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Pathogenic Mycobacteria in Water

A Guide to Public Health Consequences, Monitoring and Management

Edited by S.Pedley, J.Bartram, G.Rees, A.Dufour, J.A.Cotruvo

World Health Organization

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Contents

Preface

The World Health Organization's Water, Sanitation and Health Office, in collaboration with the US Environmental Protection Agency Office of Research and Development, is producing a series of expert workshops, reports and monographs on Emerging Pathogens in Water. This monograph was developed from the expert workshop entitled: Pathogenic Mycobacteria in Water that was held in Guildford, UK between the 18th and 20th September 2002. The sponsors of this workshop were the US EPA Office of Research and Development.

A total of 20 experts from 7 different countries, including those with expertise in medicine, clinical and water microbiology, epidemiology, regulatory policy, public health and water engineering, examined the range of issues associated with pathogenic mycobacteria in current and potential human-water interfaces.

Other workshops and publications in the series include:

- Heterotrophic Plate-Counts and Drinking-Water Supply
- H_2S Method for Detection of Faecal Contamination
- Severe Outcomes from Infectious Diseases Associated with Recreational Water Contact
- Respiratory Transmission of Faecally Excreted Viruses

- Toxic Cyanobacteria in Water
- Waterborne Zoonoses: Identification, Causes and Controls

These are all available from the World Health Organization website (www.WHO.int/water_sanitation_health/) or from the International Water Association (www.iwapublishing.com).

Non-tuberculous pathogenic mycobacteria, including those collectively referred to as *Mycobacterium avium* complex (MAC) have been associated with a variety of adverse health outcomes, including Crohn disease, cervical adenitis and pulmonary and skin infections. The bulk of interest in MAC infection in relation to water management revolves around the exposure of immunocompromised individuals (in particular HIV positive populations) to MAC in domestic water supply. The issues addressed included:

- the state of current knowledge on MAC
- health outcomes in high-exposure occupational groups
- examining the postulated causal link between *Mycobacterium avium* sub-species *paratuberculosis* (MAP) and Crohn disease
- risk assessment and risk management
- infections associated with non-tuberculous pathogenic mycobacteria
- analytical methods for the isolation and identification of non-tuberculous pathogenic mycobacteria
- environmental sources and routes of exposure
- controls, treatments and disinfection regimes

This monograph contains the results of the workshop deliberations largely based on the technical materials prepared by the participants prior to the workshops and the resultant scientific discussions. Each of the chapters in the monograph resulted from presentations by the experts in the various sessions of the workshop. The presentations were subsequently revised in the light of other presentations and the debate and discussion that reflected the collective experiences of the participants. The final monograph is the product of the Expert Workshop, all its chapters were redrafted and peer reviewed by members of the expert group and the editors.

We hope that you will find the document enlightening in describing the complex issue of non-tuberculous pathogenic mycobacteria and the role of water as a milieu for survival and transmission of infections associated with these organisms.

Acknowledgements

The World Health Organization wishes to express its deep gratitude to all those whose efforts made the production of this book possible. The international experts who attended the meeting on Pathogenic Mycobacteria in Water in Guildford, UK, in September 2002, many of whom contributed to individual chapters in this book, reviewed drafts, and supported the editorial work, comprised the following:

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Executive Summary

Mycobacteria are a large group of microorganisms that inhabit a diverse range of natural environments: some species are capable of infecting humans and animals. Apart from the highly significant mycobacterial pathogens, we may presume that the majority of people in the water industry, until recently, would not be familiar with environmental mycobacteria. Nevertheless, environmental mycobacteria are a frequent cause of infection, and there is a growing body of evidence to show that water is a significant vehicle for the transmission of these organisms.

The importance of the Pathogenic Environmental Mycobacteria (PEM), and especially the *Mycobacterium avium* Complex (MAC), was recognised with the discovery of disseminated infection in immunocompromised people, particularly people with HIV and AIDS. Yet there are many other forms of disease, both minor and serious, that are caused by PEM. This book provides a comprehensive review of PEM, the different types of disease that they are known to cause, and their distribution in water and other parts of the natural environment. It explores the role of water as a vehicle for the transmission of these pathogens and concludes with a discussion of the issues surrounding the control of PEM in drinking-water and the

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assessment and management of risks. The book is a valuable resource for sanitary and clinical microbiologists, public health professionals, water resource managers, water engineers, piped public water suppliers, regulators of water quality and facilities managers in medical and commercial buildings.

The history of human development and the growth of civilisation contains frequent reference to the role of infectious disease in forging social structures and deciding the fate of nations. Critical analysis of the historical record shows that at least some of the major infectious diseases circulating in the world today have existed relatively unchanged for several centuries, possibly even longer. In addition to these readily identifiable diseases, we may assume that there would have been in circulation a much greater number of other pathogens, causing a range of diseases that are unrecognisable from contemporary records or that were of insufficient virulence to be visible above the background of other social hazards. Notwithstanding the long, stable history of a few infectious diseases, the relationship between humans and pathogens is not static and from time-to-time new pathogens appear and present fresh challenges for society. In a brochure that accompanies this series of publications (Emerging Issues in Water and Infectious Disease, WHO, 2004) we are told that pathogenic microorganisms are subject to the same rules of evolution as all other organisms. The interaction between the pathogen and its environment leads to a modified, possibly more virulent strain of the pathogen, or to the emergence of a previously unrecognised form of the pathogen. As new types of pathogen emerge appropriate public health measures must be developed to control the spread of disease. Frequently, the public health response will impact the management of water resources, especially as drinking-water reserves and for recreational and agricultural use. The brochure highlights four drivers of emerging issues in water and infectious disease:

- New environments.
- New technologies.
- Scientific advances in water microbiology.
- Changes in human behaviour and vulnerability.

