from 15 skin biopsies of erythema migrans in Missouri with samples from 15 biopsies of erythema migrans in the northeastern United States (48). The investigators used the Steiner silver stain to identify spirochetes and the PCR with primers for the *ospA* gene to specifically identify *B. burgdorferi* in the biopsy specimens. Eight of 15 of the northeastern biopsy specimens but none of the 14 from Missouri were positive by PCR. In six of the Missouri biopsy specimens, spirochetes were observed by silver staining. Histologic examination of biopsy specimens of erythema migrans from the northeastern United States revealed perivascular lymphocytic infiltrate in the papillary and upper reticular dermis; the perivascular infiltrate contained plasma cells. In contrast, plasma cells were not observed in the biopsy specimens from Missouri.

Felz et al. prospectively studied patients presenting with erythema migrans rashes at a family medicine practice clinic serving Georgia and South Carolina (27). Twenty-three patients presenting to the clinic had a solitary rash that met the Centers for Disease Control and Prevention's case definition criterion for erythema migrans. Fourteen (61%) of the patients were men, and their ages ranged from 25 to 69. May through August were the the most common months of onset of the rash illness; no case had an onset during November through March. A tick bite was reported by 91% of the patients. In contrast, fewer than half of patients with Lyme disease in the northeastern United States had noted an *I. scapularis* bite prior to disease onset (reviewed in reference 11).

The diameters of the annular and oval rashes ranged from 5 to 20 cm, with a mean of 9.6 cm (27). No patient had secondary rashes, and no patient had other evidence of late disseminated Lyme disease, such as Bell's palsy, carditis, meningitis, or monoarticular arthritis. All lesions were photographed and biopsied, the biopsy samples were cultured, and histopathologic and PCR analyses for *B. burgdorferi* infection were carried out. Patients were treated with doxycycline and monitored for clinical resolution of the illness and with repeat serologic assays. Seven patients (30%) had some evidence of *B. burgdorferi* or other *Borrelia* sp. infection by one or more laboratory assays. A single culture (4%) from the biopsy specimens yielded a spirochete, but this was *B. garinii* and likely attributable to acquisition of the infection during travel to Europe, not in the United States. The biopsies of three patients (13%) contained spirochete-like forms by histologic examination with silver stain. Five patients (22%) had positive PCR findings with primers for flagellin DNA sequences, but these products were not sequenced to confirm the identity of the sequence as that of *B. burgdorferi*. PCR using primers for the *ospA* gene was negative for all biopsy specimens. There was not a statistically significant difference between the patients and control subjects in the results of serologic assays for antibodies to *B. burgdorferi*. Overall, laboratory evidence of *B. burgdorferi* infection was completely lacking in 16 patients (70%). In this uncontrolled study, the investigators noted that almost all the patients had a resolution of the lesions and associated constitutional symptoms within a week of starting antibiotic therapy. Sequelae were not noted during follow-up.

In their report of an outbreak of erythema migrans in central North Carolina, Kirkland et al. describe fourteen cases that occurred during two summers at an outdoor camp (39). Of the 14 individuals with solitary rashes of ≥5 cm in diameter, 10 were residents and 4 were staff. The rashes were erythematous with central clearing and irregular borders. Several of the cases reported that the rash began from a small papule at the site of a tick bite. Most of the rashes were on the legs or trunk, and several cases noted that the rashes were tender or pruritic. One person had a second lesion near the first within several days of the onset of the first lesion. Only one patient had a fever, but the majority had one or more constitutional symptoms, including headache, musculoskeletal pain, or fatigue. Ten (71%) patients recalled a prior tick bite at the site of the skin lesion; the median interval between tick removal and observation of the rash was 12 days. In the instance when the tick was available for examination, it was identified as *A. americanum*. The results of assays of paired sera from eight of the patients were not consistent with infection by *B. burgdorferi*, *R. rickettsii*, or *E. chaffeensis*. Cultures for *Borrelia* spp. from biopsy specimens of skin lesions of five patients were negative.

In retrospect, the first published report of a case of this new erythema migrans disease in the southern United States may have been in 1986 (76). For that case, I collaborated in immunohistologic identification of a spirochete in the skin with a *Borrelia* genus-specific monoclonal antibody. The patient presented in 1984 with a "sharply defined, slightly pruritic patch of mildly papular erythema, 15 cm in diameter" on the upper back (76). As we wrote, "The lesion had started approximately 2 weeks previously at the completion of a surveying and camping trip in a wooded area of western North Carolina, an area in which transmission of *B. burgdorferi* has not been documented. Initially the patient had noted a small, erythematous, slightly pruritic papule that then expanded rapidly over the next 2 weeks" (76). The patient was otherwise asymptomatic. Examination of the rash revealed only mild tenderness; the periphery was more brightly erythematous and raised than the central area. The patient was treated with tetracycline and within the 3 days the patient noted that the lesion had resolved. The clinical history and course in this case are consistent with the reports of other cases of erythema migrans in this region of the country.

A skin biopsy from the periphery of the lesion in this case revealed a moderately dense superficial and inflammatory cell infiltrate, which was predominantly lymphocytes. By Warthin-Starry silver staining, several spirochetes in the epidermis were seen. Immunofluorescent staining with the monoclonal antibody revealed a few spirochetes about 10 μm long in the papillary dermis near the dermoepidermal junction (Color Plate 9 [see color insert]). Staining with a control monoclonal antibody was negative. Sera obtained at the first visit and 6 weeks later were negative by indirect immunofluorescence assay for antibodies to *B. burgdorferi*.

B. lonestari and Related Spirochetes

The increasing evidence of the existence of a non-Lyme disease, erythema migrans illness in the southern United States led to further studies of *A. americanum*, the exposure to which was a prominent risk factor for the disease. This tick's

geographic range coincided with the area from which the reports of the illness were coming, and this tick was known as a frequent biter of humans throughout much of the southern United States (96). The study of the outbreak at the summer camp in North Carolina found that 97% of 588 ticks collected from vegetation were *A. americanum*, 2% were *Dermacentor variabilis* (the American dog tick), and only 2 (0.3%) were *I. scapularis*. Of 540 ticks removed from people at the camp, 95% were *A. americanum*, 5% were *D. variabilis*, and none were *I. scapularis* (39). The diagnostic investigation of the North Carolina study and the other studies did not reveal *B. burgdorferi* in the skin lesions, and the serologic support for the diagnosis of *B. burgdorferi* was equivocal at best.

Although *A. americanum* was the tick most closely associated with the illness in this region, this tick is not competent to be a vector of *B. burgdorferi* (61, 70, 84, 92). More recently, Piesman et al. demonstrated that *A. americanum* was refractory to *B. burgdorferi* strains isolated in the southern United States (83). Yet from various locations in the geographic distribution of *A. americanum*, approximately 2% of collected *A. americanum* ticks had spirochetes in their midguts (26, 50, 52, 53, 89, 93). Most of the investigators in these reports identified the organisms using polyclonal antiserum from rabbits immunized with whole *B. burgdorferi* cells (7). However, such reagents would not have been specific for *B. burgdorferi*; antibodies in such antisera would bind to other species of *Borrelia*, including relapsing fever species (78).

To identify possible etiologic agents for this disorder, my colleagues and I examined *A. americanum* ticks collected in Missouri, Texas, New Jersey, and New York (13). Using a genus-specific antiserum, which was similar to what was described above, to identify infected ticks, we confirmed that approximately 2% of the ticks had spirochetes. The organism could not be cultivated in medium that supports the growth of most *Borrelia* spp. (9, 24). *Borrelia* genus-specific oligonucleotides for the flagellin and 16S rRNA genes were used for amplification of DNA from the infected ticks and control samples. Products were obtained from ticks containing spirochetes by microscopy but not from spirochete-negative ticks. Sequences of partial genes from spirochetes in Texas and New Jersey ticks were nearly identical for flagellin and 16S rRNA genes. Phylogenetic analysis showed that the spirochete was a *Borrelia* species distinct from previously characterized members of this genus, including *Borrelia burgdorferi*. We designated the organism *B. lonestari* for the lone star tick (14). At the time the most closely related species was the newly discovered species *B. miyamotoi*. Armstrong et al. independently identified *B. lonestari*-like spirochetes in *A. americanum* ticks from Maryland (4).

B. miyamotoi was discovered in *I. persulcatus* ticks and rodents in Japan (30). It is cultivable, but it has not been recovered from humans or associated with a human disease. Hamase et al. showed that *B. miyamotoi* had outer membrane proteins that were very similar to the antigenically variable outer membrane proteins of the relapsing fever *Borrelia* spp. (31). Subsequently, Rich et al. showed that *B. theileri*, the agent of bovine borreliosis, is closely related to *B. lonestari* and to a lesser degree to *B. miyamotoi* (90). *B. theileri* was first observed in the blood of infected animals a century ago and has not been cultivated.

A fourth member of this cluster of species, and at this point unnamed, was recently identified in 2.2% of 712 *I. scapularis* ticks collected in Connecticut, Rhode Island, New York, and New Jersey, a region of high endemicity for *B. burgdorferi* infection (95). Of the 712 examined ticks, 112 (15.7%) were infected with *B. burgdorferi*. The organism was identified using PCR with primers for the 16S rRNA gene and for the flagellin gene. Like *B. lonestari* and *B. theileri*, the Connecticut organism has not been cultivable. Scoles et al. demonstrated the complete transstadial transmission of the organism from larvae to adult (95). The investigators also demonstrated that the organism can be horizontally transmitted among ticks through *Peromyscus leucopus* mice. The rate of horizontal transmission among ticks was lower than what was observed with *B. burgdorferi* under the same conditions.

Figure 1 shows the phylogenetic relationships based on 16S rRNA sequence between these four species, as well as the other new species and selected old species. Three major clusters are represented. One group contains the Lyme disease agents as well as closely related species of no or uncertain pathogenicity or risk to humans. These organisms are transmitted by *Ixodes* species among prostriate hard ticks. The second group comprises the relapsing fever species, *B. anserina*, and *B. coriaceae* and, with exception of *B. recurrentis*, is transmitted by argasid ticks, primarily of the genus *Ornithodoros*. The third group is newly recognized and is monophyletic. This third group is made up of *B. theileri*, *B. lonestari*, *B. miyamotoi*, and the new *Borrelia* sp. of *I. scapularis* ticks. Although it is more distant from members of the Lyme disease group than from those of the relapsing fever group, the species of the third major group are transmitted by hard ticks—prostriate ticks in the cases of *B. miyamotoi* and the new *Borrelia* sp. and metastriate ticks in the cases of *B. theileri* and *B. lonestari*. It is possible that the *I. scapularis* organism is synonymous with *B. miyamotoi*.

While the infectious and pathogenic potential for humans of *B. theileri*, *B. miyamotoi*, and the *I. scapularis Borrelia* sp. are unknown, a recent report by investigators of the Centers for Disease Control and Prevention and New York Medical College indicates that *B. lonestari* is infectious and pathogenic for humans (35). The case report describes a male patient who presented with an abdominal rash for 4 days and fatigue. He was a resident of an area in New York where Lyme disease was endemic but shortly before presentation had been traveling in Maryland and North Carolina. In both areas he had walked in grassy areas. On examination the patient was afebrile and had 19-by-11-cm and 4-by-3-cm annular erythematous lesions on the abdomen. Near the center of the larger lesion was an engorged female *A. americanum* tick. The tick and a skin biopsy specimen were cultured and subjected to PCR assay with *Borrelia*-genus specific primers as well as primers specific for *B. burgdorferi*. No microorganism was recovered from the tick or skin biopsy specimen by cultivation. From both the tick and skin biospy PCR products were obtained with the genus-specific primers but not the *B. burgdorferi*-specific primers. Whereas the sequences of the products were nearly identical to the sequence of the homologous genes of *B. lonestari*, there were several mismatches in the alignment of the PCR product sequences with the comparable sequences of *B. miyamotoi* and *B. burgdorferi*. ELISAs and Western blot assays of acute- and convalescent-

phase sera were negative by standard criteria for antibodies to *B. burgdorferi*. However, this erythema migrans patient, like some others in Missouri, had antibodies to the flagellin of *B. burgdorferi*, a possible indication of infection with another species of *Borrelia*.

NEW RELAPSING FEVER GROUP SPECIES

While most of the attention of the last 2 decades has been on Lyme disease and related disorders, improved cultivation techniques and PCR assays have allowed further characterization of the diversity and distribution of isolates of relapsing fever *Borrelia* spp. For instance, tick-borne relapsing fever in the United States was thought to be limited to areas west of the Mississippi River until Breitschwerdt and colleagues recovered *Borrelia* spp. from two ill dogs in Florida. The isolates have gene sequences that place them very close if not identical to *B. turicatae*, a southwestern U.S. species (18). The organism clusters with other New World relapsing fever species (Fig. 1). Anda et al. reported the recovery of a new species of relapsing fever *Borrelia* from the blood of patients suffering relapsing fever-like illness in Spain, as well as from the implicated argasid tick vectors (1). The organism is pathogenic for mice, but it has not yet been cultivated in the laboratory. The gene sequences of this *Borrelia* sp. place it within the Old World subgroup of relapsing fever species, but it is clearly distinct from an example of *B. hispanica*, *B. crocidurae*, and *B. persica*, three other possible etiologic agents. Shanbay et al. reported the detection of a *Borrelia* sp. in Egypt in *Ornithodoros savignyi* argasid ticks. The organism appears to be distinct from *B. crocidurae*, another African species, in tick specificity studies (97). More recently, Thomas et al., in collaboration with my laboratory, identified a relapsing fever *Borrelia* sp. as the cause of a fatal illness in the endangered northern spotted owl in the northwestern United States (104). PCR was used to amplify three gene sequences from liver tissue obtained post mortem. The sequences indicate that the organism is closely related to *B. hermsii*, a relapsing fever species enzootic in the area, but there are also sequence positions that distinguish the organism from all other strains of *B. hermsii* (J. Bunikis, S. M. Rich, and G. Barbour, submitted for publication). *B. hermsii* was not previously known to infect birds in nature.

Another spirochete that phylogenetically clusters with the relapsing fever *Borrelia* sp. group is *B. coriaceae* (32, 45). The spirochete has been found in *O. coriaceus* ticks in northern California (32, 45, 46). It is a possible agent of epizootic bovine abortion, but to date there has not been direct evidence that *B. coriaceae* causes this disease (110).

COMPLEMENT AND VERTEBRATE HOST RANGE FOR *BORRELIA* SPP.

The last study indicates the importance of studies of host range for estimates of pathogenic potential for humans, domestic animals, and wildlife. Before gene sequencing and DNA hybridization and before the organisms could be cultivated for biochemical charcterization, the classification of *Borrelia* spp. was largely deter-

mined by certain biologic features of the organism: which species of tick were competent as vectors for the spirochete and which types of animals the spirochete could infect (12). The reasons for restricted associations between a *Borrelia* sp. and a certain tick remain to be determined, but recent studies have provided insight into host range limits for a given *Borrelia* sp. and vertebrates. The differential sensitivity of a *Borrelia* sp. to different types of animal sera had been noted in the past (reviewed in references 8 and 12), but the basis for the susceptibility of a strain to one type of serum but not to another was not known.

In 1997 Breitner-Ruddock et al. reported that in an assay of complement-dependent lysis in nonimmune serum, five *B. afzelii* strains were serum resistant while six strains of *B. garinii* were serum susceptible (17). Strains of *B. burgdorferi* generally fell between the two other species in serum susceptibility. The authors suggested that serum resistance was due to the absent or transient formation of the terminal complement complex. The greater serum susceptibility of *B. garinii* in comparison to *B. afzelii* and *B. burgdorferi* was also demonstrated by van Dam et al. (105). The finding of greater serum susceptibility of *B. garinii* than *B. afzelii* is paradoxical because *B. garinii* is more likely than *B. afzelii* to be isolated from the cerebrospinal fluid, while *B. afzelii* is the species more commonly recovered from the skin (108).

The findings of Kurtenbach et al. on complement susceptibility and the host ranges of different Lyme disease group species are less perplexing (42, 43). These investigators found that while *B. garinii* was generally more susceptible than *B. burgdorferi* and *B. afzelii* to mammalian serum and complement, *B. garinii* was more resistant than *B. burgdorferi* and *B. afzelii* to bird serum and complement. *B. valaisiana* resembled *B. garinii* in this regard, and like *B. garinii*, *B. valaisiana* uses birds as reservoris. In a study in Britain both *B. garinii* and *B. burgdorferi* were found in rodents, but only *B. burgdorferi* was transmitted to xenodiagnostic ticks. The majority of *I. ricinus* ticks recovered from pheasants were infected with *B. garinii* or *B. valaisiana* (42).

Differential complement susceptibility may also explain the situation for *B. burgdorferi* in California. Although there is focal transmission of *B. burgdorferi* in the state (101), the overall incidence of human infections in the regions of endemicity is lower than in areas of high endemicity in the northeastern United States (11). The lower incidence is in part attributable to a lower prevalence of infected *I. pacificus* ticks in the areas of California where they are enzootic. *I. pacificus* ticks feed on lizards as well as on rodents; however, unlike native rodents of California, lizards of the area could not be infected with *B. burgdorferi* (44). Indeed, lizard blood was found to kill *B. burgdorferi* spirochetes upon exposure in the ticks themselves or in an in vitro assay (47). In studies with the blood of the western fence lizard (*Sceloporus occidentalis*) and from the southern alligator lizard (*Elgaria multicarinata*), Kuo et al. determined that the bactericidal activity of the lizard blood involved a component of the alternative complement pathway (41).

CONCLUSIONS

The studies reviewed here reveal a wider diversity and distribution of *Borrelia* spp. than was appreciated 20 or even 10 years ago. While many of these newly

discovered species undoubtedly pose little threat for humans, the very presence of additional species in an area where Lyme disease is reported prompts more care in species identification and investigations of the ecology of the new agent and its vector and reservoirs. Reporting of the occurrence of *B. burgdorferi* in a region that was not previously known to have transmision of the agent can provide the rationale for diagnosing Lyme disease among the region's residents, when perhaps this is unwarranted. Much of the literature about *B. burgdorferi* in the southern and western United States is being reevaluated, now that considerable diversity of the Lyme disease group of species in these areas has been revealed.

Yet as Kirkland et al. concluded, "erythema migrans-like rash illness should no longer be considered definitive evidence of early Lyme disease" (39). In this and other articles, the authors have appended "-like" to the end of erythema migrans or Lyme disease. There is justification for distinguishing between the clinical features of the rash disorders associated with *Ixodes* ticks and with the lone star tick, but, as Melski points out, the term "erythema migrans" could be as appropriately applied to the rashes in the southern United States as to the distinctive rashes around Lyme, Connecticut (64). *B. lonestari* is now recognized as another possible etiologic agent of erythema migrans. *B. lonestari* is distant enough from *B. burgdorferi* and the other Lyme disease species to expect that the standard serologic assays for Lyme disease will not be adequate for confidently detecting infection with this organism. A polyclonal antiserum against *B. burgdorferi* was used to first identify *B. lonestari* in the ticks, but the reactions with this reagent were more qualitative than quantitative. With whole-cell ELISAs, quantitation distinguishes the controls from the definite cases of infection. Although the majority of patients with an erythema migrans rash illness associated with *A. americanum* have had antibodies to the flagellin of *B. burgdorferi*, this reaction by itself is insufficient for interpreting a test as positive.