In order to inform the public about these emerging issues and to provide guidance to the water sector about the management options that are available to moderate their effects, the WHO and USEPA have initiated a series of meetings at which leading experts from different disciplines discuss the issues and work towards a consensus about the risks to public health and the measures required to control their impact. PEM have been identified as a group of microorganisms that are widespread in the environment and which appear to be an emerging cause of waterborne disease. This book is the final output from a meeting of experts held in Guildford, UK, in September 2002. The meeting brought together microbiologists, water quality

experts, public health experts and epidemiologists to present and analyse our knowledge of PEM and the particular issues surrounding their presence in water. The first drafts of the chapters were prepared from the presentations made by the experts. Subsequently, the chapters were redrafted and reviewed by members of the expert group, the editors, and other contributors.

The chapters in the book have been grouped into three sections, although this is not manifest in the contents. In chapters 2 to 5 the reader is introduced to the biology of PEM and is shown how this governs the ecology of the organisms. This section also describes the particular difficulties associated with the isolation, enumeration and identification of PEM and shows how a combination of classical and modern analytical methods are being used by laboratories to detect the organisms. In chapters 6 to 10 the book focuses on the range of infections caused by PEM and the role of water in the transmission of these infections. Chapters 10 and 11 form the final section of the book and deal with the particular issues in water treatment and water quality management that are presented by the characteristics of PEM. Every chapter is concluded with a list of the key research issues that, in the opinion of the authors, should be tackled in order to improve our understanding of the organisms, their ecology, their pathogenicity, and the role of water in the transmission of disease.

We have described earlier the concept of emerging issues in water and infectious disease and the key issues that form the theme of this series of books. Throughout this book you will find many examples of how PEM have gained prominence by exploiting the changing interface between humans and the environment, which is embodied in the key issues listed above. Chapter 1 provides a comprehensive summary of PEM and introduces topics that are dealt with in depth by the authors of later chapters. It is in this chapter that the reader will find the most conspicuous links with the key issues of water and infectious disease.

The relationship between PEM, in particular MAC, and the environment is developed in chapters 2 and 3. It is clear from the reviews presented in these two chapters that the ecology of PEM is complicated and poorly understood. As well as existing as a free-living resident of many environments, PEM may also form a symbiotic, or parasitic, relationship with certain species of amoebae and insect. These relationships may be fundamental to the transmission and pathogenicity of PEM. Furthermore, the unusual chemical structure and hydrophobicity of their cell wall leads to the organisms being concentrated at the interface between water and air; a particular issue for the dispersal of the organisms in aerosols. Another important aspect of the ecology of PEM is illustrated by the example of cervical lymphadenitis (chapter 7). Until recently, the principal cause of cervical lymphadenitis in children was *Mycobacterium scrofulaceum*; however, several groups of workers have reported a rapid shift in the main etiological agent of disease from *M. scrofulaceum* to *M. avium*. A significant feature of this shift is that it has

occurred concurrently in the UK, USA and Australia, and that it coincides with a sudden decline in the occurrence of *M. scrofulaceum* in water. The reason for this sudden change is not understood.

The authors of chapter 1 note that MAC was first recognised as a problem in people with HIV and AIDS. This theme is expanded in chapter 7, where the appearance and spread of disseminated MAC infection is discussed in the context of the emergence of HIV and AIDS and the vulnerability of individuals with impaired immunity: an example of changes in human behaviour and vulnerability. For these people, PEM in water and soil is a particular hazard, and exposure to these environments, for example at swimming pools or even contact with some potting composts, may give rise to a special risk of infection. The subject of changes in human vulnerability is also tackled by some of the other authors.

A common thread running through the book is the emergence of PEM as a problem organism in water systems. There can be little doubt that MAC and other PEM have existed in the aquatic and soil environments for a long time; in other words, they are not a recent contaminant. Yet it is only relatively recently that their significance for public health has been appreciated. This point can be illustrated by reference to Buruli Ulcer (BU), a disease caused by *Mycobacterium ulcerans*, which is described in chapter 8. The first description of disease symptoms consistent with BU was made in 1897, but it was not until 50 years later that the etiological agent was identified and described. Since 1980, evidence has emerged of the true extent of the disease and it is now the third most common mycobacterial infection after TB and leprosy. The authors of chapter 8 report the results of recent studies that suggest a link between *M. ulcerans* and a biting, aquatic insect. Based upon these observations we can speculate that BU is a vector-borne disease and that water may be an important environment for the proliferation of the vector. The authors of chapter 8 point out that this new development offers options for water resource management that may control the spread of the disease. In the context of the key emerging issues in water and infectious disease, BU embodies a number of themes, but principally it is an example of scientific advances in microbiology that have provided the tools that were necessary to detect *M. ulcerans* in aquatic insects.

The possible role of water in the transmission of Crohn Disease (CD) has stimulated a fascinating debate between microbiologists, public health professionals, and water quality scientists, which is reviewed in chapter 6. Currently, the link between *M. avium* subspecies *paratuberculosis* (MAP) and the waterborne transmission of CD has not been proven, but there is a body of epidemiological and microbiological evidence that would suggest that a link does exist, and that further research is needed. We await with interest the resolution of this debate. Once again, the possible role of water in the transmission of a pernicious disease is being unravelled. However, as new information is published

the possibilities for controlling the disease through effective water quality management strategies may become apparent.

Several of the chapters portray PEM as an opportunistic pathogen: a pathogen that can infect a compromised host but would not normally infect a healthy host. Yet these are not trivial infections as chapters 7 to 10 demonstrate. Once the organism has established an infection treatment can be very difficult, requiring the use of a combination of drugs for an extended period of time. Relapses are not uncommon. In some cases the treatment may involve surgical removal of the infected area. Water plays a significant role in the infection process. In chapter 8 the authors cite examples of people contracting PEM infections by contact with water used in a beauty treatment salon. One patient was left with a permanent scar on her leg. There is also evidence to suggest that body piercing may be a cause of PEM infection. These examples show how different uses of water, brought about by changing lifestyles, can present new challenges for public health protection, as well as emphasising the need for water users to be mindful of the possible risks associated with some of the more unconventional uses of water. Similarly, medical and dental establishments are not immune from opportunistic infections with PEM. Bronchoscopes that have been cleaned with water contaminated with PEM have been shown to be a cause of pulmonary infection (chapter 9).

New analytical methods are being developed all the time in all fields of microbiology. During the past 20 years, methods that use the detection, amplification, or sequencing of genetic material have emerged as major tools for analysts specialising in medical and environmental microbiology. These advanced methods have provided essential evidence to confirm PEM as a cause of some infections and for discovering new environmental reservoirs of the pathogen (for example, *M. ulcerans*). Yet the confirmation of PEM in many infections still relies upon the classical methods for culturing the organism in the laboratory. Chapter 5 describes the state of the art methods available for the isolation and identification of PEM, but points out that although many of the methods are accurate and precise, the first step in diagnosis often requires the growth of the organism on selective media. Similar limitations apply to the recovery of PEM from environmental samples. The chapter concludes that further work is required to improve the analytical methods that are available to microbiologists to expedite diagnosis and improve environmental monitoring.

Modern, rapid methods of analysis that detect specific nucleic acid sequences in the PEM are possible because of the major developments in our understanding of the biology and genetics of the organisms. In chapter 4, the authors expand upon the earlier descriptions of the ecology of MAC and other PEM and show how this can be affected by the biology of the organisms. The description of the genetics of the PEM provides an important insight into the many factors that control the ecology of the organisms.

Two factors control the presence and level of PEM in drinking-water distribution systems: ingress and regrowth. From our understanding of the biology and ecology of PEM (chapters 2, 3 and 4) we may surmise that drinking-water treatment will not eliminate PEM but, if operating satisfactorily, will significantly reduce the numbers that may be present in the source water to a level that represents a negligible risk to the general population. Ingress of PEM through leaks in the distribution system, and the regrowth of PEM in biofilms are potentially more significant events for drinking-water quality management. These issues are addressed in chapter 11.

The biology and ecology of PEM render them highly resistant to chlorine and the other chemical disinfectants used for the treatment of drinking-water. Chapters 1, 2 and 3 demonstrate that many sources of water, in particular surface water, will be contaminated with some species of PEM. Consequently, the early stages of water treatment – flocculation, sedimentation and filtration – are the most important barriers to the transfer of PEM into the water distribution system. Any breach of these treatment steps will not be remedied by chemical disinfection of the water. Even if PEM are removed from the source water, the potential for contamination to occur through breaches in the pipework, either from a burst water main or during maintenance work, is high. Once inside the network, PEM can readily integrate into the biofilm where they become an established member of the microbial community. Some studies carried out in drinking-water distribution systems have shown that slow growing PEM can be found at densities greater than 4000 per cm^2 in the surface biofilm, creating a potentially high frequency and level of exposure.

The book concludes by discussing the use of water management strategies for controlling exposure to PEM from water systems. In chapter 12, the authors argue that water safety plans (WSPs) can be an effective method for the control of PEM in water. To support their argument, the authors draw heavily upon the example of Legionella and the procedures that have been developed to control Legionella in water systems. WSPs are an important aspect of the 3rd edition of the WHO Guidelines for Drinking-Water Quality and the PEM are used to demonstrate how the risk assessment and management framework of a WSP can be an effective mechanism for the control of waterborne pathogens.

This book provides an in-depth review of PEM as an emerging waterborne pathogen, and examines their significance for public health and water resource management. PEM offer many challenges to the microbiologist for the development of improved analytical methods and monitoring procedures; to the public health sector to determine the full extent of the disease caused by PEM, their epidemiology and clinical management; and to the water sector, where PEM present significant problems in treatment and control. PEM highlight how holistic approaches to the management of water supplies may be the best, even the only option that is available to the water sector.