There is also now some justification for proposing that erythema migrans in the northeastern United States may also have another etiology besides *B. burgdorferi*. The northern limits to the range of *A. americanum* include New York, New Jersey, Connecticut, and Rhode Island, states with high incidences of Lyme disease. Although the infectious potential of the *B. miyamotoi*-like organisms of *I. scapularis* for humans remains to be determined, the occurrence of these organisms in the same tick that transmits *B. burgdorferi* suggests that some of the rash illnesses in the Northeast are not Lyme disease but infection by a different type of *Borrelia* (95). In one of the phase III field trials of an OspA vaccine to prevent Lyme disease, the investigators' endpoints were not only clinical Lyme disease but also seroconversion of antibodies to *B. burgdorferi* antigens (99). After three doses the vaccine was 100% effective in preventing seroconversion but only 76% effective in preventing clinical Lyme disease. Using a sensitive culture procedure and PCR, the investigators did not recover *B. burgdorferi* in about one-fifth of the erythema migrans lesions that were biopsied. Approximately 1:5 was the ratio of *B. burgdorferi* to the *B. miyamotoi*-like organism in *I. scapularis* ticks in northeastern states (95). These findings suggest that some of the cases called "Lyme disease" in the trial had another etiology besides *B. burgdorferi*.

If the *B. miyamotoi*-like spirochete and *B. lonestari* cause human disease in North America, it is possible that other representatives of this newly recognized third group of *Borrelia* spp. cause disease elsewhere in the world. I predict that the list of *Borrelia* species will further lengthen during the next decade and that the wide distribution of these successful parasites in nature will be recognized.

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Emerging Infections 5
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Chapter 11

Emerging Perspectives on Human Babesiosis

Anne M. Kjemtrup and Patricia A. Conrad

Babesiosis is a zoonotic tick-borne disease, with the natural acquisition of human infections most often the result of interaction with established enzootic cycles. A number of factors have contributed to the emergence of human babesiosis, including increased awareness among physicians, changing ecology, and an increased population of immunocompromised individuals susceptible to infection.

Babesial parasites have special historical significance because the babesial agent of Texas cattle fever, characterized by Smith and Kilborne in 1893, was the first arthropod-transmitted pathogen described in vertebrates (118). Smith and Kilborne assigned the agent the generic name, *Pyroplasma* ("flame shaped"), reflecting the morphology often assumed by the parasite in the erythrocytes. Starcovici later assigned the generic name, *Babesia*, in honor of Victor Babès, who first saw and described babesial parasites in cattle in Romania (3, 125). The first evidence that humans could be infected with babesial parasites was reported in 1904 by Wilson and Chowning (141), who saw piriform, intraerythrocytic inclusions like those described by Smith and Kilbourne, in the blood of patients with Rocky Mountain spotted fever in the western United States. They called this agent *Pyroplasma hominis*. The first definitive case of human babesiosis, caused by the cattle parasite *Babesia divergens*, was identified in a Yugoslavian farmer in 1957 (116). Since then, three distinctly different babesial parasites have been recognized as the primary agents of human disease. In Europe, *B. divergens* infection of humans has been sporadically reported since 1957 but appears to be increasing, with over half of the 31 cases in the literature having been reported since 1985 (27, 44, 82). In the United States, human babesiosis was first recognized in California in 1968 (111). One year later, infection with *Babesia microti* was identified in a spleen-intact woman in the eastern United States (139). Since 1982, over 300 human cases of babesiosis have been reported in the eastern United States, providing the impetus to make babesiosis a reportable disease in some states (140). In the western United

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States, nine human cases have been attributed to the WA1-type babesial parasite, which is distinctly different from *B. microti* but closely related to small babesial parasites of wildlife and dogs in California (63, 97, 103, 134).

The life cycle of *Babesia* spp. begins when a competent ixodid tick takes a blood meal from an infected host. Depending on the species of *Babesia*, transmission in the tick vector is either transovarial (from adult female to eggs), or transstadial (from larva to nymph or nymph to adult) (85). Regardless of the type of transmission, it takes 1 to 3 days following tick attachment for the parasites to migrate to the salivary glands, where they are inoculated into the host. Once in the host, the parasites directly invade erythrocytes. The intraerythrocytic parasites multiply to form two to four separate merozoites (85). Two of these forms together are called the "paired form," while four together are commonly referred to as a tetrad or "Maltese cross" form. The host erythrocyte is destroyed as the rapidly reproducing parasites leave the cell to invade other erythrocytes.

BABESIOSIS IN EUROPE

The documentation of a human babesiosis case in a splenectomized Yugoslavian farmer in 1957 (116) was the first evidence of the zoonotic potential of *Babesia* spp. Since 1957, 31 more human cases of endogenously acquired *Babesia* infections have been identified in Europe; 23 of these cases were caused by *B. divergens* (27, 44). More than half of these cases were reported from France and the British Isles, likely due to the awareness of local physicians rather than being a reflection of an increased prevalence of disease (44).

The populations most at risk for acquiring babesiosis are farmers, foresters, campers, and hikers who frequent rural cattle-raising areas. Splenectomy is the main risk factor for human disease caused by *B. divergens*. At least 26 of the 31 (84%) known cases have been in patients who had been splenectomized for various medical reasons (27, 44, 82). However, serological evidence of human exposure to *B. divergens* without concomitant disease has been documented in France (43). Thus far, *B. divergens* human infection has not been reported in human immunodeficiency virus (HIV)-positive individuals.

Babesiosis due to *B. divergens* has an acute onset in splenectomized patients and is considered a medical emergency. One to three weeks following infection, patients present with severe intravascular hemolysis with hemoglobinuria and jaundice. Persistently high fevers of 40 to 41°C, chills, and sweats occur with myalgia (often lumbar and abdominal pain), headaches, vomiting, and diarrhea. This nonspecific clinical presentation can be easily confused with malaria (81). On physical examination, the liver may be moderately enlarged and painful, and signs of pulmonary edema may also be present. In severe cases, renal failure can occur with oliguria due to hemoglobin deposition in the glomeruli. Without treatment, or in cases where treatment is instituted late in the course of disease, general organ failure may occur 4 to 7 days after the initial hemoglobinuria, resulting in death.

Babesia bovis, *Babesia canis*, *B. microti*, and *Babesia* of unknown species have been implicated in human disease in Europe. Identification of these agents, how-

ever, was based on morphological characteristics and antigenic reactivity alone (16, 17, 58, 82).

BABESIOSIS IN THE UNITED STATES

B. microti

The first case of human babesiosis in an individual from the eastern United States was reported in 1969 and attracted the interest of the medical community because it was a fulminating case in a spleen-intact patient. The etiologic agent was identified as the rodent babesial parasite, *B. microti* (139). The disease was called "Nantucket fever" after 13 subsequent cases occurred between 1973 and 1976 on Nantucket Island, off the coast of Massachusetts (110). With over 40 more cases of Nantucket fever identified in the following decade, it became evident that a focus of *B. microti* endemicity was established along the eastern seaboard, particularly on Nantucket Island, Mass., and Long Island, N.Y. (6, 121). Similar areas of foci of endemicity have been identified in Connecticut, Wisconsin, and Minnesota (72, 127, 102). Intriguing case reports suggest that the geographic range of *B. microti* may extend further south, with recent case descriptions detailing cases from New Jersey (30) and an account in 1976 of a subclinical case from Georgia (46). A review of the literature indicates that more than 300 human cases of babesiosis due to *B. microti* have been identified in the United States since the index case (52, 72, 130, 140). Many more cases of *B. microti* are probably subclinical and therefore go undetected (68).

The biology of *B. microti* was investigated on the islands off the eastern seaboard of the United States. The white-footed mouse (*Peromyscus leucopus*) was identified as the primary reservoir because it had the highest prevalence of *B. microti* infection, was the most abundant small mammal in the area, and hosted the greatest number of the suspected tick vector, *Ixodes scapularis* (syn *I. dammini* [91]), immature stages (99). *Microtus pennsylvanicus* was a potential secondary reservoir. *P. leucopus* mice are carriers of *B. microti*, possibly with lifelong infection (130), and have been shown to be the major reservoir host in other areas of *B. microti* endemicity (1, 2, 127).

I. scapularis was shown to be a competent vector for transmitting *B. microti* by using a hamster model (120). The life history of *I. scapularis* covers 2 years and follows an inverted pattern, such that nymphs feed in the spring, preceding the period of maximum larval feeding in the late summer (55, 122). *B. microti*-infected nymphs infect naïve *P. leucopus* mice, which are subsequently fed on by uninfected larvae, resulting in a highly efficient maintenance of *B. microti* within the tick and reservoir species (122). The 2-year life history strategy of *I. scapularis* also buffers the tick population from host density disturbances (130).

White-tailed deer serve as hosts primarily for adult *I. scapularis*, although all life stages are found on the deer (100). Deer are not competent reservoirs for *B. microti* (100); however, they are important for the maintenance of *I. scapularis*.

Coinfection with other tick-borne agents has served to both confuse and enlighten the understanding of these diseases. The first clinical studies of human

babesiosis described erythema chronicum migrans-like lesions in the early stages of disease (5). In fact, this expanding rash is strongly suggestive of Lyme disease, caused by the spirochete *Borrelia burgdorferi* (126), and not a clinical sign of babesiosis. The sylvatic cycle of *B. microti* follows closely that of *B. burgdorferi* (123). Both the reservoir host, *P. leucopus*, and humans are commonly found to be coinfected with these agents (1, 2, 4, 65). Indeed, coinfection appears to aggravate both Lyme disease and babesiosis in humans (68, 71). The only fatality associated thus far with *B. burgdorferi*, the agent of Lyme disease, was in a patient coinfected with *B. microti* (80). Recent investigations of the zoonotic rickettsial tick-borne agent of human granulocytic ehrlichiosis also implicated *P. leucopus* as a reservoir (78). Understanding the epidemiology of *B. microti* has benefited the study of the epidemiology of these other tick-borne agents and also revealed that *B. microti* is part of an important zoonotic tick-borne disease complex.

B. microti infection presents with a spectrum of clinical manifestations, from asymptomatic to acute and fatal disease. In spleen-intact, immunocompetent people, an infection with *B. microti* may be inapparent or appear as a transient, undiagnosed flu-like illness (109). Alternatively, some infections become chronic and may manifest themselves only when the patient becomes immunocompromised or is splenectomized (68, 117). Typically, symptoms of babesiosis caused by *B. microti* are less acute than those caused by *B. divergens* and begin 1 to 4 weeks after the bite of an infected tick. The onset is gradual, with malaise, myalgia, anorexia, fatigue, and shaking chills being the most common signs. These signs are often followed by fever (>38°C), nausea, and vomiting (108, 140). In severe cases, hemoglobinuria may occur (102). Other physical findings less frequently noted are pulmonary edema, which may occur after the initiation of treatment (11); splenomegaly; hepatomegaly; and jaundice (140). Laboratory abnormalities include anemia, thrombocytopenia, leukopenia, and elevations of the liver function enzymes, including lactate dehydrogenase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin (60, 108, 140). Clinical features of babesiosis are similar to those of malaria, not including the fatal cerebral form of *Plasmodium falciparum* malaria, which does not occur with human babesiosis (8, 19).

Asplenic patients infected with *B. microti* often suffer severe babesiosis (132); however, unlike infections with *B. divergens*, the absence of a spleen is not a requirement for disease. In a recent summary of cases, only 3 out of 22 symptomatic patients were asplenic (68). Risk factors for severe disease include advanced age (≥55 years old) (108, 140) and immunocompromising conditions such as AIDS (34) and immunosuppresive drugs administered after organ transplants or chemotherapy for cancer (33, 117).

In addition to tick bite, two other routes of transmission exist. *B. microti* has been documented in over 20 cases of blood transfusion-acquired babesiosis since 1980 (84, 57) and, after *Plasmodium*, is the second most common parasitic infection acquired by blood transfusion (115). Babesial parasites can be transmitted in a variety of blood components—including erythrocytes, both liquid and frozen, and platelets, which usually contain a small number of erythrocytes (84). The implicated donors were all asymptomatic at the time of donation and in many of the cases

did not recall a tick bite (28, 84, 88). This underscores the subclinical nature of many *B. microti* infections: silent infections can persist from months to years (68).

Currently, the only method used to prevent contamination of blood supplies with babesial parasites is a predonation question asking donors if they have ever had babesiosis. Those that answer in the affirmative are excluded from donating due to the possibility of persistent infections (84). Other proposals, such as disallowing those from areas of endemicity to donate and not accepting donations during the summer months from areas of highly endemic disease, have been dismissed since these restrictions would not eliminate chronically infected individuals (84) but would exclude too many healthy donors (38). In 1997, investigation of five transfusion-transmitted cases of babesiosis identified three *B. microti*-infected donors, representing three units of blood out of half a million units donated, corresponding to an incidence of 6 per 1 million units of collected blood (84). With such a low incidence, eliminating blood donations from areas of endemicity would unnecessarily exclude uninfected donors. Promising chemical and physical manipulations of blood donor products to inactivate microbes (including *Babesia* spp.) are currently being developed and investigated (101). Physicians in areas of endemicity are advised to include babesiosis as a differential diagnosis for febrile illness following blood transfusion, particularly in elderly, immunocompromised, or splenectomized individiuals.

Transplacental transmission is a second nontick route of infection. Only two human cases have been convincingly documented (29, 90). In addition, there is one reported case of maternal infection with *B. microti* during pregnancy, followed by successful treatment, resulting in no transmission to the fetus (104). Other cases of perinatal babesiosis originating from transfusion or tick bite (112, 142) suggest that the spectrum of clinical disease seen in adults also occurs in infants.

WA1 *Babesia*: a New Human Piroplasm

Endogenously acquired human babesiosis in the western United States was first reported from California in 1968 and was the second human case of babesiosis ever described (111). Attempts were made to identify the parasite via antibody tests against 10 *Plasmodium* spp. as well as against known veterinary piroplasms: *B. canis* of dogs and *Babesia* (syn. *Theileria*) *equi* and *Babesia caballi* of horses. The patient's serum reacted against the babesial antigens but did not react against the *Plasmodium* spp. In 1981, another Californian case in an asplenic patient was described (13). This case was remarkable for having a high parasitemia (30%). No other cases were described from the western United States until 1991 to 1994, when seven human cases attracted medical attention. In California, a soldier participating in field exercises developed fever, chills, myalgia, and neck pain (59). Examination of blood smears revealed parasites in 1.2% of the erythrocytes, some forming the characteristic small babesial tetrad or Maltese cross form. Antibody tests demonstrated a titer of 1:80 for *B. microti* and a titer of 1:640 for canine small piroplasm in California (20, 61). (These titers were previously reported incorrectly as 1:8 for *B. microti* and 1:64,000 for the Californian canine piroplasm due to a publication error [59]). This patient was designated CA1.

In the same month, another human case of babesiosis was observed in Washington State (103). At 41 years of age, this patient did not have typical risk factors for fulminating babesiosis: he was not elderly, splenectomized, or immunocompromised, nor had he traveled outside Washington for many years. Nonetheless, he suffered from the typical flu-like illness and from pulmonary congestion. Antibody testing again revealed a very high titer of the Californian canine piroplasm (1:2,560), and inoculation of his blood into a hamster resulted in an unusually virulent infection (103). Molecular characterization of the small piroplasm parasites in his blood revealed that this organism was more closely related to the Californian canine piroplasm than to *B. microti* (134). This newly described agent was called WA1. Three more cases of human babesiosis in California patients (CA2 to CA4) were described from 1992 to 1993 (15, 97), and one of these (CA3) was fatal. These patients, with the exception of patient CA3, had extremely high (1:5,120) titers of antibody to the WA1 isolate and no titer of antibody to *B. microti* (97). Patient CA3 did not develop a detectable titer of antibody to any babesial antigens, presumably because his fatal disease progressed extremely rapidly (97). All of the infecting agents involved in Californian cases were shown by molecular characterization techniques to be closely related to the WA1 agent (134).

The WA1-type *Babesia* can also be transmitted via blood transfusion. In 1994, a WA1-type agent was recovered from an elderly surgical patient in Washington state who had received numerous blood transfusions (patient WA2) (51). The identified donor (subject WA3) was asymptomatic, and no parasites were identifiable on examination of his blood smear. Yet blood collected from this individual 7 months after the implicated donation date and inoculated into a hamster resulted in the isolation of WA1-type parasites (51). More recently, in 2000, a fifth human case of WA1-type *Babesia* was identified in California in a premature infant who had received multiple blood transfusions from the father and an anonymous donor (A. M. Kjemtrup, B. Lee, C. L. Fritz, C. Evans, M. Chervenak, and P. A. Conrad, submitted for publication). Babesial parasites were recovered from the healthy asymptomatic anonymous donor (subject CA6) 2 months after the implicated donation. Identification of two asymptomatic blood donors (subjects WA3 and CA6) underscores the fact that people can acquire and maintain persistent babesial infections with parasitemia so low that the infection remains subclincal yet transmissible by blood donation.

The small number of documented WA1-type human infections precludes a description of typical epidemiological and clinical patterns. Those infected individuals in California showing fulminating babesiosis were either asplenic (patients CA1 to CA4) or immunocompromised (patient CA5). Those from Washington and the asymptomatic blood donor in California (subject CA6) were spleen intact. All WA1-type babesiosis patients demonstrated typical babesial flu-like symptoms, including fever, chills, myalgia, and vomiting. Anemia in the WA1-type cases was nonexistent to moderate, with the exception of patient CA3 who had a hematocrit of 17% at the time of hospital admission (15). Some patients progressed to serious conditions, including disseminated intravascular coagulation (patient CA4), pulmonary congestion of varying degrees (patients WA1, CA3, and CA4), and renal insufficiency (patient CA4). Hemoglobinuria was noted in patients WA1, CA3, and

CA4 (15, 97, 103). These findings suggest that clinical disease in WA1-type cases does not completely result from erythrocyte cell destruction.

Studies with animal models of both *B. microti* and the WA1-type agent demonstrate that the WA1 agent has a different pathogenesis than *B. microti*. Intraperitoneal inoculation of infected whole blood into Syrian golden hamsters (*Mesocricetus auratus*) is a common method for isolating and identifying babesial parasites (12, 31). Inoculation of *B. microti* into hamsters can result in high (60 to 90%) parasitemia, anemia, and splenomegaly; however, the parasitemia subsequently decreases to undetectable levels within 3 to 6 weeks, and the hamsters survive, showing few if any clinical signs (22, 143). Hamsters inoculated with the WA1 agent become depressed, dyspneic, lethargic, and paretic and show porphyrin-stained secretions on the rostrum and forelimbs within 5 to 7 days after inoculation. Parasitemia of up to 90% results in severe anemia, thrombocytopenia, proteinuria, and hemoglobinuria. The infection is almost always fatal within 5 to 8 days postinoculation. On histopathologic examination, hamsters show marked dilation of blood vessels in the lungs, liver, spleen, kidneys, and brain (25). Splenomegaly in *B. microti*-inoculated hamsters has been attributed to the proliferation of phagocytic cells and extensive extramedullary hematopoiesis. WA1-infected hamster spleens show similar changes; however, expansion of the splenic white pulp and mesenteric lymphadenopathy suggest strong antigenic stimulation of lymphoid proliferation and clonal expansion (143). Pulmonary and hepatic phlebitis, resulting in disseminated intravascular coagulation, thrombosis, infarction, and edema, are typical of WA1, but not *B. microti*, infections in hamsters (143).

The mouse models of *B. microti* and WA1 infections exemplify the differential pathogenicity of the two parasites. In a C3H/HeN mouse model, inoculation of *B. microti* resulted in low parasitemia with few if any clinical signs, while inoculation of WA1 at one-eighth of the dose of *B. microti* resulted in moderate parasitemia, dyspnea, and death (49). Postmortem changes in the lungs of the WA1-infected mice showed pulmonary edema and intravascular margination of leukocytes, whereas no pulmonary changes were seen in *B. microti*-infected mice. These findings suggested that the pulmonary changes in WA1-infected mice were responsible for the respiratory failure, which was the most likely cause of death.