List of acronyms and abbreviations

AFB	acid fast bacillus
AOC	assimilable organic carbon
BCG	Bacillus Calmette-Guerin
BDOC	biodegradable dissolved organic carbon
bp	base pairs
BSA	bovine serum albumin
BU	Buruli Ulcer
CCL	contaminant candidate list
CD	Crohn disease
CDC	Centers for Disease Control and Prevention
CF	cystic fibrosis
cfu	colony forming units
CPC	cetyl pyridinium chloride
CR	congo red
DNA	deoxyribonucleic acid
ELISAs	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
G+C	guanine plus cytosine
GAC	granular active carbon

GPL	glycopetidolipids
H&E	haematoxylin and eosin
HAART	highly active antiretroviral therapy
HACCP	Hazard Analysis Critical Control Point
HPC	heterotrophic plate count
IFN-gamma	interferon gamma
IMS	immunomagnetic separation
IS	insertion sequences
ISH	*in situ* hybridization
ITS	internal transcribed spacer
JD	Johne disease
kb	kilobases
MAA	*Mycobacterium avium* subsp. *avium*
MAC	*Mycobacterium avium* complex
MAIS	*M. avium-intracelluslare-scrofulaceum*
MAP	*M. avium* subsp. *paratuberculosis*
mb	megabases
mce	mycobacterial cell entry
MLST	multi-locus sequence typing
MTB	*Mycobacterium turberculosis*
NTM	non-tuberculous mycobacteria
NTU	nephlometric turbidity units
OSHA	Occupational Safety & Health Administration
PCR	polymerase chain reaction
PEM	pathogenic environmental mycobacteria
PFGE	pulsed-field gel electrophoresis
PPD	purified protein derivatives
PRA	PCR restriction analysis
PVC	polyvinyl chloride
RFLP	restriction fragment length polymorphism
RGM	rapidly growing mycobacteria
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCOTS	selective capture of transcribed sequences
SDS	sodium dodecyl sulfate
ssGPL	serovar-specific gylcopeptidolipids
TB	tuberculosis
TU	turbidity units
USEPA	US Environmental Protection Agency
WHO	World Health Organization
ZN	Ziehl-Neelsen

1

Introduction

G. Nichols, T. Ford, J. Bartram, A. Dufour and F. Portaels

1.1 ENVIRONMENTAL MYCOBACTERIA

Environmental mycobacteria can be found in diverse environments and most appear to exhibit a saprophytic lifestyle. However, some have the ability to infect animals, birds and humans, and have evolved mechanisms by which they can invade and grow within host cells. Because the number of organisms shed back into the environment from infected animals can be relatively small, and heavy and widespread colonization of some environments occurs, it remains rather unclear what role animal/human infection plays in the life-cycle of many of these organisms.

Because these organisms are widespread in the environment, and there is little evidence that person-to-person transmission is common, there is an implicit assumption that environmental mycobacterial infections derive from water, food, the environment or contact with animals. There is evidence to support this assumption in many cases, although the source of infection in most

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remains unclear. A variety of mycobacterial species causing human disease have been linked to contaminated water (Table 1.1). However, some of these links can result from diagnostic uncertainty associated with differentiating contamination of patients or their specimens from human disease caused by environmental mycobacteria; a topic that is dealt with in later chapters.

Members of the MAC are responsible for the majority of non-*M. tuberculosis* mycobacteria infections in developed countries (Horsburgh 1996). The main presentations of MAC are lymphadenitis in children, respiratory infection in the elderly, respiratory, intestinal and disseminated disease in HIV-positive people and infections in people with other immunocompromising conditions. In areas of developing countries where *M. ulcerans* is endemic, ulcerative disease can cause severe disability (refer to Chapter 8).

1.2 NOMENCLATURE AND TYPING

As methods for differentiating strains improve, the need to accurately type and name organisms becomes important for our understanding of the disease epidemiology. With environmental mycobacteria this is no less important. Much of the literature in the last twenty years contains terms like MAC and MAIS for a group of organisms with different properties and virulence characteristics. MAC includes *M. intracellulare*, *M. avium* and its subspecies MAA, MAP and *M. avium* subspecies *sylvaticum*. Because MAP is very slow growing, fastidious and requires special media for its isolation, the term MAC is usually used to indicate members of the complex other than MAP, although strictly speaking MAP is a member. *M. lepraemurium* is related to *M. avium* and causes a leprosy like disease in mice, rats and cats (Rojas-Espinosa & Lovik 2001). Like MAP it is difficult to cultivate and very slow growing.

The MAIS complex includes *M. scrofulaceum*, *M. avium* and *M. intracellulare*, although MAIS is a less commonly used term. The term *M. avium-intracellulare* is for most purposes identical to MAC. One of the difficulties of using this nomenclature is that differences in the individual species have not been highlighted and therefore the epidemiology is less clear. There is support for strains from humans and pigs being reclassified as *M. avium* subspecies *hominissuis* because of their differences from bird strains, and this reclassification may be useful in revising our understanding of the epidemiology of this group of diseases (Mijs *et al.* 2002). Another example where typing can be useful is with MAP. There are typing differences between the organisms found in sheep and cattle and a lack of clarity about the zoonotic origin of organisms derived from humans.

Table 1.1 Evidence of pathogenic *Mycobacterium* species in water identified by selected references

Species	Natural waters	Drinking water	Drinking water biofilm	Sewage	Hospital water	Hospital equip-ment	Hot water systems	Recrea-tional water	Injections	Indus-trial water	Damp build-ings	Mine water
M. bohemicum	Torkko *et al.* (2001)											
M. chelonae subsp *abscessus*									Zhibang *et al.* (2002)			
M. fortuitum	Jin *et al.* (1984)			Jin *et al* (1984)								
M. genavense		Ristola *et al.* (1999)			Hillebrand-Haverkort *et al* (1999)							
M. immunogenum										Wallace *et al.* (2002)		
M. intracellulare			Falkinham *et al.* (2001)					Saito & Tsuka-mura (1976)				
M. kansasii		Kaustova *et al.* (1981)			Wright *et al* (1985)				Domergue *et al.* (2001)	Chobot *et al.* (1997)		
M. malmoense	Portaels *et*											

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Species	Natural waters	Drinking water	Drinking water biofilm	Sewage	Hospital water	Hospital equip-ment	Hot water systems	Recrea-tional water	Injections	Indus-trial water	Damp build-ings	Mine water
	al. (1995)											
M. marinum												Slosarek *et al.* (1994)
M. terrae	Tuffley & Holbeche (1980)	Jin *et al.* (1984)	Schulze-Robbecke *et al.* (1992)		Lockwood *et al.* (1989)			Dailloux *et al.* (1980)	Zenone *et al.* (1999)		Huttunen *et al.* (2001)	
M. tusciae		Tortoli *et al.* (1999)										
M. xenopi	Torkko *et al.* (2000)	Sniadack *et al.* (1993)			Wright *et al.* (1985)	Bennett *et al.* (1994)	Wright *et al.* (1985)	Slosarek *et al.* (1994)				

1.3 UNDERSTANDING THE DISEASE

Primary *M. avium* infections, including non-tuberculous lymphadenitis (inflammation/swelling of the lymph nodes), can occur in children, although infection is more common in patients with pre-existing pulmonary disease. Most diagnosed infections occur in people who are severely immunocompromised. Infections can affect the respiratory and gastrointestinal tracts and may produce a generalized infection. MAA is the most frequent mycobacterial subspecies isolated from patients with AIDS and often causes serious disseminated disease: *M. intracellulare* is more common in immunocompetent people (Guthertz *et al.* 1989).

1.3.1 The epidemiology of environmental mycobacteria

As a result of the dramatic increase in HIV-associated MAC infection in developed countries, MAC has received more attention than many other environmental mycobacteria. Disease can result from infection by one or more strains of different species (Conville *et al.* 1989), serotypes (Dawson 1990) or genotypes (Arbeit *et al.* 1993; Mazurek *et al.* 1997) of MAC, sometimes in combination with a mycobacterium other than MAC (Levy-Frebault *et al.* 1987; Falkinham 1996), including MTB (Epstein *et al.* 1997; Raju & Schluger 2000). HIV-positive patients frequently have a variety of concurrent infections, such as respiratory co-infection with *Pneumocystis carinii* (Raju & Schluger 2000). The observation that higher rates of disseminated MAC in AIDS patients occur in developed rather than developing countries is probably due to differences in both exposure and immunity (von Reyn *et al.* 1996) and high rates of infection with MTB.

Mycobacterial infections linked to contaminated hospital water have been recognized for many years (Wallace *et al.* 1998), and MAC has been isolated from hospital waters (Graham *et al.* 1988), particularly hot water systems (du Moulin *et al.* 1988). However, diagnostic difficulties can result from contamination of patients or their specimens by the use of non-sterile water during sample processing (Stine *et al.* 1987; Graham *et al.* 1988). It is likely that hospital waters represent a source of infection for immunocompromised patients (du Moulin *et al.* 1988; Peters *et al.* 1995). du Moulin *et al.* (1988) and Gurtler (1994) have suggested that contaminated showers may be a source of infection for HIV patients, although, in contrast, a case-control study of the risk factors for MAC in HIV-positive patients found that showering was protective (Horsburgh *et al.* 1994). A study of mycobacteria in swimming pool water in Finland found no MAC among the many mycobacteria isolated (Iivanainen *et*

al. 1999c), but MAC were recovered from pools and spas in the Netherlands (Havelaar *et al.* 1985). MAC infections have also been linked to hot tub use (Embil *et al.* 1997; Mangione *et al.* 2001), and there is some evidence that use of water transported over long distances represents an increased risk for infection (du Moulin *et al.* 1985).

A range of environmental mycobacteria have been recovered from bronchoscopes, bronchoscopy specimens (Dawson *et al.* 1982; Stine *et al.* 1987) and other clinical specimens from patients whose infections are related to the use of bronchoscopes. Mycobacteria have also been isolated during pseudo-epidemics where contaminated bronchoscope washers have been implicated as the source of infection (Gubler *et al.* 1992; Sniadack *et al.* 1993; Maloney *et al.* 1994; Wallace *et al.* 1998; Kressel & Kidd 2001; see also Chapter 10).

A prospective cohort study of AIDS patients in developed and developing countries found patient rates of disseminated MAC were 10.5-21.6% in New Hampshire, Boston and Finland compared to 2.4-2.6% in Trinidad and Kenya ($p < 0.001$) (von Reyn *et al.* 1996a). PPD skin test reactions greater than or equal to 5 mm were present in 20% of patients from Kenya compared to 1% at other sites ($p < 0.001$). Among patients from the United States and Finland, multiple logistic regression indicated that occupational exposure to soil and water was associated with a decreased risk of disseminated MAC; whereas low CD4 count, swimming in an indoor pool, history of bronchoscopy, regular consumption of raw or partially cooked fish/shellfish and treatment with granulocyte colony-stimulating factor were associated with an increased risk of disseminated MAC (von Reyn *et al.* 1996).