Histopathological changes resulting from parasitic infections, such as infections with *Plasmodium* spp., have been attributed to host cytokine production (21). Similarly, in a mouse model of babesiosis developed by Hemmer et al. (48), in the lungs of WA1-infected mice, tumor necrosis factor alpha (TNF-α) and gamma interferon were expressed significantly more than in *B. microti*-infected mice. The intravascular margination of leukocytes was directly attributable to TNF-α, resulting in endothelial cell stimulation and the ensuing pulmonary edema, with gamma interferon acting synergistically. This suggested that the disease process of WA1 infection was probably the result of the host's own immune response rather than a direct result of the parasite itself (48). Support for the hypothesis came with the finding that TNF-α played an integral role in the pathogenesis of WA1 infections in mice genetically deficient in TNF-α receptors (TNFp55$^{-/-}$ mice) (47).

The reservoir host for the WA1-type parasite remains unknown. Piroplasm infection in small mammals has been documented in the western United States since

1968 when small babesial parasites were identified in the desert woodrat (*Neotoma lepida*), California mouse (*Peromyscus californicus*), California vole (*Microtus californicus*), California ground squirrel (*Spermophilus beecheyi*), and desert cottontail rabbit (*Sylvilagus audubonii*) in southern California (136). These parasites were not completely characterized. Genetically characterized *B. microti*-type piroplasms have been identified in rodents in coastal Alaska (35, 40). Small mammals trapped and tested in a Sonoma County community, south of the exposure site for the CA3 patient, identified yet another piroplasm in dusky-footed woodrats (*Neotoma fuscipes*). This parasite was shown to be genotypically different from WA1 or *B. microti* and was more closely related to *Theileria* spp. Hence, this parasite was named *Theileria youngi* (62). Although a reservoir species for the WA1-type parasite has yet to be identified, it is clear that there are at least three species of piroplasms circulating in sylvatic cycles in the western United States.

The close relationship of WA1 to the Californian small piroplasm parasite of dogs suggested nonrodent reservoirs for the WA1-type *Babesia*. Concomitant with the identification of the human cases in patients CA1 to CA4 (1992 to 1994), other babesial parasites were isolated in bighorn sheep (*Ovis canadensis*) (41, 133), mule deer (*Odocoileus hemionus*) (133), and captive fallow deer (*Dama dama*) (A. M. Kjemtrup, unpublished data) in California. There was an intriguing geographic coincidence in the exposure sites of the infected humans with the wildlife populations from which morphologically similar babesial parasites were isolated and/or serologic antibodies to small *Babesia* were detected (64). Serum from all of the western human cases of infection (patients WA1 and CA1 to CA4), with the exception of patient CA3, had elevated titers of antibodies to babesial isolates from wildlife as shown using the indirect fluorescent antibody test (IFAT) (Kjemtrup, unpublished data). A thorough phylogenetic analysis of the entire 18S small-subunit (ss) rRNA gene of all of the human and wildlife isolates, as well as other piroplasms, demonstrated that the isolates obtained from humans and animals in the western United States formed a single group, separate from *B. microti, B. divergens*, and *Theileria* spp. (63). Figure 1 shows the result of a similar neighbor-joining phylogenetic analysis, performed as previously described (63), of piroplasm species from California and other major piroplasm species worldwide. The piroplasm isolates from the Californian human patients were indistinguishable from some of the isolates from the western wildlife species, most notably the isolates from mule deer. These results suggested that large ungulates might serve as reservoirs for human WA1 piroplasm infection.

The tick vector for the WA1-type *Babesia* is also unknown. Several species of human-biting ticks occur in the areas where humans and wildlife were infected and include *Dermacentor variabilis*, *Dermacentor occidentalis* (in California only), *Ixodes pacificus*, and *Ixodes angustus* (24, 37, 73, 74).

Indirect evidence provided by serosurveys suggests that human infection with the WA1-type *Babesia* may result in asymptomatic infections. In the initial WA1 report, 4 of 83 neighbors (4.8%) of patient WA1 had elevated (1:256) antibody titers to the WA1 antigen, no reactivity to *B. microti*, and reported episodes of undiagnosed flu-like symptoms during the previous year. However, blood from these people did not infect hamsters (103). In California, human serosurveys dem-

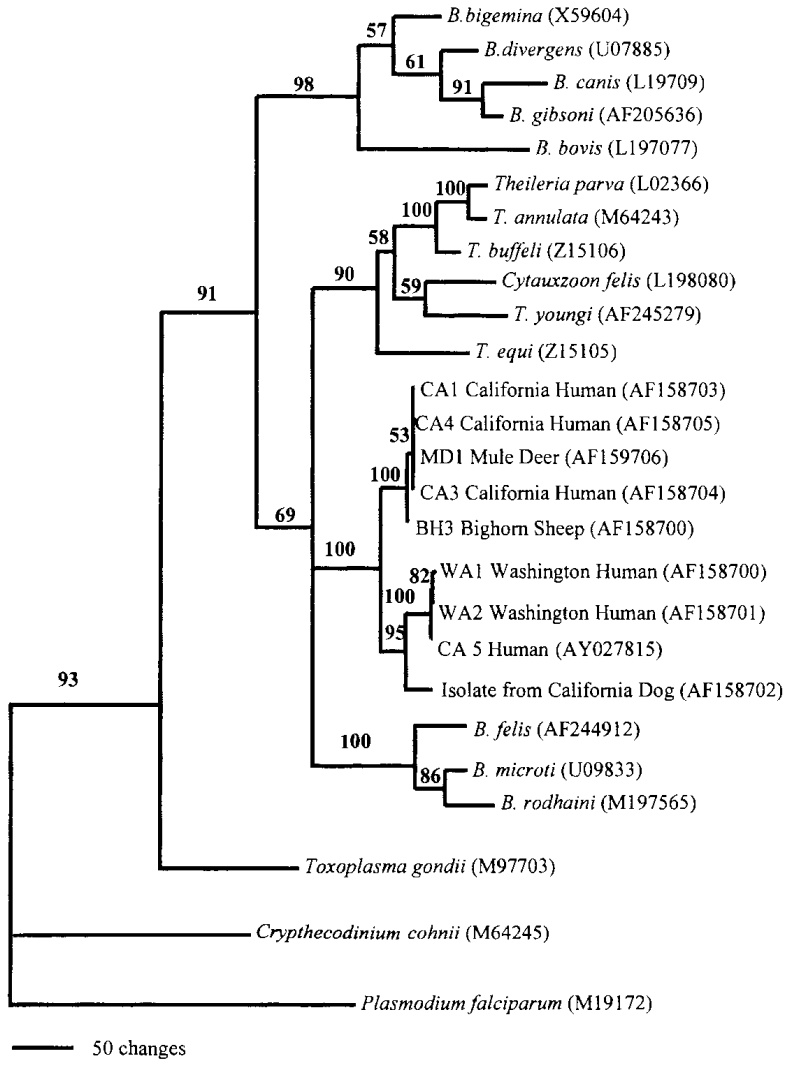

Figure 1. Phylogenetic tree inferred from neighbor-joining analysis of piroplasm species using an alignment of the entire 18S ss rRNA gene sequence. Numbers above the lines represent the percentage of replicates out of 500 bootstrap samplings in which the given branching pattern was obtained. GenBank accession numbers for the sequences used are noted in parentheses after the isolate name.

onstrated elevated (≥1:320) WA1 antibody titers in people from Monterey County at the same military fort where the case of patient CA1 occurred (2 of 51 subjects [3.9%]); from Sonoma County, both north (1 of 115 subjects [0.9%]) and south (37 of 219 subjects [16.9%]) of the exposure site of patient CA3; and from a convenience sample from blood bank donors from an urban area in California (25 of 124 subjects [20.6%]) (36, 97). Again, none of the samples had detectable

antibodies to *B. microti*. The WA1 IFAT follows the same methodology as the *B. microti* IFAT, which has been carefully validated with a positive cutoff titer considered to be 1:80 or 1:160, depending on the laboratory (69). Infection status of people with elevated titers of antibody to WA1 is not clear. Positive cutoff values for the WA1 IFAT have not been established due to the limited number of confirmed cases of WA1 infections available for test validation. Nonetheless, a positive cutoff titer of ≥1:320 has been suggested based on follow-up samples from known infected patients (patients WA1 to WA3 and patients CA1 and CA2). These patients showed elevated (1:160 to 1:1,024) antibody titers for 1.5 years after treatment (51, 97, 103), with one elevated titer persisting up to 4 years after treatment (P. Conrad, unpublished data). As with *B. microti* clinical infections, the clinical cases of WA1-type babesiosis probably represent a small percentage of people who are actually infected, and splenectomized or immunocompromised people may act as sentinels for the disease agent.

B. divergens-Like Parasites

In 1992, a 73-year-old splenectomized man in Missouri, who rarely traveled outside the state and never out of the country, was hospitalized with fever, rigor, thrombocytopenia, dry cough, headache, and joint pain (50). Babesiosis was diagnosed based on the identification of characteristic intraerythrocytic babesial forms on a blood smear. Antigenic and genetic characterization identified a *B. divergens*-like parasite. There were some antigenic differences between the Missouri human isolate and a European *B. divergens* strain, and the Missouri isolate did not infect jirds or hamsters, animals generally susceptible to infection with *B. divergens*. Unfortunately, as is often the case with European *B. divergens* infections, this infection was fatal.

HUMAN BABESIOSIS THROUGHOUT THE WORLD

Awareness of human babesiosis has prompted the identification of new human cases worldwide. Reports of human babesiosis around the world underscore the likelihood that many such cases go undetected, possibly because of unfamiliarity of physicians with the disease.

Outside Europe and the United States, human infection with *Babesia* spp. of domestic animals has been described. Three well-documented asymptomatic cases in humans were identified in Mexico by parasite isolation via hamster inoculation (94). Sera from these infected humans were tested against *B. canis* antigen and reacted weakly. Fluorescent antibody conjugates specific for *B. canis* or *Babesia bigemina* stained the parasites isolated from the humans. A species identification of these isolates was not made. In 1981, babesial isolates were obtained from two acute, fatal human cases, and from three other infected humans with undescribed clinical signs, in Mozambique (105). The parasites were identified via blood smear examination, isolation in hamster, and blood culture. In Africa, sporadic human infections with *Babesia* spp. have also been reported from the northern regions, including cases in Egypt (87) and the Canary Islands (92). These studies emphasize

that awareness of the parasite can lead to identification of human infection in previously unrecognized areas of endemicity and that human babesiosis is probably more widespread than is currently known.

In malarial regions, human babesiosis may be misdiagnosed as infections with *Plasmodium* spp. due to the similar clinical presentations of these diseases, similar morphologies of the parasites on blood smears, and possible coinfection with both agents. In the Sudan, while monitoring for malaria infections, blood smears from 20 human patients out of 137 examined (14.5%) demonstrated intraerythrocytic merozoite forms more consistent with *Babesia* spp. than *Plasmodium* spp. (128). Similarly, in South Africa, two human cases initially diagnosed as malaria were later recognized as cases of human babesiosis after reexamination of blood smears revealed merozoites in erythrocytes without pigment but with nonstaining vacuoles and many multiply infected erythrocytes, all of which are charactersitic of babesial infections (14). Coinfection with *P. falciparum* could not be ruled out in one case. The possibility of coinfection with babesial and malarial agents was described for an infant from the Ivory Coast who was quite ill with a parasitemia of 35% (138). Medical and veterinary experts could not agree whether the infection was due to a *Babesia* sp. or a *Plasmodium* sp. Atypical malaria cases should be evaluated for the potential of a babesial infection (76, 124).

In Taiwan, investigation of rodent *Babesia* spp. culminated in the identification of a case of human babesiosis. *Babesia* spp. morphologically consistent with *B. microti* were described from rodents in Taiwan in 1977 (135). In the same year, Hsu and Cross (54) showed that a few residents of Taiwan were seroreactive to *B. microti*. Recently, a case of human babesiosis with mild clinical signs was ascribed to a babesial agent closely related to *B. microti* (114). Rats in the same area were shown to have high rates of seroreactivity to the isolate. Two out of 32 (6.3%) neighbors of the infected individual showed elevated titers of antibody to the Taiwanese isolate of *B. microti*.

FACTORS RESPONSIBLE FOR EMERGING STATUS

Emerging diseases have been defined as "new, re-emerging, or drug-resistant infections whose incidence in humans has increased within the past 2 decades or whose incidence threatens to increase in the near future" (56). By virtue of the number of human cases described since 1980 (>300), human babesiosis can be considered an emerging disease. The reasons for the increased incidence of human babesiosis are complex, most likely involving ecological factors, increased awareness of the disease, and an increased number of susceptible individuals, such as those infected with HIV.

Over the past several decades, white-tailed deer populations in the northeastern United States have expanded tremendously, partly due to conservation efforts and partly due to changes in landscape from farms to forested, semiurban areas (119). The expansion of the deer range concomittantly resulted in an expansion of their ectoparasites, particularly *I. scapularis* (119). Though refractory to infection with *B. microti*, white-tailed deer are the primary host for adult *I. scapularis* (100). The increased number of large mammal hosts for adult ticks has facilitated the spread

of *I. scapularis* in the northeastern United States (123). The density of nymphal ticks appears to be an important factor for *B. microti* infection in rodents. In the northeast state of Rhode Island, *B. microti* was only detected in rodent populations where *I. scapularis* nymphs could be collected by flagging techniques at a rate greater than 21 ticks per h (83).

Desirability of rural living and outdoor activities has permitted a greater exposure of humans to the infected vectors (89). Residential and recreational activities in rural tick-infested areas contribute to the increased exposure of humans to babesial agents in the United States and Europe (32, 36, 131). Indeed, on Nantucket Island, in the summer at the height of babesial transmission, the human population increases from 7,000 winter residents to 35,000 residents (131). Thus, human encroachment into rural areas and increased tick densities have contributed to the "spillover" of babesial agents from enzootic cycles (26).

The emergence of other tick-borne agents, such as *B. burgdorferi*, the bacterial agent of Lyme disease, and the rickettsial agent for granulocytic ehrlichiosis, has increased both physician and public awareness of babesiosis and tick-borne diseases in general (79, 113, 137). In the United States, coinfection with these agents has highlighted the need for physicians to test for multiple agents when presented with an apparent tick-borne disease (71, 89, 129). A quick search of the Internet reveals no fewer than 10 websites, of both medical and lay-public quality, discussing human babesiosis. Thus, rapid access to medical information via the Internet has also facilitated public awareness of the disease.

An increased older population, the increasing number of HIV-positive individuals, and the population of people receiving immunsuppresive drugs for cancer chemotherapy will all serve to increase the number of human babesiosis cases. Increased age (≥55 years old) is an important predictor for both the clinical manifestation and severe outcome of infection with *B. microti* (86, 140). Four cases of *B. microti* infections in HIV-positive individuals or in AIDS patients have been reported in the literature (7, 34, 77, 93). All cases either had severe outcomes or had persistent parasitemia that had to be treated with long-term clindamycin and quinine or doxycycline and azithromycin. It is important for physicians to be aware of the clinical manifestations of babesiosis, particularly in AIDS patients. In light of the immunological studies on mice indicating the important role of $CD4^+$ T cells in controlling parasitemia (47), persistent parasitemia in patients with low T4-cell counts is not surprising. Human babesiosis has also been documented in patients who were on immunosuppressive drug therapy for organ transplants (33, 45, 117). An increasing population of immunocompromised individuals will result in an increase in the number of human babesiosis cases.

PRESENT METHODS OF DIAGNOSIS

Definitive diagnosis of babesiosis is based on the visualization of intraerythrocytic parasites on Wright-Giemsa-stained thin blood smears under oil immersion (Color Plate 10 [see color insert]). Intraerythrocytic merozoites, in general, measure 1 to 3 μm in diameter (85). Color Plate 10 demonstrates the common morphologic forms of WA1 (and *B. microti*) parasites in a blood smear from a WA1-infected

hamster. Common morphologic forms include ring forms, the tetrad or Maltese cross form, and teardrop shapes called piroplasms. Occasionally, extracellular merozoites or amorphous or amoeboid forms are visible. Experienced microscopists can identify babesial infections at sensitivities of 1 parasite per 10^5 to 10^6 erythrocytes (9). Giemsa stain examination of thin blood smears is a rapid method of particular importance for the diagnosis of *B. divergens* infections where a quick diagnosis is essential for survival (10).

In *B. microti* infections, parasitemias range from 1 to 20% of the erythrocytes in normosplenic individuals and up to 80% in splenectomized individuals (106). Patterns of morphology of WA1-type infections resemble those of *B. microti* infections (97). The morphology of intraerythrocytic *B. divergens* parasites is similar to that of the WA1-type and *B. microti* parasites (44), with the exception that the paired forms are typically found at an obtuse or diverging angle, usually in a central position in human erythrocytes (130). Parasitemias in human infection with *B. divergens* range from 1 to 50% of the erythrocytes (44).

It is difficult but not impossible to differentiate *Babesia* and *Plasmodium* spp. on Giemsa-stained blood smears. Ring forms of *Babesia* may appear similar to *Plasmodium* spp.; however, *Babesia* spp. lack hemozoin deposition in their cytoplasm. Identification of the tetrad forms and extracellular merozoites of *Babesia* spp., or of the distinctive schizonts and gametocytes of *Plasmodium* spp. when present, are used to differentiate these genera (10).

The IFAT remains the diagnostic test of choice for detection of antibody against babesial parasites. The IFAT for *B. microti* has been standardized for the northeastern United States, where the test is known to perform with 88 to 96% sensitivity, 90 to 100% specificity, 69 to 100% positive predictive value, and 69 to 99% negative predictive value (69). For acute infections, the use of fluorescein isothiocyanate-labeled anti-human immunoglobulin M has been found to be even more sensitive for detecting *B. microti* infections (67). The IFAT for *B. divergens* is useful for retrospective evaluation, since antibodies to *B. divergens* do not develop until 7 to 10 days after the onset of hemoglobinuria (44). The antigens of *B. divergens* also cross-react with several *Plasmodium* and *Babesia* spp. (44). The WA1 IFAT has been used to screen potentially infected individuals (36, 97); however, a positive cutoff titer has not been clearly established. Results of this test on humans outside of areas of babesial endemicity need to be interpreted with caution, since sensitivity and specificity values change with population prevalence (107).

Alternative serologic tests, such as an enzyme-linked immunosorbent assay using recombinant antigens, are being developed (53, 75) and may provide a tool for rapid screening of a large number of samples. Soluble whole-parasite antigen has been used for enzyme-linked immunosorbent assays for *B. divergens* for screening of cattle and was found useful for the detection of recent infections only in these hosts (18). Recombinant antigens offer the most promise for rapid, sensitive, and specific tests (9).

Inoculation of susceptible animals with whole blood from an individual with a suspected case of infection is a diagnostic technique primarily useful in academic settings. Hamsters are classically used to isolate *B. microti* (12, 39), while gerbils and calves are used for *B. divergens* isolation (42). WA1-type infections have been

identified via hamster inoculation only (103). Inoculation of the animal requires monitoring for infection via blood smear examination for up to 6 weeks (130). Thus, although this system is fairly sensitive (300 organisms per ml of blood for *B. microti*) (31), it is time-consuming and expensive.