MAC have been isolated from a variety of supermarket foods (Argueta *et al.* 2000), and the apparent similarity between some food and clinical isolates suggests that food may be an important source of infection (Yoder *et al.* 1999). MAC isolates can be relatively resistant to heating and will survive pasteurization if present in sufficient numbers in raw milk (Grant *et al.* 1996). Not surprisingly, MAC have been recovered from raw and pasteurized milk (Hosty & McDurmont 1975). As part of an epidemiological study of patients with MAC and AIDS in San Francisco, food samples from the houses of patients were cultured for MAC. The organism was recovered from only 1 of 397 food samples, suggesting that this was not the principal route of transmission (Yajko *et al.* 1995). A significant association has been found between MAC infection in HIV-positive people and the consumption of hard cheese (Horsburgh *et al.* 1994). MAC have also been recovered from cigarettes (Eaton *et al.* 1995), and it has been suggested that this may contribute to MAC disease in smokers (Falkinham 1996). There is also some evidence that MAC infections are more common in people such as miners and farmers who are exposed to dust (Falkinham 1996).

1.3.2 Risk factors

People throughout the world are exposed to environmental mycobacteria. However, substantial differences in exposure occur at different ages, as measured by skin tests, and in different geographical regions. The overall picture suggests greater exposures in developing countries. While exposure to environmental mycobacteria may confer some resistance to infection with *M. tuberculosis,* it also appears to reduce the effectiveness of the BCG vaccine in protecting against *M. tuberculosis*. The links between HIV and TB within the developing world in particular make it important to determine whether environmental mycobacteria are contributing indirectly to the burden of disease associated with the HIV pandemic. In the developed world, MAC disease has been a significant contributor to the morbidity and mortality associated with HIV. However, since the introduction of HAART to control HIV replication, MAC disease has become less of a clinical problem in these patients.

1.4 PATHOGENIC MYCOBACTERIA IN WATER

In 1997, Hunter reported that about eight species of mycobacteria had been associated with waterborne transmission of human disease (Hunter 1997). These species included *M. avium* complex, *M. fortuitum, M. gordonae, M. marinum, M. scrofulaceum, M. terrae, M. ulcerans,* and *M. xenopi.* Today the list continues to grow, with the possible addition of *M. chelonae, M. immunogenum, M. abscessus, M. kansasii, M. ulcerans, M. szulgai, M. simiae, M. palstre* and MAP. There has been an increase in the number of potentially pathogenic mycobacterial species whose transmission route is associated with water (Table 1.1). This, in part, is due to industrial and institutional exposures that have resulted in respiratory infections that have previously been misdiagnosed. In some cases, the causative agents had yet to be described, e.g. *M. immunogenum* in metal-working fluids (Shelton *et al.* 1999). No doubt further species will emerge as water-related infections as both our epidemiological and monitoring tools continue to improve.

1.4.1 Water supply

Water remains an important potential source of human exposure to environmental mycobacteria. Organisms such as MAP, although difficult to recover from environmental samples, are excreted in large numbers in the faeces of infected animals and are likely to be present in source waters that are abstracted for drinking-water.

Some species, such as *M. kansasii,* can colonize cold water distribution systems whilst *M. xenopi* and *M. avium* are more commonly associated with hot water systems. In contrast, *M. marinum* in swimming pools or aquaria can cause infection of skin abrasions. Contamination of liquids that are injected can cause abscesses (*M. chelonae, M. fortuitum*), and other iatrogenic infections are linked to contaminated endoscope washers and renal dialysis fluid.

The difficulty with investigating the waterborne transmission of mycobacteria is firstly that the infections are generally sporadic (there are some outbreaks), secondly there are a variety of sources of exposure other than water, and thirdly that the typing schemes that are routinely available are not sufficiently discriminating to confidently identify whether isolates from the environment are the same as those from associated patients.

1.4.2 Recently reported cases of waterborne mycobacterial disease

Table 1.2 lists a sample of recently reported cases of environmental mycobacterial diseases. It is important to remember that the vast burden of Buruli ulcer (caused by *M. ulcerans*) in tropical countries is not reflected in this table. An association between water exposure and Buruli ulcer in endemic areas is suspected, but the evidence is inconclusive (refer to Chapter 8). In fact, a recent study suggests that the disease may be at least partially vectorborne, transmitted by an aquatic insect (Marsollier *et al.* 2002).

For MAP, the connection with CD is controversial (refer to Chapter 6, the article by Sechi *et al.* 2001 and response by Roholl *et al.* 2002). At present, there would appear to be no epidemiological studies linking MAP in water with human disease. From a public health stand point this is a much-needed area of research/clarification.

1.5 GLOBAL BURDEN OF DISEASE

According to the World Health Report for 2002 (WHO 2002), mortality due to infectious diseases accounts for 19.3% (10 932 000 of 56 554 000) of total deaths. In terms of the burden of disease, as measured in DALYs, the contribution from infectious diseases is 24.5% (359 377 000 of 1 467 257 000) with TB, HIV/AIDS and malaria responsible for nearly half of this burden (WHO 2002).