DNA amplification by PCR has been proposed as a highly sensitive method to detect *Babesia* infection (98). The DNA product additionally may be sequenced, allowing for species identification (96). Protocols for diagnosing human babesial infections have been most successful with *B. microti* infections, using specific primers that target the 18S ss rRNA gene (98). This method is capable of identifying three merozoites of *B. microti* from a 100-μl sample (98) and correctly identifies acutely infected patients 95% of the time (70). PCR has the added benefit that results can be obtained within 2 to 3 days, compared to 1 to 6 weeks (70) for animal inoculation. PCR using general primers is a valuable tool for the identification and characterization of WA1-type infections in the western United States (96). Development of standard PCR protocols for diagnostic purposes is not trivial; it is costly and time-consuming, and requires considerable technical expertise (95). As with any diagnostic technique, sensitivities and specificities of the tests need to be established for each laboratory in order to interpret test results accurately. In chronically infected individuals, the sensitivity of the PCR technique targeting the 18S ss rRNA gene would be low due to the small number of parasites in a sample and the small number of target genes present in *Babesia* spp. (two to four copies) (23). Although potentially very powerful, PCR is currently most useful diagnostically in conjunction with an evaluation of clinical signs and other babesial diagnostic tools, such as blood smear examination and serology.

CURRENT TREATMENT PROTOCOLS

Treatment of *B. divergens* must be rapid and aggressive. A massive blood exchange transfusion (two to three times the patient's blood volume), followed by 10 days of intravenous clindamycin (600 mg three to four times per day) is recommended (44). In patients with less than 1% parasitemia, intravenous clindamycin at the same dose may be sufficient without blood transfusion (27).

The current treatment recommendation for human infection with *B. microti* is combined oral quinine (650 mg three times per day) and oral clindamycin (1,200 mg twice per day) for 7 days (131). In severe cases, apheresis may be required to reduce parasitemia (8, 33). Individuals infected with HIV may require the addition of other drugs, such as doxycycline (200 mg per day) and azithromycin (2,000 mg per day) to the regimen, for long-term treatment (34, 93). The combination of atovaquone and azithromycin has recently been shown to be an effective treatment, with fewer side effects, for babesiosis caused by *B. microti* (66). It should be noted that even if treatment results in undetectable parasitemia and amelioration of clinical symptoms, persistent infections are common, and physicians should be aware that recrudescence of the infection may be possible (68). Moreover, treatment of nonclinical human babesiosis cases has been suggested to protect the infected individual from fulminating infection (68). Treatment of WA1-type babesial infections has followed *B. microti* recommendations.

CONCLUSIONS

Babesia spp. are the second most common blood-borne parasites of mammals after trypanosomes (130). As an expanding human population results in closer contact with animals and their diseases and ectoparasites (26), more cases of human babesiosis will likely be identified. Human infection with babesial agents was unheard of less than 50 years ago: today over 300 human cases worldwide are reported in the literature. Thus, changing ecology, increased populations of susceptible individuals, and awareness of the disease will likely result in the discovery of new foci of endemicity and new agents of human babesiosis.

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Chapter 12

Amebiasis, an Emerging Disease

Mehmet Tanyuksel, Hiroshi Tachibana, and William A. Petri, Jr.

Amebiasis is caused by a protozoan parasite called *Entamoeba histolytica* and has a variety of clinical manifestations, from asymptomatic to colitis or liver abscess. Amebiasis is estimated by the World Health Organization to be the third leading cause of death among parasitic diseases (70).

Amebiasis is spread by fecal-oral transmission; as a result, this disease has long been associated with developing countries. Today, amebiasis is commonly found in nearly all tropical and subtropical countries. However, the bulk of the morbidity and mortality due to amebiasis is in Central and South America, Africa, and the Indian subcontinent (68). Amebiasis is responsible for an estimated 50 million infections and 100,000 deaths per year worldwide (70).

Amebiasis outbreaks in developed nations have resulted from contamination of water supplies with sewage secondary to inadequate water maintenance and treatment (R. S. Barwick, A. Uzicanin, S. M. Lareau, N. Malakmadze, P. Imnadze, M. Iosava, N. Ninashvili, M. Wilson, H. Bishop, A. Hightower, W. A. Petri, Jr., and D. D. Juranek, *48th Annu. Am. Soc. Trop. Med. Hyg. Meet.*, p. 234, 1999). The peak incidence of infection, as determined by case series of patients, is in children less than 14 years old, with a second increase in adults more than 40 years old. The only large serosurvey available revealed that 8.4% of the Mexican population had been exposed to amebiasis, resulting in an estimate of 1 million cases of amebiasis and 1,216 deaths per year in Mexico (9, 48).

The incidence and impact of amebiasis have been obscured by the prior inability to accurately diagnose amebiasis. Improved diagnostic tests are beginning to overturn some of the commonly held beliefs about amebiasis.

As our experience with emerging *E. histolytica* infections increases and accurate laboratory-based diagnostic tests are employed, better management of this protozoan disease will become possible.

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LABORATORY DIFFERENTIATION OF *E. HISTOLYTICA* FROM *E. DISPAR*

Two recent developments have caused a major shift in our thinking about amebiasis as a disease. First, easy-to-use commercial kits have become available that have greatly increased the sensitivity of detection. Second, *E. histolytica* has recently been recognized to actually be two distinct species, pathogenic *E. histolytica* and an apparently nonpathogenic commensal, *E. dispar*.

Despite the identical and indistinguishable morphology of *E. histolytica* and *E. dispar*, all available data support the idea that they are indeed different species (66). There are biochemical, genetic, and immunologic differences between the two species. Microscopically, one can differentiate *E. histolytica* from *E. dispar* to some extent if ingested erythrocytes are present. But 16% of *E. dispar* and only 68% of *E. histolytica* trophozoites contain ingested red blood cells, making this at best an imperfect means to distinguish the two organisms (27). The differences between the two are more apparent biochemically. Four glycolytic isoenzymes (glucose-phosphate isomerase, phosphoglucomutase, hexokinase, and malic enzyme) exhibit different migration patterns on gel electrophoresis. These zymodemes allowed the grouping of organisms into two groups: pathogenic zymodemes, now called *E. histolytica* sensu stricto, and nonpathogenic zymodemes, now called *E. dispar* (14). The two organisms have been shown to be different at the DNA level in several ways (14, 22, 29, 43, 58, 61, 66).

1. Every gene sequenced to date has a distinct genetic sequence in *E. histolytica* versus that in *E. dispar*, typically with more than 5% divergence.
2. Distinct DNA restriction fragment patterns are observed.
3. Repetitive DNA sequences are unique.
4. The organization of the actin genes in the genomes is different.

Immunological and antigenic differences are distinguishable with the use of monoclonal antibodies. Significant systemic humoral immune responses occur only with *E. histolytica* and not with *E. dispar* infection (29, 49, 62).

Microscopy-Cytological Diagnosis

Traditionally, amebiasis has been diagnosed by microscopy (Color Plate 11 [see color insert]). Because of difficulty in readily distinguishing *E. dispar* from the morphologically similar *E. histolytica*, there is a paucity of data on the prevalence of the two organisms.

The cytologic diagnosis of amebiasis is neither specific nor sensitive. *E. histolytica* and *E. dispar* are identical in appearance. Since in most areas *E. dispar* is more common than *E. histolytica*, this makes cytologic diagnosis extremely nonspecific. While some have suggested that the presence of ingested red blood cells aids in the cytologic diagnosis of amebiasis (6), we have demonstrated that in some cases *E. dispar* is also observed to contain red blood cells (27).

Frequently, fecal leukocytes have been misidentified as *E. histolytica* cysts by laboratories. Likewise, some macrophages have been reported as *E. histolytica* trophozoites (15, 37). Microscopy also suffers from poor sensitivity, such that even in experienced hands this technique may miss 50% of infections (Table 1) (30). In one study, the specificity of *E. histolytica-E. dispar* complex as determined by microscopy was only 9.5% in community laboratories compared with the *Entamoeba* test and ProSpecT enzyme-linked immunosorbent assay (ELISA) antigen detection tests (50).

Amebic liver abscess (ALA) is difficult to diagnose by microscopic examination of abscess fluid, with microscopy of abscess fluid yielding negative results in most cases (71). In summary, microscopy is neither sufficiently sensitive nor specific for the diagnosis of *E. histolytica* infection.

Culture and Zymodemes

Stool culture followed by zymodeme analysis for *E. histolytica* is a more sensitive and reliable means of identifying this protozoan; however, stool cultures are usually not available in clinical practice, and the entire process of cultures plus zymodeme analysis takes several weeks to complete. Another disadvantage of this method of diagnosis is that culture is frequently negative because of self-treatment with metronidazole before seeing a health care provider. The advantage of culture is that it allows specific detection of *E. histolytica* and subclassification within *E. histolytica* and *E. dispar* on the basis of zymodeme pattern (16).

Regarding zymodeme analysis, determination of the isoenzyme patterns of hexokinase is very helpful. In this technique, slow-migrating bands indicate *E. dispar*

Table 1. Sensitivity and specificity of tests of diagnosis for amebiasis in patients with colitis and liver abscess[a]

Test	Colitis patients		% Sensitivity(ies) in liver abscess patients
	% Sensitivity	% Specificity	
Microscopy of:			
Stool	<60	10–50	<10
Abscess fluid	NA[b]	NA	<25
Culture with isoenzyme analysis	Less than that of antigen or PCR tests	Gold standard	<25
Antigen detection (ELISA) in:			
Stool	>95	>95	Usually poor
Serum	65 (early)	>90	~13 (late), ~100 (first 3 days)
Abscess fluid	NA	NA	~100 (before treatment)
Antigen detection in saliva (ELISA)	ND[c]	ND	70
PCR (stool)	>70	>90	ND
Antibody detection in serum (ELISA)	>90	>85	70–80 (acute phase), >90 (convalescent phase)

[a]Data are from references 29 and 48.
[b]NA, not available.
[c]ND, not done.

isolates, whereas fast-migrating bands show the presence of *E. histolytica* isolates. In one study of German travelers from tropical and subtropical regions, 18 of 60 subjects were found to be infected with *E. histolytica* by using hexokinase isoenzymes. This was confirmed by PCR analysis in 16 of the cases, with PCR analysis not performed in 2 cases (67).

The utility of zymodeme analysis is illustrated in a second study from South Africa, which revealed that *E. dispar* does not cause seropositivity. A total of 94 to 100% of infected patients with pathogenic zymodemes (*E. histolytica*) were strongly seropositive, compared to 2 to 4% of subjects with nonpathogenic zymodemes (*E. dispar*) (32).

In a series of studies in Dhaka, Bangladesh, Haque et al. found excellent agreement in the sensitivity and specificity of *E. histolytica* detection between cultures with or without zymodeme analysis, PCR, and antigen detection (23, 24, 26, 27).

Antigen Detection

Differentiation of *E. histolytica* from *E. dispar* can also, and perhaps most practically, be accomplished by antigen detection. Currently there are several antigen detection tests commercially available for in vitro diagnostic use. Most of these tests do not distinguish *E. histolytica* from *E. dispar*, but the TechLab *E. histolytica* II test detects solely *E. histolytica* (26).

Antigen Detection in Stool

Several kits marketed for antigenic diagnosis of *E. histolytica* are currently available. The ideal antigen detection test must be highly sensitive and specific, especially in early infection. Currently, commercial ELISA-based kits and their specificity are the Merlin Optimum S antigen detection kit, which has been reported to detect the serine-rich *E. histolytica* protein (SREHP); the Alexon kit (ELISA), which utilizes anti-*E. histolytica* polyclonal antibodies, which do not differentiate between *E. histolytica* and *E. dispar* (40); and the ProSpecT ELISA, which detects *E. histolytica* and *E. dispar* but cannot distinguish pathogen from nonpathogen (50).

TechLab produces the *Entamoeba* test (which detects the Gal-GalNAc lectin of both *E. histolytica* and *E. dispar*), and the *E. histolytica* II test (which detects the Gal-GalNAc lectin of *E. histolytica* but not *E. dispar*). The *E. histolytica* II kit is more sensitive than the first-generation TechLab *E. histolytica* antigen detection kit (26).

The TechLab kits that specifically detect *E. histolytica* in stool specimens make use of four epitopes distinct for *E. histolytica* on the 170-kDa subunit of the galactose-inhibitable adherence lectin, which also contains at least two epitopes found in both *E. dispar* and *E. histolytica* (49). This lectin, an important surface adhesin, is highly conserved in *E. histolytica* strains isolated throughout the world.

Culture and zymodeme analysis were compared to the TechLab *E. histolytica* II test for stool specimens from 1,164 asymptomatic preschool children in Dhaka, Bangladesh. Culture and zymodeme analysis identified *E. histolytica* infection in 16 children. All 16 were also positive by the *E. histolytica* II test. In addition, the antigen detection test identified 34 additional *E. histolytica* infections missed by culture. All 50 stool specimens positive by antigen detection were also tested by

PCR, which identified *E. histolytica* in 43 of 50 specimens. The authors concluded that the TechLab *E. histolytica* test was more sensitive than the previous "gold standard" of culture plus zymodeme analysis for the detection of *E. histolytica* infection (26).

Other studies from Egypt have confirmed that detection of fecal 170-kDa antigen is the preferred method for detection of *E. histolytica* and *E. dispar* (4).

Antigen Detection in Serum

Detection of the Gal-GalNAc lectin antigen in serum can be used to identify *E. histolytica* infection. The advantage of antigen detection in serum is multifold. It is a more sensitive method than detection of antilectin antibody for the early diagnosis of ALA (26). It is also more specific (1, 26) and uses a well-defined antigen, the Gal-GalNAc lectin (26, 39). Unlike antibody detection tests, it can also be used as a test of cure (26). A disadvantage of this method is that the sensitivity of this method is significantly decreased in ALA patients by 1 week after initiation of antiamebic therapy (3). Using the TechLab *E. histolytica* II test, Haque et al. detected *E. histolytica* in serum samples from 22 out of 23 ALA patients before treatment with the antiamebic drug metronidazole, a sensitivity of 95.7%. Testing following metronidazole treatment significantly decreased the detectability of Gal-GalNAc lectin in the sera of ALA patients, with only 10 to 75 patient specimens testing positive, a sensitivity of 13.3%. Detection of lectin antigen may be possible in liver abscess pus of ALA patients. Three out of three (100%) specimens without prior metronidazole treatment and 8 of 24 (33.3%) specimens from patients with prior metronidazole treatment had detectable levels of lectin antigen (26).

Salivary antigen has also been tested as a predictor for invasive disease. It was found that assay for the presence of lectin in saliva had moderate sensitivity (65.8%) and high specificity (97.4%) in early infections (<1 week of amebic colitis) (3). The sensitivity of this assay appears to be lower than that of antigen detection in serum.

PCR

The main advantage of PCR amplification and detection of small rRNA genes or other *E. histolytica*-specific DNA sequences is the potential for improved sensitivity over other methods. However, the presence of inhibitors of the PCR in stool has to date offset this potential advantage, with PCR proving to be less sensitive in our hands than antigen detection for identification of *E. histolytica* in stool (26).

Another advantage of PCR is its application to formalin-ether-sedimented samples. The DNA equivalent of five cysts was enough to act as a template for detection by PCR (54). PCR is also useful for the detection of *E. histolytica* in abscess fluids from liver and brain (47, 64). Even in the samples from patients with prior treatment with metronidazole, 12 of 12 liver abscesses scored positive (64).

PCR-based methods are extremely powerful tools for genetically typing different amoebic strains (40). However, the expense and relatively sophisticated technology required for PCR to date have made it ill suited for use in developing countries where amebiasis is prevalent.

Antibody Detection: Serology

Several serologic techniques (e.g., ELISA, indirect hemagglutination assay [IHA], and latex agglutination [LA] assay) are a useful adjunct in the diagnosis of amebiasis and useful in epidemiologic studies. However, some serologic assays are less sensitive than antigen detection in early infections. Because serologies remain positive for months to years after exposure to the parasite, serology is not a specific marker for acute infection in all cases.

An easy-to-use and inexpensive method of blood antibody detection is IHA (9, 56). This assay is useful for screening large numbers of samples; however, its low sensitivity and specificity can lead to a high number of false positives (compared to ELISA) if this assay is used alone (56).

Anti-lectin salivary immunoglobulin A (IgA) ELISA can be used to detect a secretory immune response to *E. histolytica* in both infected asymptomatic children and seropositive children. Specific anti-lectin IgA reactivity in saliva appears to be induced by *E. histolytica* infection (25).

Detection of serum IgA antibodies might prove useful for diagnosis of amebiasis, since these antibodies were found in 83.8% of amebic colitis patients. Interestingly, salivary IgA titers were found to be significantly higher in the same patients (5). In another study, salivary sIgA levels in patients with ALA were determined to be higher than those in patients with nonamebic diseases and healthy persons (33).

Anti-lectin IgM was found in one study to be present in 2.8% of uninfected controls, 13.4% of asymptomatic carriers, 55% of colitis patients, and 77% of ALA patients. The sensitivity of measuring lectin IgM levels was 55%, with a specificity of 97.2%. This assay was more sensitive in patients with colitis that had longer than a week (79.3%) but less sensitive than antigen detection (2).

In another study, 143 sera were examined by each of the three tests (ELISA, IHA, and LA assay) to determine the utility of ELISA. Study sera included sera from patients with hepatic amebiasis who underwent clinical and ultrasound examinations (43 sera), sera from patients with serologically proven hepatic diseases with different infectious causes (33 sera), and sera from control healthy people (67 sera). According to evaluation results of the three tests, IHA had the highest sensitivity, with 97.6%, while ELISA had the highest specificity, with 100% (36).

In another study comparing ELISA and LA assay, 167 serum samples collected from 76 patients with invasive amebiasis and 91 control subjects were studied by three tests: soluble-antigen ELISA (soluble *E. histolytica* extract), P1-ELISA (single recombinant *E. histolytica* protein), and membrane LA assay (*E. histolytica* membrane fraction latex aggregate). The best results were obtained with the membrane LA assay and P1-ELISA, with sensitivities of 96.1 and 86.8% respectively, and specificities of 92.3 and 96.7%. As these screening assays are rapid and easy to perform, they may be a useful tool for serodiagnosis of invasive amebiasis (38).

The potential value of amebiasis diagnostic tests based on recombinant fragments of SREHP or the 170-kDa heavy subunit of the Gal-GalNAc lectin in areas of high background seropositivity is illustrated by a study in which seropositivity to recombinant *E. histolytica* antigens of ALA patients was evaluated by three

methods: a SREHP-maltose binding protein-based test, a 170-kDa lectin CR fragment–glutathione-*S*-transferase (GST)-based test, and the IHA. Within the first week, 70% of patients tested as reactive when the SREHP-maltose binding protein-based test was used, 85% of patients tested as reactive when the 170 CR-GST-based test was used, and 100% of patients tested as reactive by IHA. Virtually all (19 of 20) patients whose titers of *E. histolytica* antigen were ≥1:512 were seropositive by IHA. After 180 days, none of the samples were seropositive by SREHP, and only three were seropositive by 170 CR-GST. The authors concluded that serologic tests based on recombinant antigens had a greater ability to distinguish recent from past infections (59).

Riboprinting

A useful tool for the rapid identification and assessment of relatedness between species in a broad range of organisms, riboprinting allows us to discriminate among closely related species of *Entamoeba* by restriction enzyme site polymorphisms in PCR-amplified small-subunit rRNA genes (13). It has revealed unsuspected variation in classically defined *Entamoeba* species and has helped support the validity of other species and species groups previously defined by morphology alone (12). The application of riboprinting techniques may therefore provide a better understanding of the epidemiology of amebiasis, including different modes of transmission.

Strain-Specific Identification

Strain-specific identification is important to ascertain if virulence, hepatotropism, or immunity differs for different *E. histolytica* strains. Stable polymorphisms in several *E. histolytica* genes allow strain-specific identification (11, 71).

In one study, authors detected both size and restriction site polymorphisms among the isolates within an area of endemicity in Bangladesh. After a combination of the nested PCR results and *Alu*I digestion of the PCR products of the SREHP gene, 34 distinct patterns in 54 clinical isolates were identified, demonstrating that polymorphism is extensive within a single region. Also, it has been shown that there is genetic diversity within *E. histolytica* isolates from a population with endemic infection, as reflected in SREHP gene polymorphism (5a, 20).

APPLICATIONS OF *E. HISTOLYTICA*-SPECIFIC TESTS TO EPIDEMIOLOGY

Due to the recent discovery that *E. histolytica* could be divided into two species, *E. histolytica* and *E. dispar*, there are few prevalence studies of *E. histolytica* alone. This number is further reduced because not all of the reportedly specific tests are actually specific (S. L. Reed, Editorial response, *Clin. Infect. Dis.* **30:**959–961, 2000). Therefore, there is not a good picture of the prevalence of *E. histolytica*, as current reports vary greatly depending on the methods employed in the studies and the characteristics of the group being used for the study (e.g., age, living conditions,

and prevalence of other infections). In this section we will review the fragmentary knowledge of the prevalence of *E. histolytica* in developing and developed nations.

Developing Countries

Since many people in developing countries suffer from poor living conditions with unsafe drinking water and food, prevalence of infection is high. In these countries, rapid, simple, quantitative, and highly sensitive and specific diagnostic methods are useful and important for clinicians due to difficulties in clinical diagnosis of amebiasis. Sometimes both microscopy and culture underestimate the prevalence of *E. histolytica* and *E. dispar* infection (24), and sometimes these techniques overestimate and overdiagnose (15). Amebic culture and isoenzyme analysis are highly accurate methods which are of special value in distinguishing *E. histolytica* from *E. dispar*; however, they are time-consuming and impractical for routine diagnostic laboratories (27, 50).

The length of time for which precipitating antibodies persist following *E. histolytica* infection can be estimated from the results of a longitudinal survey (5-year study) in which 100% of ALA patients were found to be seropositive at enrollment in South Africa. After 3 years, 40% remained seropositive. A 5-year study revealed that annual seropositivity ranged between 10.4 and 17.6%, while the annual prevalence of *E. histolytica* infection ranged between 2.1 and 3.5%. The annual prevalence of *E. dispar* was 6.6 to 9.1% (31).

Braga et al. utilized several methods to assess the prevalence of *E. histolytica* in a slum community in Fortaleza, Brazil (8). Overall, 8.0% of the stool samples were found microscopically to contain *E. histolytica*, 10.6% of subjects tested positive using the *E. histolytica* antigen detection test (TechLab), and 19.6% of subjects obtained a positive result with the *Entamoeba* kit, which detects both *E. dispar* and *E. histolytica* (TechLab). Colonization with *E. histolytica* increased with age, with 8.5% of 1- to 5-year-old children colonized and 13.7% of individuals over the age of 45 years colonized. An ELISA performed to detect the presence of anti-GalNAc lectin IgG antibodies revealed a seroprevalance of 19.7% among all groups. When this test was performed on individuals colonized with *E. histolytica*, 19.5% were seropositive, as were 25% of individuals colonized with either *E. dispar* or *E. histolytica* and 18.2% of the individuals whose stools did not contain *Entamoeba*. Seropositivity and stool colonization with either of the *Entamoeba* species exhibited no correlation (8).

In the Pernambuco region of northeast Brazil, 47 of 47 isolates obtained in five locations were identified as *E. dispar* by zymodeme analysis and by PCR followed by restriction enzyme digestion (65). In another study in Recife, Pernambuco, Brazil, 87 of 634 individuals were *E. histolytica-E. dispar* positive as shown by stool examination, but only 1 of 558 was seropositive as shown by gel diffusion precipitin test, suggesting that this region is endemic for *E. dispar* infection (34). Zymodeme analysis revealed that five (9.3%) of 54 isolates were *E. histolytica* in Manaus and Belem. The other 49 isolates were *E. dispar*. The prevalence of *E. histolytica* and *E. dispar* seems to differ in regions of northern and northeastern Brazil (44).

Infections with *E. histolytica* (as determined by zymodeme analysis) occurred in family units and closely related individuals in the Durban area of South Africa (18). A study of the prevalence of *E. histolytica* in a semirural area of endemicity south of Durban found that microscopy yielded a prevalence approximately four times lower than that obtained by culture. One percent of healthy individuals were found to harbor *E. histolytica* (by zymodeme analysis), and the prevalence of *E. histolytica* was about 10%. Females, especially those in the 46- to 50-year age group, were found to be infected in greater numbers than males, although frequently zymodeme analysis revealed *E. dispar* (nonpathogenic types I and III) in females (17).

In rural Equatorial Guinea, a survey by Roche and Benito found that *E. histolytica-E. dispar* was the most common protozoan parasite in stool as shown by microscopic examination. The prevalence of this parasite (*E. histolytica-E. dispar*) increased with age. A correlation between positive stool specimens and the quality of drinking water treatment existed. Unlike that of *Giardia lamblia*, the presence of *E. histolytica* was found to significantly correlate with diarrhea (55).

Overall prevalence of asymptomatic colonization with *E. histolytica-E. dispar* complex in Dhaka, Bangladesh, was found to be 8.0% as determined by antigen detection with the *Entamoeba* test and the *Entamoeba histolytica* test (TechLab). Using microscopy, the prevalence was found to be 5%, and culture yielded a value of 4.2%. When investigating asymptomatic infection, it was revealed that the majority of subjects (21 children) were infected with *E. dispar* (zymodeme I), while 8 children were infected with *E. histolytica* (zymodeme II). The occurrence of *E. histolytica-E. dispar* complex infection in children less than 3 years old was uncommon. The prevalence in both sexes was found to be similar as determined by three different techniques—microscopy, culture, and the antigen detection test—with female-to-male prevalence ratios of 0.7, 1.0, and 0.9, respectively (24).

In a prospective study in Dhaka, Bangladesh, of the prevalence of *E. histolytica* and *E. dispar* in asymptomatic preschool children 24 to 60 months old, with a median age of 19 months, the prevalence as determined by *Entamoeba* antigen detection (TechLab ELISA) was found to be 4.7% for *E. histolytica* and 12.6% for *E. dispar*. Similar to previous results, the rates of prevalence of both sexes were similar, with a ratio of 0.9 (as determined by antigen detection) (23).

In 1,872 individuals from 14 communities in the northern Philippines, 137 cases (7.3%) of *E. dispar* and 18 cases (0.96%) of *E. histolytica* were detected by PCR using DNA extracted from formalin-fixed stool samples. The 5- to 14-year-old age group was the most affected. There was no significant difference in the sex distribution of *E. histolytica*, while *E. dispar* had a higher prevalence in females (9.2%) than in males (5.7%) ($P < 0.01$) (53).

IHA was used to detect the presence of serum antibodies to *E. histolytica* in a representative sample of the Mexican population. Analysis of 67,668 serum samples revealed an 8.4% prevalence of anti-*E. histolytica* antibodies. A decreasing trend in seroprevalence was observed with increasing age, from 11.0% in the 5- to 9-year-old age group to 5.2% in the 45- to 49-year-old age group. Females had a slightly higher seroprevalance than males, reported as 9.3% and 7.1%, respectively (9).

Another study demonstrated the accuracy of detection of antibody in serum in the diagnosis of *E. histolytica* infection by using it in conjunction with microscopy and ELISA of stool specimens. Braga et al. (7) tested a representative sample of the population of a northeastern Brazil slum, an area with highly endemic *E. histolytica*. Of 14 stool samples identified as *E. histolytica-E. dispar* by microscopy, 4 samples were identified as *E. histolytica* upon Gal-GalNAc lectin ELISA of the stool sample, with the remaining 10 identified as *E. dispar*. Sera of the 14 patients were subjected to Gal-GalNAc lectin ELISA, and three of the subjects with *E. histolytica*-positive stool samples were seropositive. Only one of the subjects with an *E. dispar*-positive stool sample was seropositive for *E. histolytica* (7).

Serum antibodies against *E. histolytica* were detected by ELISA prepared from axenic culture of *E. histolytica* HM1-1MSS in 60,538 serum samples obtained from a representative sample of the Mexican population. A male-to-female seropositivity ratio of 1:1.25 was detected, with males comprising 24,887 of the subjects at 3.9% seropositivity and females comprising 33,371 of the subjects at 4.9% seropositivity. Children under the age of 10 years had the lowest prevalence of seropositivity (3.5%). Increasing population density correlated with an increase in seropositivity, with prevalence rates in metropolitan, urban, and rural areas at 5.2%, 4.6%, and 3.7%, respectively (21). When only one technique, IHA in the following example, is used, there is a chance that seropositivity will be overreported. Sánchez-Guillén et al. used IHA to determine seroprevalence in donors selected from areas with various population densities in the state of Puebla in Mexico, and they found 8.6% of the subjects to be seropositive. However, when both ELISA and IHA were used, this value decreased to 6.4% (56).

In another study, 31 stool specimens (33%) of 94 schoolchildren (age range, 7 to 14 years) in Durban, South Africa, were positive for *E. histolytica* by isoenzyme analysis. Only one of these produced a pathogenic zymodeme pattern (zymodeme II) (57).

Industrialized Nations

Men who have sex with men in North America exhibit an increased prevalence of *E. dispar* infection due to fecal-oral transmission. A total of 120 specimens (92 from British Columbia, Canada, and 28 from Ontario, Canada) that were identified as *E. histolytica-E. dispar* microscopically were analyzed by zymodemes. This study contained samples from the following groups: homosexuals (34 isolates), heterosexuals (63 isolates), children under the age of 15 years (nine isolates), and subjects of unknown sexual orientation (14 isolates). The zymodeme distribution of the 120 isolates was as follows: 78 isolates contained zymodeme I (*E. dispar*), 7 isolates contained zymodeme II (*E. histolytica*), 22 isolates contained zymodeme III (*E. dispar*), 2 isolates contained zymodeme XIV (*E. histolytica*), 8 isolates contained zymodeme XVII (*E. dispar*), 2 isolates contained zymodeme XVIII (*E. dispar*), and one isolate contained zymodeme XXI (*E. dispar*). *E. histolytica* only, and not *E. dispar*, was found in eight heterosexual subjects and one subject whose sexual orientation was unknown (51).

Twenty-five patients out of twenty-five were found to be seropositive for *E. histolytica* by the gel diffusion precipitin test, complement fixation test, IHA, indirect fluorescent-antibody assay (IFA), or ELISA at the Tokyo Metropolitan Bokutoh General Hospital, Tokyo, Japan. Of these patients, 11 had colitis, 9 had liver abscess, 4 had colitis with liver abscess, and 1 had liver abscess with brain abscess. Zymodeme types of *E. histolytica* isolated from four colitis patients were *E. histolytica* zymodeme II (three patients) and *E. histolytica* zymodeme XIV (one patient) (46).

Institutionalized individuals in developed nations are at risk for amebiasis. Of 77 mentally retarded patients hospitalized with diarrhea in one of Italy's psychiatric institutions examined, 26 (33.7%) had *E. histolytica* and/or *E. dispar* in their stool specimens. Specimens from 16 of these subjects were positive on Robinson medium for *Entamoeba* spp., and 11 of these cultures were subjected to isoenzyme analysis. All isolates had *E. histolytica* pathogen zymodemes (zymodemes II, XII, and XIV). All the patients were identified as *E. histolytica* positive. However, seven patients had higher antibody titers (>1/400) as shown by IFA (19).

In another study from Japan, 13% of the mentally retarded patients (78 of 620) had *E. histolytica* cysts or trophozoites present in their stools as seen by microscopic examination. In addition, 164 (26.5%) of these patients were seropositive by IFA. However, to assess the status of amebiasis, zymodeme studies of isolated amebae were performed. Nine of the 10 amebic isolates obtained from cyst carriers had the same *E. histolytica* pathogenic zymodeme type (zymodeme II), and only one isolate had an *E. dispar* nonpathogenic zymodeme (zymodeme I) (41).

Another study of an *E. histolytica* outbreak in Japan revealed that among mentally retarded patients, a statistically significant age-related difference in rates of seropositivity existed. While the overall prevalence of seropositivity was 37.9% (72 of 190) by IFA, seronegative boys were 18.6 ± 6.0 years of age and seropositive boys were 16.2 ± 5.0 years of age. This trend was more apparent when stool examinations were used as a diagnostic test. Twenty percent (38 of 190) of subjects were stool positive, with the mean age of stool-positive boys being 15.6 ± 3.4 years and the mean age of stool-negative boys being 19.1 ± 6.5 years ($P < 0.02$). No statistically significant difference in rates of infection between boys and girls was observed. High rates of *E. histolytica* infection in the mentally retarded were associated with idiosyncratic activities including pica and fecal play (42).

A random serosurvey of patients found that antibodies to *E. histolytica* were significantly more common in the sera of mentally retarded patients than in the sera of other patient groups, including geriatric and psychotic patients (37).

The prevalence of *E. histolytica* among 565 institutionalized psychiatric patients, of whom 61.6% were male and 38.9% were female, was 2.0% in three hospitals in northern Taiwan. Infection was most common between the ages of 17 and 65 years. Five of six patients who were positive for *E. histolytica-E. dispar* microscopically were positive for *E. histolytica* antigen in stool by ELISA (ProSpecT). Eleven patients who did not have amebiasis as seen by parasitological examination and ELISA of stool were found to be seropositive for *E. histolytica*. One cyst carrier without *E. histolytica* stool antigen was shown to be seropositive with an Amebiasis Serology Microwell ELISA kit (LMD Laboratories Inc.). A positive finding was

obtained for two patients by all three techniques (parasitological examination, ELISA of stool, and ELISA of blood) (10).

Twenty-seven samples with a pathogenic zymodeme (*E. histolytica*) were found among 50 amebic isolates obtained from seropositive asymptomatic cyst carriers in institutions for the mentally retarded in Japan (63). As in Europe (19), interestingly all the samples were isolated as *E. histolytica* in this study (63). These results support the idea that *E. histolytica* infections are a problem among institutionalized people.

In Japan *E. histolytica* is common in homosexual men (35, 45, 46). A total of 25 human immunodeficiency virus-infected individuals, including 22 homosexual or bisexual males, were diagnosed as positive for *E. histolytica-E. dispar* at the Tokyo Metropolitan Komagome Hospital (G. Masuda, A. Ajisawa, M. Negishi, A. Imamura, H. Tachibana, T. Takeuchi, and M. Saito, Presentation, IXth Int. Congr. Parasitol, 1998). Their medical problems were 9 cases of colitis, 14 cases of liver abscess with or without colitis, and two instances of being asymptomatic cyst passers. Two AIDS patients presented with serious clinical manifestations; one had ALA complicated with a subcutaneous amebic abscess, and the other had amebic colitis with an advanced amebic perianal abscess. In another hospital, 41 (24%) of 169 human immunodeficiency virus-infected male homosexuals were positive serologically for *E. histolytica.*

E. dispar was detected by PCR in stool samples from 41 of 101 (42.6%) wild and 84 of 133 (63.2%) captive macaques in Japan, while *E. histolytica* was not detected, suggesting that the amebae of macaques are not the source of human infection in Japan (52, 60). Subtyping analysis will make it possible to clarify the origin of *E. histolytica* distributed in Japan.

Even a short trip to tropical or subtropical countries of endemicity is sufficient for amebiasis to occur. One study investigated 105 cases of travelers returning from regions of endemicity and residents of those regions, with infection with *E. histolytica* and/or *E. dispar* identified by microscopic detection. Twenty-one of 60 isolates were identified as *E. histolytica* by PCR and hexokinase isoenzyme, including isolates from 13 of 26 infected patients with amebiasis-like symptoms. Fifteen of these patients were serologically positive for antibodies to *E. histolytica* by ELISA. The remaining 45 isolates had a nonpathogenic zymodeme (67).

In another study, IFA and ELISA were used to determine the prevalence of *E. histolytica* in German travelers and male homosexuals. One hundred nine subjects (4.0%) out of 2,700 travelers returning from the tropics had microscopically observed *E. histolytica-E. dispar.* The prevalence of *E. histolytica* was 16.3% (52 of 320) in homosexual men. The mean ages of the two groups were 34 (range, 18 to 63) years and 36 (range, 22 to 54) years, respectively. Zymodeme analyses were performed for 61 travelers and 26 homosexual men. Five of the traveler subjects' isolates contained a pathogenic zymodeme that correlated serologically to antibodies to *E. histolytica* upon IFA and ELISA. Zymodemes detected in these cases were XIX (four cases) and II (one case). Only three of the five infected travelers were seropositive (69). So far, these studies have concluded that amebiasis is a not uncommon intestinal protozoan disease of short- or long-term travelers, particularly those visiting areas of endemicity.

The other important group to affect the epidemiology of amebiasis in the United States is the immigrant population, especially Hispanic and Pacific Islander immigrants and Indochinese refugees. Most cases of amebiasis in the United States reported to the Centers for Disease Control and Prevention have been in immigrants (28).

CONCLUSIONS

We are left with a situation where groups at risk for amebiasis have been identified, but data on prevalence of infection and disease are at best incomplete. The wider application of the new generations of diagnostic tests specific for *E. histolytica* should provide opportunities to address these issues in the near future.

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Chapter 13

Bioterrorism: a Real Modern Threat

Michael T. Osterholm

The use of microbial agents as intentional weapons against humans, animals, and plants goes back to earliest recorded history. However, at no time in history have the potential consequences of microbial agents' possible use been more catastrophic. Today the use of biologic weapons has as much potential to cause massive casualties with a single event as a nuclear weapon, if not more. For example, the Office of Technology Assessment calculated that 100 kg of anthrax spores spread over Washington, D.C., could kill from 1 million to 3 million people under the right weather conditions. In contrast, a 1-megaton nuclear warhead would kill from 750,000 to 1.9 million (11).

Throughout history, biologic agents have been used as weapons in the context of military action, thus leading to the concept of biologic warfare. However, today the highest likelihood for their use is against unsuspecting civilian populations, that is, for bioterrorism.

DEFINITION OF TERRORISM AND BIOTERRORISM

According to the yearly Federal Bureau of Investigation report titled *Terrorism in the United States*, (http://www.fbi.gov/publications/terror/terris.htm) "There is no single, universally accepted definition of terrorism. It is defined in the *Code of Federal Regulations* as '. . . the unlawful use of force and violence against persons or property to intimidate or coerce a government, the civilian population, or any segment thereof, in furtherance of political or social objectives'."

Bioterrorism is defined as the use of biologic agents to intentionally produce disease or intoxication in susceptible populations to meet terrorists' aims. The methods and motivations for such use vary; however, the common denominator is the potential for substantial morbidity and mortality and creation of the highest order of human panic.

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HISTORICAL PERSPECTIVE

Throughout history, epidemics have demonstrated the potential danger of uncontrolled infectious diseases. Despite our enormous advances in sanitation, anti-infective treatments, immunizations, and molecular technology, widespread panic is common when even limited outbreaks of serious infectious diseases occur in our communities. Recent examples of such reactions can be seen in association with the appearance of West Nile virus in the eastern United States and outbreaks of bacterial meningitis among college students. It is against this backdrop of human fear that the use of biologic agents must be considered (12).

Warfare with biologic weapons has been employed many times, dating back to earliest history. While preparing for naval battles against King Eumenes in 184 B.C., Hannibal ordered that earthen pots be filled with "serpents of every kind" and hurled onto the decks of enemy ships. In 1346, the Tartar army catapulted bodies of dead plague victims over the walls of Caffa (in modern Ukraine). An epidemic of plague followed as the Genoese mercenary forces retreated from the city.

Smallpox was used as a biologic weapon by the British army during the Pontiac Rebellion in 1763. They provided the Delaware Indians, a group loyal to the French, with blankets and handkerchiefs from the "Smallpox Hospital" at Fort Pitt. The subsequent outbreak of smallpox that occurred among the Indians was likely related.