Table 1.2 Waterborne outbreaks of disease caused by environmental mycobacteria, or where water is strongly implicated in transmission

Organism	Disease	Source	Nos. infected	Reference
MAC	Pulmonary disease	Hot tub	Family of 5	Mangione *et al.* 2001
MAC	Pulmonary disease	Hot tub	Family of 5	Embil *et al.* 1997
MAC	Cutaneous infection	Circulating bath water	3	Sugita *et al.* 2000
MAC	Hypersensitivity pneumonitis	Hot tub	2 case studies	Rickman *et al.* 2002
M. fortuitum	Furunculosis	Whirlpool footbaths at a nail salon	110	Winthrop *et al.* 2002
M. fortuitum	Respiratory tract colonization	Hospital ice machine	19	Labombardi *et al.* 2002
M. fortuitum	Respiratory tract colonization	Hospital ice machine	47	Gebo *et al.* 2002
M. chelonae	Pseudo-outbreak	Contaminated endoscopy washer	-	Kressel & Kidd 2001
M. chelonae	Cutaneous abscesses	Tap water contaminated instruments in liposuction	34	Meyers *et al.* 2002
M. immunogenum	Hypersensitivity pneumonitis	Metal removal fluids	Several case studies	Shelton *et al.* 1999
M. abscessus	Sporotrichoid dermatosis	Public bath	2 case studies	Lee *et al.* 2000a
M. marinum	Cutaneous infection	Aquarium management	3 case studies	Dorronsoro *et al.* 1997
M. marinum	Ulcerated nodule	Aquarium	14 month old girl	Speight & Williams 1997
M. kansasii	Cellulitis	Swimming at a beach	1 immuno-compromised patient	Hsu *et al.* 2002
M. ulcerans	Ulcerative disease	Irrigation waters	29	Ross *et al.* 1997
M. szulgai	Keratitis	Intraoperative contamination from ice water	5	Holmes *et al.* 2002
M. palstre	Cervical lymphadenitis	Potential for water exposure	1 child + veterinary and natural water isolates	Torkko *et al.* 2002

In the USEPA's Mycobacteria: Drinking-water fact sheet (EPA, 2002), the CDC estimates that 1.8 out of 100 000 individuals are infected by non-AIDS related mycobacterial diseases each year in the USA. The burden within the American AIDS population has been as high as 50% (primarily MAC) (Horsburgh 1991), although antiretroviral therapy has reduced this rate in recent years (Horsburgh *et al.* 2001). Two and a half million people are estimated to be living with HIV in industrial countries (United States, Canada, Australia, New Zealand, Japan, and Western and Eastern Europe), most of who have or will have access to antiretroviral therapy. In developing countries, where HIV/AIDS prevalence can exceed 30% in the adult population (e.g. Botswana, Lesotho, Swaziland and Zimbabwe) (AIDS 2002), access to antiretroviral therapy can be extremely limited. In fact, the AIDS 2002 Barcelona report suggests that a total of only 158 168 people have received or are receiving antiretroviral therapy in "low and middle income" countries (AIDS 2002). However, the burden of mycobacterial diseases may not be as high as in developed countries, at least for MAC-related infections. This is thought to be due, in part, to the relatively short survival times of people after conversion to AIDS (Maartens 2002).

Based on CDC estimates for the USA, the global burden of non-AIDS related mycobacterial diseases would exceed 110 000 per year. However, applying American infection rates to other countries may be very unrealistic, particularly where nutritional and other indicators of health status suggest high susceptibility to disease. Of particular note are the numerous endemic areas for Buruli ulcer (at least 32 countries) caused by *M. ulcerans*. Although considered the third most common mycobacterial infection of humans after TB and leprosy, the actual burden of this disease is unknown (http://www.who.int/gtb-buruli/). Potential transmission by an insect vector raises specific issues related to public health and the potential to manage the disease (refer to Chapter 8).

At the end of 2001 over 40 million adults and children were reported to be living with HIV/AIDS: over 71% in sub-Saharan Africa. Although at present, rates of infection with MAC within the HIV/AIDS population in developing countries may be lower than in developed countries, any improvement in longevity of AIDS sufferers may place them at greater risk from these opportunistic infections.

People in different parts of the world appear to be exposed to MAC and other environmental mycobacteria in different ways (von Reyn *et al.* 1993, 1996). Evidence of exposure in Africa is based on skin tests (von Reyn *et al.* 1993, 1996; Fine *et al.* 2001) and INFγ responsiveness to MAC PPD using whole blood (Black *et al.* 2001). The evidence suggests that people in Malawi are commonly exposed to environmental mycobacteria. The growing evidence that environmental mycobacterial exposure reduces the ability of people to develop an effective immune response to TB following BCG vaccination means that

protection programmes may be influenced by exposure. This has clear public health implications in determining the effectiveness of BCG vaccination programmes (Black *et al.* 2001; Fine *et al.* 2001). It remains unclear whether differences in such immunological tests are a good measure of exposure to MAC or represent the extent of sub-clinical disease.

Estimating the amount of infection and disease associated with environmental mycobacteria is both difficult and inherently inaccurate. This is true even in HIV where disease caused by MAC can affect a significant proportion of the population. The extent of MAC contamination of the environment suggests that people are commonly exposed to this and other environmental mycobacteria but do not necessarily develop an immune response following such exposures. The substantial regional variation in reported infections with different environmental mycobacteria makes global generalization problematic.