By the 20th century, biologic warfare had become a science. The Germans developed and used a number of animal and human pathogens in World War I; it is unclear how successful these efforts were in causing disease. During World War II, the Japanese military used biologic agents in the Soviet Union, Mongolia, and China. Japan's Imperial Unit 371 used at least 3,000 prisoners of war as guinea pigs for biologic weapons testing. It is estimated that more than 1,000 of these prisoners died in experiments with pathogens causing anthrax, botulism, brucellosis, cholera, dysentery, meningococcal infection, and plague.

Following World War II, biologic weapons programs in the United States, Canada, the Soviet Union, and the United Kingdom continued to expand until the late 1960s. The U.S. government officially ended its weapons program in 1969. The United Kingdom and Canada ended their programs shortly thereafter. In 1972, the Biological and Toxin Weapons Convention was signed and ratified by 140 nations. Despite signing that document, the Soviet Union began a massive buildup of its biologic weapons capabilities through the late 1980s. At least 10 other nations also expanded their efforts in this area during this time. The implications of the former Soviet program are far-reaching, as there is clear evidence that both former researchers and the weapons they developed are in the hands of other nations and terrorist groups (1, 6, 12).

Today, the prospect of biologic warfare is still a concern; however, the greater risk is bioterrorism against unsuspecting civilians.

CRITICAL ELEMENTS NECESSARY FOR A BIOTERRORISM EVENT

There are three critical elements necessary for a bioterrorism event to occur; potential perpetrators, availability of biologic agents, and technical means to dis-

seminate these agents. If any one of these elements is missing, an event will not occur. However, their presence does not guarantee that an attempted attack will be successful. With the recent developments in aerosol particle technology, the increased access to high-level training in microbiology, and the rapidly changing landscape of world politics, many believe success is more likely.

Potential Perpetrators

What sort of person or group would actually carry out a bioterrorism attack? Unfortunately, there are numerous possibilities, making it difficult for international or domestic intelligence and law enforcement agencies to intercept such plans in advance. We must be prepared for a wide range of potential perpetrators who have an equally wide capability to produce and disseminate a variety of infectious agents.

Today, there is a general belief among many professionals and the general public that bioterrorism will not happen in the United States. This belief is related to several factors, including the opinions that (i) biologic weapons have seldom been used among civilian populations, (ii) their use is morally repugnant and therefore unlikely, (iii) the propagation of pathogens and their dissemination among the civilian population is technologically difficult, and (iv) the concept of a nuclear winter-like biologic terrorism event is unthinkable and thus dismissed. However, the necessary expertise and potential motivation to commit a bioterrorist act has been documented among various levels of terrorist organizations. The potential for bioterrorism can no longer be dismissed (12).

There are at least six categories within the spectrum of potential perpetrators who are capable of and willing to conduct a bioterrorism attack: state-sponsored groups, insurgent rebels, doomsday/cult-type groups, nonaligned terrorists, splinter groups, and lone offenders.

Today, at least a dozen nations either possess or are actively pursuing offensive biologic programs (3). Between 1970 and 1990, the Soviet Union developed an extensive arsenal of biologic weapons and considerable expertise in their development and dissemination (1). With the breakup of the Soviet Union, and specifically the bioweapons program, it is highly likely that there has been widespread dissemination of the knowledge necessary to create such weapons to other potential terrorist groups and individuals. In addition, the materials needed to construct bioweapons are relatively inexpensive and readily available. The potential for subnational and individual terrorists to obtain the technical expertise and materials to carry out a bioterrorism attack is real (12).

Potential Agents

For any weapon to be considered effective and usable, it must have four main attributes. First, it must be within the economic and practical means of the perpetrator(s). Second, the weapon must be capable of reaching the intended target. Third, it must cause limited collateral damage, in particular to those staging the attack. And finally, use of the weapon must result in the desired outcome, usually death. No other weapon created by even the richest and most technologically sophisticated countries can match infectious agents in each of these categories.

Characteristics of the Ideal Agent

Many known infectious agents cause disease in humans, animals, and plants, although only a very limited number of agents are effective as weapons against humans. In addition, there are unique biologic weapons that can be effective against animals or plants only.

An ideal potential bioterrorism agent has the following six characteristics: it is inexpensive and easy to produce; can be aerosolized (1 to 10 μm diameter); survives sunlight, drying, and heat; causes lethal or disabling disease; and results in person-to-person transmission; and either it has no effective treatment or prophylaxis or none is available.

Agent Classification

On the basis of the above characteristics, several groups have devised lists of biologic warfare or terrorism "threat agents." The North Atlantic Treaty Organization *Handbook on the Medical Aspects of NBC Defensive Operations*, which addresses potential warfare, lists 31 pathogens. However, only a limited number can be grown and dispersed effectively to cause a large number of serious illnesses. In 1994, a group of former Russian biologic warfare experts disclosed that they had worked with 11 such agents. The four agents highest on their list were those causing smallpox, plague, anthrax, and botulism. In 1998, the Johns Hopkins Center for Civilian Biodefense Studies considered potential agents that present the greatest risk for transmission, and have the ability to infect large numbers of civilians and cause death. They concluded that smallpox and anthrax had the highest risk potential. Other diseases of serious concern were plague, botulism, tularemia, and viral hemorrhagic fever. In 1999, the Centers for Disease Control and Prevention (CDC) convened a group of experts to determine the critical biologic agents for use in bioterrorism (9). The CDC effort identified three categories (A, B, and C) of potential biologic agents (Table 1).

Means of Agent Dissemination

A delivery system for biologic agents can be as simple as a juice bottle or a vial with dried anthrax spores thrown against a wall to break and release the dried powder. However, it can also be much more. At the same time that the broad expansion of biomedical training has created a large population of medical scientists capable of cultivating pathogens, advances in other fields such as aerosol particle technology have created numerous possibilities for dual-use technologies that can be inexpensive dissemination weapons. The advances in aerosolized particle technology pose new and substantial possibilities and thus increase the risk of an at least moderately successful bioterrorist attack (12).

A biologic agent can be introduced into the human body by several routes: aerosol (the inhalational route), ingestion of contaminated food or water (the oral route), injection through the skin (the percutaneous route), or absorption through or placement on the skin (the dermal route).

Aerosol Inhalation

The aerosol inhalational route of entry into the body is the most important means of transmission for a majority of the most virulent and pathogenic biologic weap-

Table 1. CDC bioterrorism agent classification system

Category A	Category B	Category C
High-priority agents include organisms that: • can be easily disseminated or transmitted from person to person • cause high mortality and have the potential for major public health impact • might cause public panic and social disruption • require special action for public health preparedness Category A agents include: • Variola major (smallpox) virus • *Bacillus anthracis* (anthrax) • *Yersinia pestis* (plague) • *Clostridium botulinum* toxin (botulism) • *Francisella tularensis* (tularemia) • filoviruses (Ebola virus [Ebola hemorrhagic fever], Marburg virus [Marburg hemorrhagic fever]) • arenaviruses (Lassa virus [Lassa fever], Junin virus [Argentine hemorrhagic fever])	Second-highest-priority agents include those that: • are moderately easy to disseminate • cause moderate morbidity and low mortality • require specific enhancements of CDC's diagnostic capacity and disease surveillance Category B agents include but are not limited to: • *Coxiella burnetii* (Q fever) • *Brucella* species (brucellosis) • *Burkholderia mallei* (glanders) • alphaviruses (Venezuelan encephalomyelitis virus, eastern and western equine encephalomyelitis viruses) • ricin toxin from *Ricinus communis* (castor beans) • epsilon toxin of *Clostridium perfringens* • *Staphylococcus* enterotoxin B A subset of category B agents includes patholgens that are spread by food or water. These patholgens include but are not limited to: • *Salmonella* species • *Shigella dysenteriae* • *Escherichia coli* O157:H7 • *Vibrio cholerae* • *Cryptosporidium parvum*	Third-highest-priority agents include emerging pathogens that could be engineered for mass dissemination in the future because of: • availability • ease of production and dissemination • potential for high morbidity and mortality and major health impact Category C agents include but are not limited to: • Nipah virus • hantaviruses • tick-borne hemorrhagic fever viruses • tick-borne encephalitis viruses • yellow fever virus • multidrug-resistant *Mycobacterium tuberculosis*

ons. To maximize the potential impact of the aerosol route of agent delivery, the agent should be of a particle size that can be carried for long distances by prevailing winds or indoor ventilation systems and can be inhaled deeply into the lungs of the intended victims. In this regard, the ideal particle size is 1 to 5 μm in diameter. Particles larger than this will either settle out onto the ground or be filtered out in the upper respiratory tract of individuals who inhale them. Since particles of this size are typically not detected by persons inhaling them, most agent releases, if unannounced, will go unnoticed. Biologic agents disseminated by the aerosol route can be delivered in either wet or dry form. Dried powders composed of very small particles tend to have better dissemination and have advantages in storage. However, production of dried-powder agents also requires an increased level of technological sophistication.

Equipment to disperse both wet and dry aerosols can be readily purchased on the Internet. Dozens of Web sites offer information on new and used crop-dusting planes and equipment that can be fitted to almost any plane or truck. Most of the equipment on these sites or from other commercial sources has nozzles that can set the droplet size precisely and produce a highly controlled wet spray. Dry powder devices, while less available, can still be purchased by a consumer. In addition, technology is readily available to enable someone to build an effective dispersing device the size of a heat thermostat box. One website even provides a guide to the area that one could expect to cover using various wet and dry particle sizes—from 1,000-μm particles that travel only 4 ft to 0.5-μm particles capable of drifting almost 400 miles (12). The knowhow to build or obtain state-of-the-art dissemination equipment has been greatly facilitated by the Internet.

Ingestion

To date, the only documented examples of multiple-victim bioterrorism events in the United States are related to the intentional contamination of food. The first U.S. case involving the use of a biologic agent occurred in 1984, when members of the Rajneeshee cult in The Dalles, Oreg., intentionally contaminated salad bars in area restaurants with *Salmonella enterica* serovar Typhimurium. More than 750 cases of illness were documented (13). The second episode of the use of a biologic agent in the United States to intentionally cause disease occurred in a Texas hospital. A disgruntled laboratory employee took cultures of *Shigella dysenteriae* from the hospital laboratory and contaminated muffins and donuts placed in the staff break room on a specific day. Severe acute diarrheal illness developed in 12 of 45 laboratory staff; *S. dysenteriae* was isolated from the stool in eight, and four were hospitalized. Because of the unusual cluster of illnesses among coworkers, an investigation was quickly launched. *S. dysenteriae* was found in uneaten muffins, and the genetic fingerprint analysis showed that it was identical to the *S. dysenteriae* found in the workers. The laboratory employee who committed the act was identified; she acknowledged that she intentionally contaminated the food items (10).

Fortunately, only a very few of the infectious agents that cause food or waterborne diseases are likely to make the average person severely ill. Nevertheless, public health officials must consider intentional contamination as a possible cause of any food or waterborne outbreak that occurs today.

PREPAREDNESS ACTIVITIES

A bioterrorist attack on U.S. civilians is both plausible and technologically doable. Even distinguished national commissions have concluded that such an attack is likely to occur (3). It is clear that the outcome of such an attack could be an epidemic with the potential to cause an unprecedented public health emergency. In addition, the panic and fear associated with this attack will be nationwide. Yet, there are currently many gaps in our bioterrorism preparedness activities. To date, most of the nation's resources made available through federal support have been directed toward the military or first responders (i.e., police and firefighters) at the state and local levels. Some of these individuals are the primary responders to explosive device or chemical terrorism acts; however, they would assume a secondary role in a bioterrorism event.

Historically, infectious disease outbreaks are first recognized and the core response occurs at the local level. State and local public health agencies, together with local health care organizations, hospitals, and other health care system partners, serve as the first, second, and third lines of response. Nonetheless, at the local level, only limited preparedness activities are taking place that include planning and drill exercises accurately reflective of the likely scenario of a biologic-agent-related outbreak and a coordinated public health and healthcare system response.

Each community must begin planning—as an entire community—for its response to a potential biologic terrorism event. This means leaders in public health, healthcare services, and medical and nursing communities, as well as the traditional first responders, which include police, fire, and other emergency management officials, must coordinate all activities. Preparedness planning must address 14 critical components (Table 2).

These critical components of bioterrorism planning activities will be carried out by multiple agencies and organizations at all levels of government as well as in the private sector. Since some components will require federal action before state or local officials can respond (e.g., the provision of adequate supplies of vaccines

Table 2. Critical components of bioterrorism planning activities[a]

Community-based surveillance and medical diagnosis, including laboratory support
Delivery of medical services
Infection control and occupational health
Epidemiologic investigation
Mass prophylaxis and immunization
Criminal investigation
Communications
Security
Fatality management
Residual hazard assessment mitigation
Emergency management operations and coordination
Legal issues
Resource and logistic support
Infrastructure support

[a] Data are from reference 4.

and anti-infective agents), officials at the local level must consider the sequence and priority of preparedness activities both with and without federal support. In other words, what do you do if federal support is—or is not—readily available? At a minimum, bioterrorism response plans must be developed and coordinated at the regional level rather than at individual hospitals, local health departments, or city governments. This is necessary because even a limited attack could involve hundreds to thousands of cases in addition to many more "worried well." Unless coordinated regional support is provided immediately, a single agency, institution, or government body will be quickly overwhelmed. Thus, a regional or statewide incident command system must be in place. (For additional discussion of the incident command system, see below.) This is likely to pose difficulties for most healthcare professionals, because they lack knowledge of such systems. In contrast, most traditional first responders base their command and control on the incident command system.

Recently, three groups have published documents to assist with federal, state, and local bioterrorism preparedness and response planning. Various elements of the above-listed critical components are addressed in these documents.

In April 1999, the Association for Professionals in Infection Control and Epidemiology (APIC) and the CDC developed the Bioterrorism Readiness Plan: a Template for Healthcare Facilities (5). As the authors state, "This document is not intended to provide an exhaustive reference on the topic of bioterrorism. Rather it is intended to serve as a tool for infection control (IC) professionals and healthcare epidemiologists to guide the development of practical and realistic response plans for their institutions in preparation for a real or suspected bioterrorism attack." This document does not address the need for the incident command system approach or how to respond using that approach. The guideline also lacks specificity regarding many critical components of bioterrorism planning activities.

In April 2000, the CDC produced a second document titled *Biological and Chemical Terrorism: Strategic Plan for Preparedness and Response* (2). This document provides a road map for the following focus areas that the CDC will address by 2004: preparedness and prevention, detection and surveillance, diagnosis and characterization of biologic and chemical agents, and response and communication. Few specifics are provided, making this document of only limited assistance to local and state authorities. In addition, the document combines preparedness and response planning for both biologic and chemical agents. Unfortunately, many current preparedness programs for biologic agent attacks are not comprehensive because they use the hazardous materials (HAZMAT) model as a planning framework for community response (14). The current CDC document is complicated in that virtually all infectious disease outbreaks (e.g., bioterrorism attacks) are handled by local or state health departments, while most chemical events (e.g., chemical attacks) are under the jurisdiction of HAZMAT teams, which are typically firefighter or police related. Effective responses to these very different events require separate training, separate medical service delivery plans, and even unique approaches to unresolved legal issues.

The third plan was produced by the U.S. Army Soldier and Biological Chemical Command in August 2000 (4). This is the most comprehensive and detailed of all

the federal planning documents produced to date. The critical components of bioterrorism planning activities are addressed. All planners of local and state preparedness and response activities should consider this document as they develop regional or statewide plans.

To date, the best opportunity to assess our nation's capacity to respond to a bioterrorism attack occurred in association with the May 2000 TOPOFF drill. This drill was mandated by Congress to test the readiness of federal, state, and local officials to respond to three separate events involving biologic, chemical, and radiologic agents. In short, the biologic event, which simulated the release of an aerosol of *Yersinia pestis* in Denver, demonstrated numerous and serious shortcomings in our current response capacity (7, 8). Exercises like this can be very instructive in regard to our preparedness and response shortcomings. However, unlike simulated acute events such as plane crashes or chemical terrorist attacks, biologic attacks will likely unfold over weeks or longer. Since drills are unlikely to last for more than several days, our future biologic terrorism response capabilities will not be challenged sufficiently by such exercises to fully understand our weaknesses.

CONCLUSIONS

The risk of a biological terrorism event in the United States in the near future is real. With recent developments in aerosol particle technology, increased access to high-level training in microbiology, and the rapidly changing landscape of world politics, we can no longer ignore the potential for such an event. To date, the medical and public health communities have had only limited input to our nation's terrorism preparedness and response activities. This must change, if we are to have any hope of minimizing the impact of a potential catastrophic event in the community involving the intentional release of a biologic agent. In addition, the need for stockpiles of specific vaccines, such as smallpox and anthrax, as well as appropriate antimicrobial agents, must be given the highest priority.

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Chapter 14

Bioterrorist Threats: What the Infectious Disease Community Should Know about Anthrax and Plague

Thomas V. Inglesby

The majority of this chapter addresses the following questions. If anthrax or plague were used as a biological weapon, what would be the expected epidemiology? What would be the clinical manifestations? How would a diagnosis be made? What is the recommended treatment? And what is the role of vaccination?

Before turning to these questions, I first briefly take up two other questions: Could anthrax or plague be used as a weapon? Would anthrax or plague be used as a biological weapon? I will argue that the answer to the first question is unquestionably yes and that the answer to the second question is quite possibly yes.

Anthrax and plague are widely and appropriately considered two of the most serious potential biological weapons. In the program of the former Soviet Union, anthrax and plague, with smallpox, were the three top-rated biological weapons. These three weapons earned the title of *strategic* weapons, weapons capable of destroying whole populations, a distinction also used to describe nuclear weapons (30). A World Health Organization analysis of 1970 also described the destructive capacity of anthrax and plague (34). The recently released Critical Agent list from the Centers for Disease Control and Prevention (CDC) also reaches the same conclusion (8). Perhaps the most grim report was published by the congressional Office of Technology Assessment in 1993. This is an unclassified report which concluded that if 100 kg of powdered anthrax was released upwind of Washington, D.C., the result could be the deaths of up to 300,000 to 3,000,000 people (26).

To underscore that this is not science fiction, here are a few more facts to consider. In a recent article describing the Iraqi biological weapons program, there is a photo of two 1,500-liter biological fermentors (36). They could have been used to make a variety of legitimate pharmaceutical products, but they were not. Ultimately, the Iraqi government admitted to making 8,000 liters of anthrax with a

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spore concentration of 1 billion spores per milliliter. Judy Miller and W. Broad, of the *New York Times*, recently published a photo of a bioweapon production building in Kazakhstan (24). In the former Soviet Union, during the height of bioweapon production, tonnage quantities of anthrax and plague and other biological agents were produced in this building (24). This facility was specially dedicated to the development of bacterial biological weapons; viral and toxin weapons were produced elsewhere. Inside of the building, a separate photo shows rows of smaller biofermentors lined up where these vast quantities of anthrax and plague were produced. This facility was shut down some time ago, but some suspect that biological weapons facilities in Russia still remain closed to Western scientists. There is much more to say about the threat of biological weapons, the availability of the technology, the explosive pace of change of biotechnology, the lack of effective arms control measures to prevent biological weapons, and other topics of great salience. These subjects, however, are beyond the scope of this chapter, so I now turn my attention to anthrax and plague.

ANTHRAX AS A BIOLOGICAL WEAPON

Before turning to anthrax as a biological weapon, a brief look at anthrax and its natural occurrence is in order. In 1880, John Henry Bell wrote in the *Lancet*: "Woolsorter's disease still continues severe and frequent as it ever was, overclouding the life of the sorter with a mysterious shadow and threatening him daily with death" (3). Anthrax is a disease caused by the bacterium *Bacillus anthracis*. Its association with black, coal-like skin lesions gives it its name *anthracis*, the Greek word for coal, from which it was derived. Outbreaks have appeared throughout human history, and it was a scourge among woolsorters at the beginning of the 20th century as well. It is also famous for its use in proving the germ theory of disease.

EPIDEMIOLOGY OF ANTHRAX

As a natural occurrence, the disease most commonly occurs in grazing animals. The largest outbreak on record is one in Iran in 1945, in which 1 million sheep died in an epizootic (20). Animal vaccination has now sharply lowered the occurrence of anthrax worldwide. Spores survive in a very harsh environment and are found in soil throughout the world. Anthrax is either sporadic, pandemic, or hyperendemic in most of the world. A number of outbreaks of anthrax have occurred among animals in the United States in the last decade. It is now a rare disease in humans (11). Fortunately, widespread herbivore vaccination and vaccination of persons at risk have led to this dramatic decline in the rate of the disease among humans. When anthrax does occur in humans, it follows exposure to animal products that are infected with anthrax. Most cases of naturally occurring anthrax are cutaneous anthrax, with 2,000 cases reported annually worldwide (4). If recognized, cutaneous anthrax can be treated quite successfully with a good outcome. Gastrointestinal anthrax is far less common but is still being reported in countries of the former Soviet Union, such as Kazakhstan. Inhalational anthrax is the least common

important manifestation of the naturally occurring form of this disease. There has been no case of inhalational anthrax in the United States in more than 20 years (11).

The epidemiology of anthrax as a biological weapon would be markedly distinct (Table 1). A number of the most important lessons regarding anthrax as a biological weapon are derived from the anthrax epidemic in Sverdlovsk, Russia, as described in a 1994 *Science* article by Meselson and colleagues (21). In Sverdlovsk, in 1979, a military facility northwest of the city accidentally released what was believed to be less than 1 g of anthrax spores into the open air. Some investigators say that it may have been as little as 100 mg of anthrax powder. Sixty-six deaths were officially reported, and there were deaths among persons who were exposed up to 4 km downwind of this release. There were deaths among animals up to 50 km downwind of the release. The majority of patients had disease onset in the 2 weeks following their aerosol exposure, but it is noteworthy that nearly one-third of the victims had symptom onset between 15 and 43 days after aerosol exposure. After one brief, small, accidental aerosol release, people began to develop symptoms of anthrax as many as 46 days later. The possibility of spore latency and subsequent disease has subsequently been well-documented in animal models (14).

PATHOGENESIS AND CLINICAL MANIFESTATIONS

Following their release as an anthrax aerosol, spores would be inhaled into the lung and then taken up by pulmonary macrophages. Macrophages would then be transported to regional lymph nodes, in this case, mediastinal lymph nodes. At

Table 1. Anthrax and plague following use of a biological weapon[a]

Characteristic	Inhalational anthrax	Pneumonic plague
Incubation period (days)	2–43	1–6
Symptoms and signs	Fever, cough, dyspnea, headache, vomiting, abdominal and chest pain	Fever, cough, chest pain, hemoptysis; rarely associated with cervical buboes
Epidemiology	Outbreak of "flu-like syndrome" in previously healthy persons; course is rapidly fatal	Outbreak of "severe pneumonia" in previously healthy persons; course is rapidly fatal
Microbiology studies	Gram-positive bacilli in blood culture	Gram-negative bacilli in blood or sputum cultures
Additional supportive laboratory studies	Chest X ray showing widened mediastinum (highly characteristic in advanced disease)	Laboratory evidence of disseminated intravascular coagulation not uncommon
Recommended antimicrobial therapy	Fluoroquinolones, tetracyclines, penicillins	Gentamicin or streptomycin, fluoroquinolones, tetracyclines, chloramphenicol
Vaccine	Six inoculations of licensed anthrax vaccine at 0, 2, and 4 wk and then 6, 12 and 18 mo	Vaccine no longer being produced

[a]Derived from references 16 and 17.

some point in time, the spores would germinate, eventually resulting in hemorrhagic thoracic mediastinitis and hemorrhagic lymphadenitis (13). Prominent symptoms of inhalational anthrax would include fever, cough, dyspnea, headache, chest pain, cyanosis, stridor, and ultimately shock. Signs, unfortunately, are relatively nonspecific and consistent with severe sepsis and meningitis. In about half of patients in historical case series, hemorrhagic meningitis, a relatively uncommon entity, would also occur (5, 21). The best mortality rate estimates for inhalational anthrax are 90% or greater for those not receiving effective prophylactic antibiotics or those not given antibiotics quite early in the disease. The time from symptom onset to death in historical series has been a median of 3 days. The chest radiograph of a person with advanced inhalational anthrax would be expected to show a widened mediastinal shadow (29). Inhalational anthrax does not cause a classic bronchopneumonia, and so purulent sputum should not be expected. The disease is termed inhalational by description of the route of acquisition of the disease. It is not truly an anthrax pneumonia; a more precise name is inhalational anthrax, an important clinical distinction (1).

DIAGNOSIS OF ANTHRAX

As for making a microbiologic diagnosis of anthrax, reliable rapid testing such as PCR, enzyme-linked immunosorbent assay (ELISA), and other rapid assays are available in very few locations, so at present, they generally could be used only for confirmatory purposes. *B. anthracis* would be expected to grow from standard blood cultures 6 to 24 h after inoculation; it would also grow from cerebrospinal fluid, and vesicular fluid, but because this is not a bronchopneumonia, it would not be expected to grow from sputum. Unfortunately, routine procedures in many laboratories across the country may identify only *Bacillus* species given the rarity of the disease. Special requests may be needed to ask the laboratory to definitively identify the organism as *B. anthracis*. *B. anthracis* cells are gram positive and have a nonmotile and nonflagellated appearance; it is a spore-forming bacillus. On staining it has a jointed bamboo rod-like appearance. It is notable for growing on ordinary medium at 37°C, with no hemolysis on sheep agar. It has a classic, unique curled hair, also known as Medusa, colonial appearance (32).

If routine microbiology procedures are not likely to identify anthrax without special request, how is anthrax most likely to be diagnosed in the community? A variety of scenarios seem most likely, all of which depend on an astute clinician, nurse, microbiologist, or other health care professional to suspect either anthrax specifically or at least that a serious epidemic is ongoing in the community. These potential clinical scenarios are as follows: in an emergency department, hospital, or clinic, the sudden appearance of multiple cases of rapidly progressive, highly lethal, "viral illness-like" illness; chest radiograph showing a widened mediastinum or gram-positive bacilli in unspun peripheral blood smears; blood culture growth of gram-positive bacilli with preliminary identification of a *Bacillus* species; and a postmortem examination showing a hemorrhagic thoracic lymphadenitis, hemorrhagic mediastinitis, or hemorrhagic meningitis (16).

TREATMENT OF ANTHRAX AS A BIOLOGICAL WEAPON

A national working group called the Working Group on Civilian Biodefense has published consensus guidelines on the treatment and public health management of anthrax and plague as biological weapons (16, 17). Balancing considerations of efficacy with concerns of possible antibiotic resistance and adverse drug effects, the working group has recommended that in a contained casualty setting—that is, a situation in which a modest number of patients require therapy—ciprofloxacin or other fluoroquinolones should be used as initial parenteral therapy until antibiotic susceptibility has been determined. Following susceptibility testing, the most widely available, efficacious, least-toxic antibiotic should be administered. In the case of anthrax, penicillin or doxycycline would be optimal. Given the possibility of prolonged spore latency and the subsequent delayed appearance of fulminant disease, a 45- to 60-day course of antibiotics was recommended by the working group. Oral therapy could replace intravenous (i.v.) therapy as soon as the patient's condition allowed.

In mass-casualty situations—that is, a situation in which the number of patients is sufficiently high that i.v. therapy is no longer possible, either because there is no more i.v. medication, because there is exhaustion of equipment, or because the logistics of parenteral antibiotics become impossible, then oral therapy would need to replace i.v. therapy. In this case, oral therapy with classes of antibiotics and for durations similar to those described above have been recommended (16). Recommendations for antibiotic prophylaxis for those determined to have been exposed to anthrax aerosol are analogous to treatment recommendations in the mass-casualty setting. Given that the chance for survival from inhalational anthrax diminishes with time after symptom onset, antibiotic prophylaxis is advised as early as possible.

VACCINATION

The U.S.-licensed anthrax vaccine is made by Bioport and was formerly made by the Michigan Department of Health. The vaccine is derived from an inactivated, cell-free filtrate of nonencapsulated strains (23). A similar vaccine was efficacious against cutaneous anthrax in one small, placebo-controlled trial that was done in the days when there was significant occupational exposure among some groups in the United States (6). It is licensed to be given as a six-dose series, given at 0, 2, and 4 weeks and then at 6, 12, and 18 months. It has been mandated for all members of the U.S. military, but it is generally not widely available for civilians at this time. There is a potential role for pre- and postexposure use of the anthrax vaccine in the civilian population, but more research is needed and is being done now. The role for the preexposure vaccination against anthrax for members of the civilian community has been taken up by the Advisory Committee for Immunization Practices.

The anthrax vaccine is a topic worthy of a plenary session or symposium in and of itself. This actually occurred in June 1999 in Frederick, Md., at the United States Army Medical Research Institute of Infectious Diseases, which held a 3-day sym-

posium on the anthrax vaccine. I refer the reader to three websites with information on the anthrax vaccine, all of which are quite instructive. The first is http://www.cdc.gov.nip/VAERS.html. This website explains the Vaccination Adverse Event Reporting system, which is the database management system for the collection of analysis and data from reports of adverse events from vaccination. This system is comanaged by CDC and the Food and Drug Administration (FDA). The second website is http://www.anthrax.osd.mil. This site explains the policies of the U.S. Department of Defense on anthrax vaccination. The third website is http://www.fda.gov/ola/anthraxvaccine.html. It contains the testimony of Kathryn Zoon of the Center for Biologics Evaluation and Research at FDA on the safety and efficacy of the vaccine.

Infection control measures for patients infected with *B. anthracis* consist of standard barrier universal precautions (2). It is important to underline the fact that there have been no cases of person-to-person transmission of anthrax. Processing of patient specimens in laboratories can occur at biosafety level 2 unless high-volume or aerosol-generating work is being done. Standard disinfection of hospital surfaces contaminated with infected body fluids should be performed with hypochlorite or by other suitable measures (2).

IS THERE A NEED FOR DECONTAMINATION?

Much has been written and stated regarding the possible need for environmental and personal decontamination following the release of an anthrax aerosol. Experiments have shown that it is *primary aerosolization*—that is, exposure to the original aerosol—that connotes the greatest risk. *Secondary aerosols*—or resuspension of anthrax—from the ground or other surfaces are far less likely to cause a threat (16). Moreover, decontamination may not even be feasible in large-scale aerosol releases of anthrax. It has been advised by the Working Group on Civilian Biodefense that environmental decontamination be considered only in areas of proven heavy spore contamination (16). The management of an anthrax hoax or threat should also be addressed, such as the situation of alleged anthrax use, for example, in letters alleged to contain anthrax organisms. The Working Group on Civilian Biodefense recommendations are that only those persons who have direct contact with the alleged substance would be deemed at risk. Those persons getting such a substance on their skin or their clothing directly should be advised to wash with soap and water and to wash their clothing with soap and water. Those with direct contact should get postexposure antibiotic treatment until the contact is proven not to be anthrax. Those without direct contact—that is, those at the next desk or in the next room or those walking down the street in front of the building—do not need decontamination and they do not need antibiotics (16). It is noteworthy that decontamination measures that have been ongoing following hoaxes around the country—washing persons with bleach solutions and/or exposing them outdoors to decontamination efforts—are not necessarily benign procedures and should be undertaken only if they are clearly indicated. These working group recommendations are in accord with those made by experts at the United States Army Medical Research Institute of Infectious Diseases.

PLAGUE AS A BIOLOGICAL WEAPON

Like anthrax, plague is a disease with a very prominent place in history. As Petrarch described in 1348, "future generations would be incredulous, unable to imagine the empty houses, abandoned towns, fields littered with the dead, solitude that hung over the world; physicians were useless" (19). By way of review, plague is a disease caused by *Yersinia pestis.* In the 14th century pandemic, it was known as the "Black Death" and was also termed "the great mortality" and "the great pestilence." That pandemic lasted 130 years. Thirteen million people in China died, and 20 million to 30 million people in Europe, one-third of the European population, died (28). A historical footnote to these events was the exclusion of all vessels from Venetian ports for 40 days, or *quarantina,* which is the origin of the term "quarantine."

EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS OF PLAGUE

As a natural occurrence, plague is an enzootic infection of rats, prairie dogs, and other rodents around the world and on all continents except Australia (27). It is now quite rare in humans; worldwide, about 1,700 cases are recorded annually (27). In the United States, there have been about 390 cases in the past 50 years. Despite this rarity, or perhaps because of it, and because of its great role in the pandemics in the past, plague still has the ability to incite terrible fear. This was in evidence in India in 1994, when a modest number of persons were reported to have plague in Surat, and more than 500,000 persons fled from the city in response. This outbreak in India had ramifications around the world. At the time, there were discussions in New York City about whether to ban air travel from India. There have been estimates of $2 billion damage to the Indian economy as a result (15).

Naturally occurring plague most commonly follows the bite of a *Y. pestis*-infected flea. Symptoms of plague include the sudden onset of fever, chills, and weakness. There are three main forms of disease. The most common form of disease is bubonic plague, the form seen in over 84% of the cases in the United States in the past 50 years (9). Buboes are tender, swollen, 1- to 10-cm, nonfluctuant lymph nodes, most commonly in the cervical, axillary, or inguinal regions, usually with overlying erythematous skin. Septicemic plague is like bubonic plague, but without the buboes to herald the disease. Septicemic plague has been the form of plague responsible for 13% of the cases in the United States in the past 50 years (9). Pneumonic plague has been quite rare in the United States, and generally it has been far less common than the first two forms of the disease. Pneumonic plague has been responsible for 2% of the cases of plague in the United States in the last 50 years (9). Pneumonic plague can be either primary or secondary. Primary pneumonic plague is the result of direct contact with respiratory droplets of a person infected with pneumonic plague; secondary pneumonic plague is the result of hematogenous spread from elsewhere in the body. Secondary pneumonic plague occurs as often as 10 to 12% of the time following primary bubonic or septicemic plague (27).

Plague used as a biological weapon would result in an epidemiologic and clinical picture distinct from that of naturally occurring plague (Table 1). It is possible that infected fleas could be used as a vector to cause plague. This occurred when the Japanese military released *Y. pestis*-infected fleas over Chinese cities in the years leading up to and during World War II (31). Bubonic disease would be the result. Clearly, bubonic plague is much more easily recognized by alert clinicians than pneumonic plague, and antibiotic treatment could be started promptly. Of more concern than the release of plague-infected fleas would be the direct aerosolization of the plague bacilli, a process that modern biotechnology now makes feasible. Direct aerosolization of plague bacilli would cause pneumonic plague, not bubonic plague. Other unusual features that would follow such an event would be the occurrence of plague in geographic locations without known prior plague occurrence, in groups of persons with no known risk factors, and in cities or regions with no rodent die-off prior to the disease in humans (6).

Following use of plague as a biological weapon, pneumonic plague could spread from person to person by respiratory droplet among those who are in close contact—that is, less than 2 m (10, 35). This is in distinction to the epidemiology of an anthrax outbreak, a disease for which person-to-person transmission has not been recorded. Outbreaks of pneumonic plague have historically been uncommon. The likelihood of person-to-person contagion appears to be dependent on both patient and environmental factors, although patient factors are less well understood. The environmental factors supporting spread would include cold temperatures and close, cramped, crowded conditions (22). The largest pneumonic plague outbreak was in Manchuria in 1910 and 1911, in which there were 60,000 cases of pneumonic plague (35). The last case of person-to-person transmission of pneumonic plague was in Los Angeles in 1924, when 1 person with secondary pneumonic plague gave primary pneumonic plague to 32 close contacts (22). None of the pneumonic plague cases occurring in the United States since that time has resulted in a single case of transmission of plague.

CLINICAL PRESENTATION OF PLAGUE

After exposure to a plague aerosol, symptom onset would be expected in 1 to 6 days and, most commonly, in 2 to 4 days (17). Symptoms would include fever, cough, and bloody or purulent sputum. Pneumonic plague would initially resemble other forms of severe pneumonia with hemoptysis. The signs would be consistent with sepsis and severe pneumonia. An unusual feature of the disease which may occur is acral necrosis of the digits or the nose. This black necrosis of the fingers is reportedly the reason that plague was given the moniker "Black Death" in the second pandemic. Only rarely would cervical buboes be expected with pneumonic plague. This lack of buboes makes pneumonic plague much more difficult to diagnose than bubonic plague. Laboratory studies would show sepsis, organ failure, and abnormal coagulation. The chest radiograph would show pulmonary alveolar infiltrates, much like other forms of severe pneumonia, such as pneumococcal pneumonia with consolidation (7). The mediastinum would not be expected to be wide, as opposed to the chest X ray of the patient dying of inhalational anthrax, for which

a widened mediastinum and the absence of consolidation or parenchymal infiltrate would be the hallmarks. Rates of mortality without rapid antibiotic treatment would be very high, and the course would be rapid (17).

DIAGNOSIS OF PLAGUE

Rapid microbiologic testing for plague is available in very few laboratories. Therefore, PCR, ELISA, or other rapid assays would be useful for confirmatory testing purposes only (18). Growth could be expected from blood cultures, sputum, or bubo aspirates. However, this would require optimum conditions and would likely require 24 to 48 h after culture inoculation. Unfortunately, automated or routine procedures in many laboratories may misidentify this culture growth unless the laboratory is specifically asked to exclude plague by the ordering physician (33). *Y. pestis* is a gram-negative, nonmotile, non-lactose-fermenting bacillus or coccobacillus. Bipolar "safety-pin" staining can be seen on staining with Wright and Giemsa stains (12). Bacterial colonies are small and grow optimally at 28°C, although identification may require up to 6 days.

Given the length of time needed to make a microbiologic diagnosis with the current technologies and procedures, how would a diagnosis of plague be made in a community following the covert release of plague as a biological weapon? A few pathways to diagnosis seem most likely, and all of these would depend on an astute clinician, nurse, microbiologist, or other health care professional who suspected plague itself or who was at least suspicious that an unusual epidemic was occurring in the community. These specific events would include the sudden appearance of multiple persons with fever, cough, hemoptysis, chest pain, and a fulminant course of illness and high rates of mortality. Clinical signs would be consistent with pneumonia or sepsis in previously healthy persons and would consist of purpuric skin lesions, acral necrosis, or, more unlikely, a cervical bubo. Laboratory studies would include blood, sputum, or bubo aspirates, with gram-negative bacilli seen on staining, possibly with bipolar staining. If a postmortem examination was undertaken, the expected pathology would include lobar exudation and necrosis of the pulmonary parenchyma (17). If any of these events occurred, the local health department and the hospital epidemiologist would need to be contacted immediately so that a rapid investigation could be initiated. The health department would in turn immediately notify CDC.

TREATMENT OF PLAGUE AS A BIOLOGICAL WEAPON

The Working Group on Civilian Biodefense has published consensus guidelines on the treatment and public health management of plague as a biological weapon (17). Balancing considerations of efficacy with concerns of possible antibiotic resistance and adverse drug effects, the working group has recommended that in a contained casualty setting—that is, a situation in which modest numbers of patients require therapy—gentamicin and streptomycin are the first-line therapy, followed by doxycycline, fluoroquinolones, and chloramphenicol (17). In a mass casualty or postexposure prophylaxis setting—that is, a setting in which parenteral therapy is

no longer possible, for whatever reason—oral therapy would be necessary. In that setting, doxycycline, tetracycline, fluoroquinolones, or chloramphenicol would be the recommended antibiotics. Details of these recommendations can be found in the 2 May 2000 issue of *JAMA*, where these recommendations are published (17).

VACCINATION

The plague vaccine is a U.S.-licensed, killed, whole-bacillus vaccine (Greer Laboratories plague vaccine, USP) which ameliorates or prevents bubonic plague. It has been used safely for individuals deemed to be at high risk of bubonic disease, including microbiologists or others deemed to be at high risk. It was given during the Vietnam War to servicemen serving in Vietnam, but evidence shows that this did not prevent or ameliorate pneumonic plague. There is no clear role for pre- or postexposure use of this vaccine in response to the use of an aerosolized plague biological weapon (17). Moreover, this vaccine is no longer being produced. There is ongoing research work in pursuit of a vaccine that would be protective against pneumonic plague.

PLAGUE AS A BIOLOGICAL WEAPON: INFECTION CONTROL

There is no precedent for a large, intentional outbreak of pneumonic plague following use of a biological weapon. Existing infection control and community outbreak control recommendations need to be considered with new challenges in mind. In its recommendations, the Working Group on Civilian Biodefense has recommended that in a community affected by a pneumonic plague outbreak following the use of a biological weapon, any person developing fever or cough would merit antibiotic prophylaxis (17). The extremely difficult task would be determining the extent of the community at risk. If that community could be defined, the working group has advised that individuals undertake a personal fever or cough watch: if fever or cough were to develop during the period of the pneumonic plague outbreak, affected persons would be advised to immediately seek antibiotic treatment.

If a person developed pneumonic plague, her or his family contacts, hospital contacts, or close work contacts—those interacting with a person at less than 2 m—should be given antibiotic prophylaxis. Hospital guidelines from the Working Group are consistent with those of the Control of Communicable Diseases in Man as well as other guidelines: patients with pneumonic plague should be isolated by using respiratory droplet precautions (2, 10). These precautions include the use of disposable surgical masks both for patients and for the health care workers caring for them. In the microbiology laboratory, simple clinical specimen processing should be performed under biosafety level 2 conditions. If high-volume or aerosol-inducing procedures are needed, then biosafety level 3 conditions are needed (25).

CONCLUSIONS

In closing, I return to the issue of what the infectious disease community should know about biological weapons. It is not clear when or if biological weapons will

be used against civilians in the 21st century. However, the facts and context are of great concern. It is clear that the technology supporting the development of mass-casualty-producing biological weapons has existed for some time and is now becoming more accessible and less expensive. It is clear that biological weapons are being developed by a number of countries around the world. It is clear that current treaty mechanisms have not prevented proliferation of this class of weapon. It is clear that biological weapons have the potential to cause tremendous illness and death.

From these conclusions, I think it is evident that infectious diseases specialists and the broader biological sciences community have the responsibility to engage in the challenges posed by biological weapons. It is time that as professionals dedicated to diminishing the suffering caused by infectious diseases, we should now become vigorously involved in trying to understand how to manage the growing power of biology, how to prevent its misuse, and how to respond to biological weapons should prevention fail.

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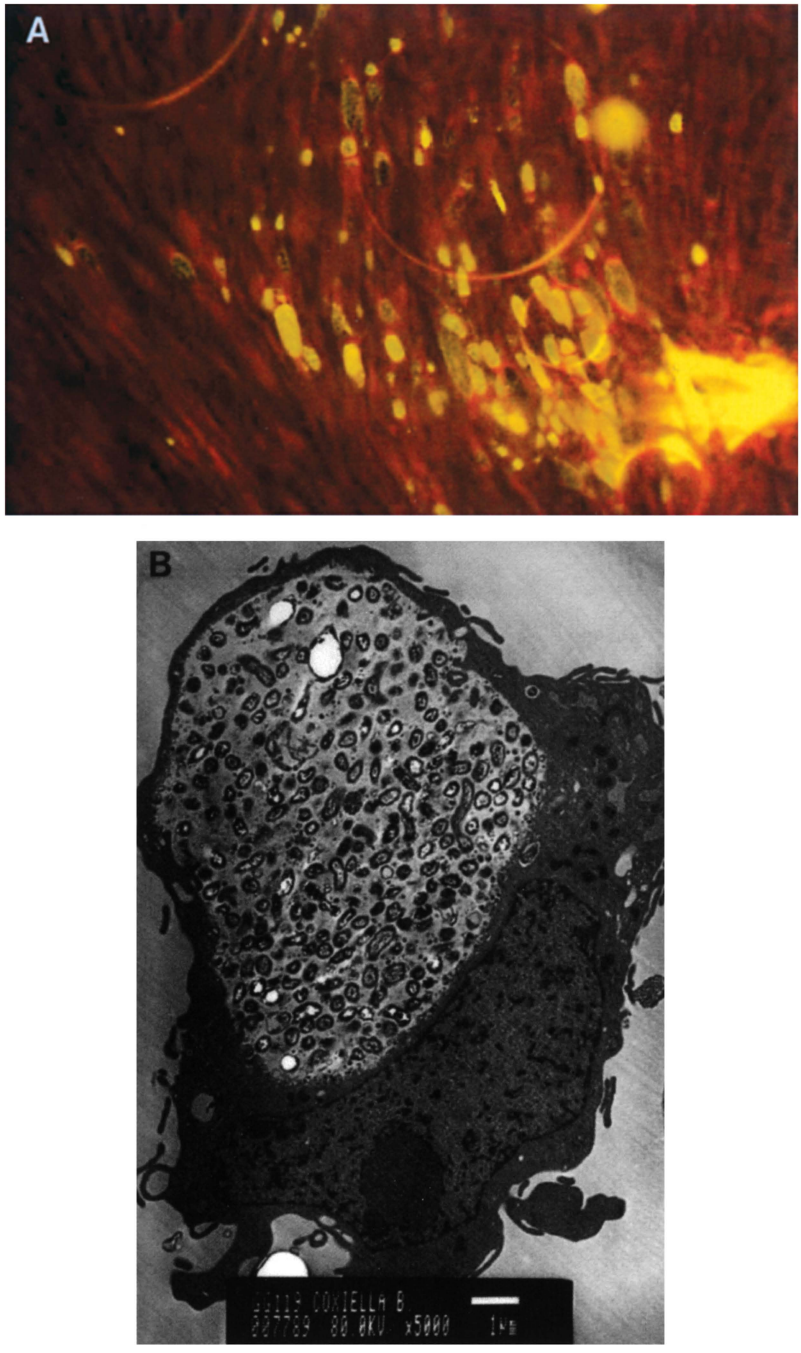
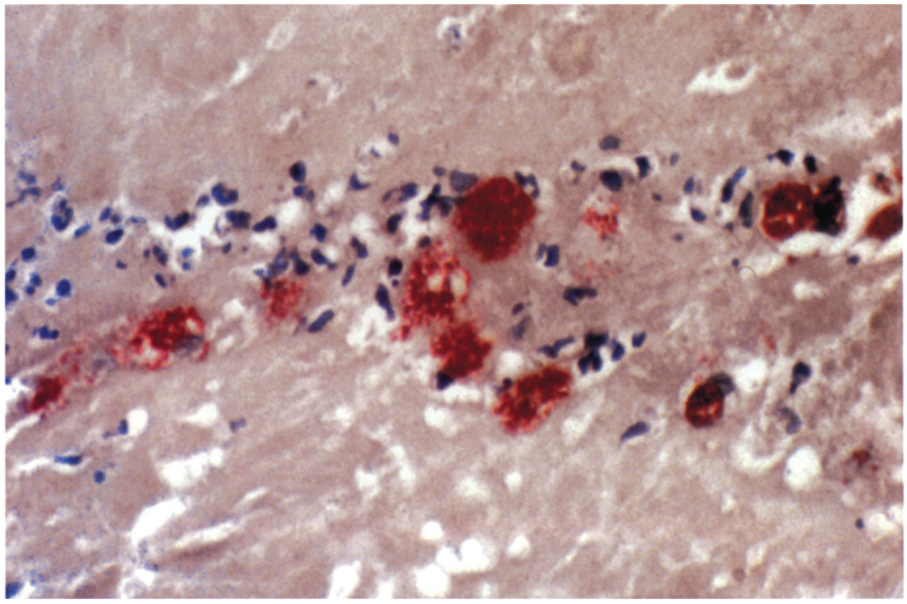
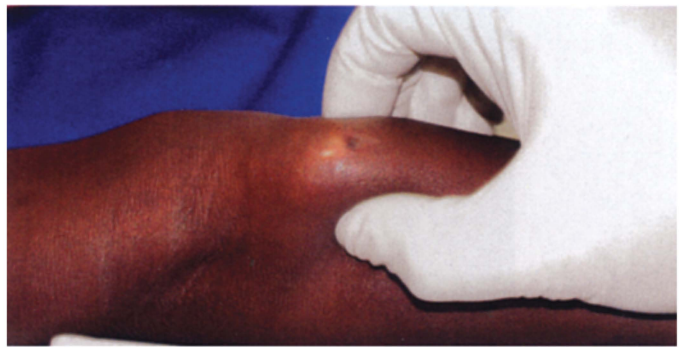


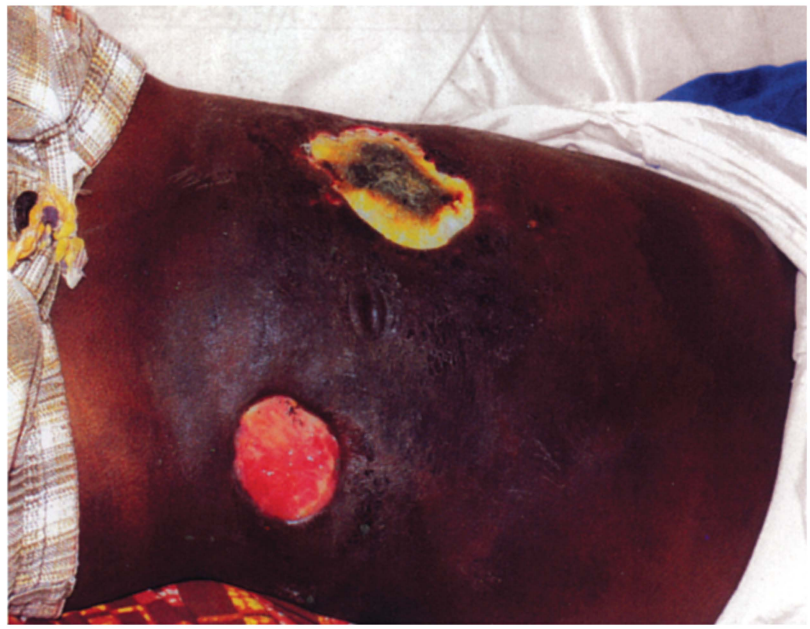
Color Plate 1 (chapter 3). (A) Culture of *C. burnetii* in shell vials detected by immunofluorescence. (B) Culture of *C. burnetii* in human embryonic fibroblasts, visualized by electronic microscopy.



Color Plate 2 (chapter 3). Detection of *C. burnetii* on a valve by immunohistochemistry.

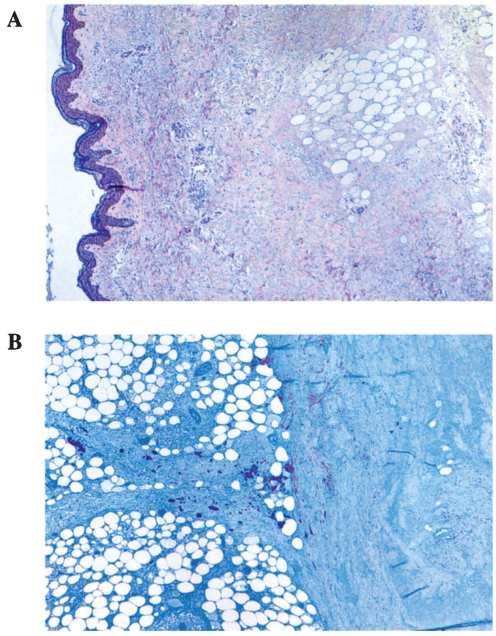


Color Plate 3 (chapter 9). Buruli ulcer nodule.

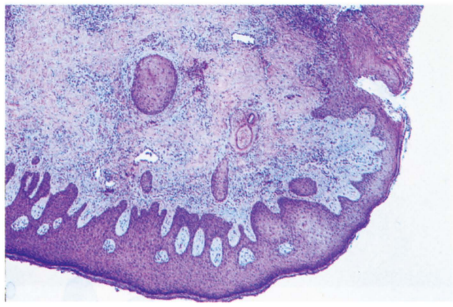


Color Plate 4 (chapter 9). Buruli ulcer. Note the undermined edges and cotton wool-like appearance of the ulcer on the patient's abdomen.

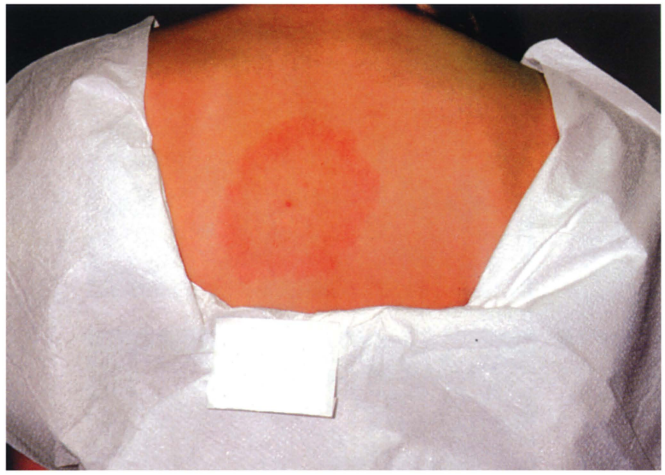
Color Plate 5 (chapter 9). Buruli ulcer scar on upper arm extending below elbow.



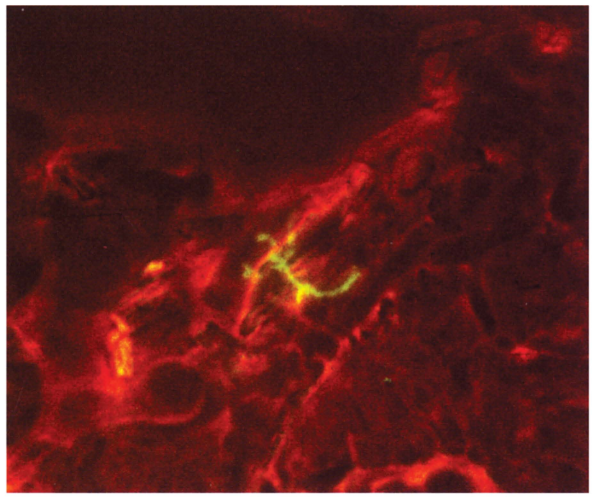
Color Plate 6 (chapter 9). Photomicrographs of nodule stage of Buruli ulcer (original magnification, ×5). (A) Hematoxylin-and-eosin stain showing subcutaneous tissue necrosis including septa and ghost adipocytes. The dermis shows elastolysis and focal inflammation around blood vessels. (B) Acid-fast stain showing clumps of red AFB in septa.



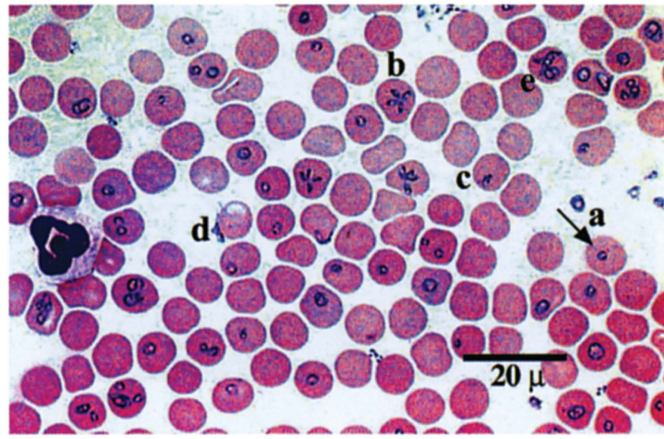
Color Plate 7 (chapter 9). Photomicrograph of ulcer stage of Buruli ulcer (original magnification, ×5). A hematoxylin-and-eosin stain shows the border of the ulcer with pseudoepitheliomatous hyperplasia and inflammation at the edge.



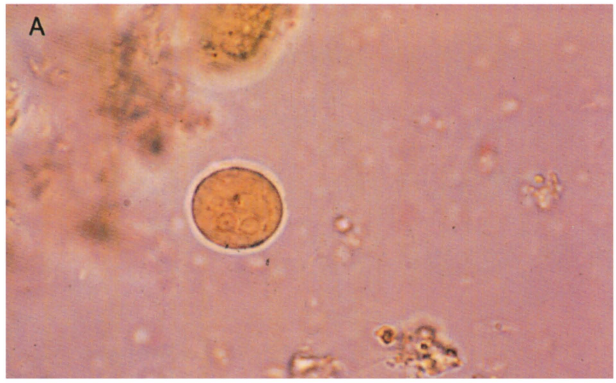
Color Plate 8 (chapter 10). Erythema migrans rash of a 12-year-old female resident of a farm in Missouri. The picture was taken on 25 May. The rash followed the bite of an *A. americanum* tick on 12 May. Photograph courtesy of Edwin J. Masters.

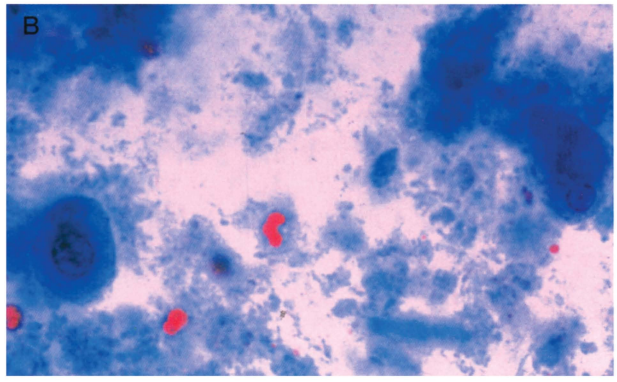


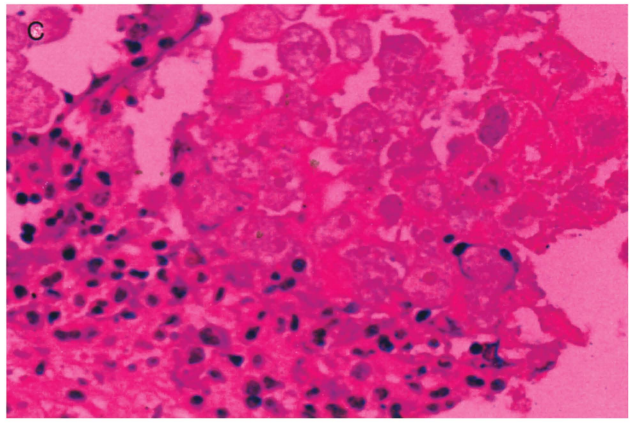
Color Plate 9 (chapter 10). A *Borrelia* sp. in the papillary dermis of a skin biopsy specimen that was subjected to indirect immunofluorescence with a *Borrelia* genus-specific monoclonal antibody. The patient developed an erythema migrans rash after travel to a wooded area of western North Carolina (see text). (This is a modified version of a figure that was published in reference 76.)



Color Plate 10 (chapter 11). Giemsa-stained thin blood smear of WA1-type parasites in hamster erythrocytes. Final magnification, ×834. a, ring form; b, tetrad or Maltese cross form; c, periform; d, exoerythrocytic merozoites; e, amoeboid form.







Color Plate 11 (chapter 12). *E. histolytica* infections in humans. The cyst (A) and trophozoite (B) forms of *E. histolytica* in stool samples and trophozoites invading colonic epithelium (C) are shown.

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