1.5.1 Crohn disease and Johne disease

The causes of CD have been the subject of much microbiological and epidemiological interest over the last 30 years. There is some microbiological and clinical data that suggests that MAP is involved with the pathology of this disease, and possibly even its causation. One reason MAP has attracted interest is its role in JD in herbivores, and the relative similarity of the pathology of the two conditions. However, CD appears to have a genetic component and causation may have multiple factors. The isolation of MAP from pasteurized cows' milk has lead to concerns that people may be exposed to this organism through the food chain. Also, as many agricultural animals excrete large numbers of MAP in their faeces, it is reasonable to assume that source waters for drinking-water supplies may be contaminated with this organism. So far, MAP has not been isolated from drinking-water in England, although molecular studies are beginning to indicate the potential presence of MAP in surface waters (Chapter 6). There are technical difficulties in isolation from both clinical and environmental samples that make confirmation of the causative role of MAP in CD and the assessment of environmental exposure difficult. It is likely that water will prove to be one of a number of sources from which people are exposed to MAP. While there is little current evidence that improving drinking-water treatment will have any impact on the incidence of CD, water companies and utilities should consider whether any action is required to adopt a more precautionary approach. A detailed discussion of the links between MAP and CD is included in Chapter 6.

1.6 CONTROL MEASURES

The preceding sections show that there is growing evidence that people are acquiring mycobacterial infections from organisms present in water, food and the environment. However, exposure to these organisms without the development of overt symptoms, but with the development of an effective immune response, is relatively common. Therefore, should we be trying to reduce or increase people's exposure in order to reduce the risks of infection?

There are clear winners and losers in this immunological conflict. People who are at a vulnerable stage of development (in particular children and the elderly) or are immunocompromised (HIV), and are immunologically naïve to the particular environmental mycobacteria, may succumb to infection if exposed. In the developed world, infection with environmental mycobacteria is sufficiently uncommon to make prevention through mass immunization an unattractive solution. We are in a position in which the majority of people must be relatively resistant to most environmental mycobacteria, given their ubiquity in the environment, and further reduction in environmental exposure is unlikely to significantly affect risk. There is, however, some rationale for reducing individual exposures to some potential sources, such as whirlpool baths and showers that might represent a significant risk of infection. For particular "at risk" populations (e.g. HIV-positive individuals) there is practical risk advice for reducing the chances of exposure to MAC.

1.7 GENOMIC AND EVOLUTIONARY PERSPECTIVES

Basic information on the characteristics of mycobacterial genomes has been important in understanding their biology, classification and taxonomy for many years (Imaeda *et al.* 1982; Bercovier *et al.* 1986; McFadden *et al.* 1987b; Antoine *et al.* 1988). The recent projects to sequence a number of *Mycobacterium* species (Table 1.3) have resulted in an expanding dataset that can be used for comparative genomics (Behr *et al.* 1999; Brosch *et al.* 2000, 2001; Cole, 2002a; Gordon *et al.* 2002; Schoolnik 2002), vaccine and drug candidate selection (Cockle *et al.* 2002; Cole 2002b) and pathogenic and evolutionary genetics (Brosch *et al.* 2001; Fitzgerald & Musser 2001). There is extensive gene loss in the strictly parasitic mycobacterial species (e.g. *M. tuberculosis, M. leprae*) (Brosch *et al.* 2001) and IS may be responsible for these losses (Ho *et al.* 2000).

Table 1.3 *Mycobacterium* genome sequencing projects

Organism	Genome size/characteristics	Institution sequencing genome
M. avium 104	The genome is about 5.48 mb long and has a G+C content of 68.5%	The genome sequence is nearing completion at the Institute for Genomic Research
M. bovis AF2122/97	The genome is 4 345 492 bp long, with a G+C content of 65.63%.	The Sanger Institute with the Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, and the Veterinary Laboratories Agency. Funded by Beowulf Genomics and the Ministry of Agriculture Fisheries and Food.
M. leprae	The complete sequence is 3 268 203 bp in length with a G+C content of 57.8%. There are 1604 protein-coding genes and 1116 pseudogenes.	The Sanger Institute with the Unité de Génétique Moléculaire Bactérienne, Institut Pasteur. Funded by the New York Community Trust.
M. marinum	The genome is approximately 6.589 Mb with a G+C content of around 65%. At present there are 138 601 reads totalling 59.714 Mb and giving a theoretical coverage of 99.99% of the genome. At present, there are 88 contigs > 1kb (65 contigs > 2kb).	The Sanger Institute with the University of Washington, Seattle; the Unité de Génétique Moléculaire Bactérienne, Institut Pasteur; Monash University and Monash Medical Centre, Australia; the Department of Microbiology, University of Tennessee, Knoxville. Funded by Beowulf Genomics.
M. microti OV254	A minimal set of 50 bacterial artificial chromosome clones that covers almost all of the genome of *M. microti* OV254	
M. paratuberculosis K10	The genome sequencing is now complete, and the annotation is in progress. The genome is a single circular chromosome 4 830 869 bp in length, with 69.30% G+C content, and contains over 4000 open reading frames.	University of Minnesota funded by the US Department of Agriculture – Agricultural Research Service.
M. smegmatis MC2 155		The Institute for Genomic Research funded by National Institute of Allergy and Infectious Disease.
M. tuberculosis H37Rv	The sequence is 4 411 529 bp long with an overall G+C content of 65.6%, and with 3924 predicted protein coding genes. (Cole *et al.* 1998)	The Sanger Institute with clones supplied by the Unité de Génétique Moléculaire Bactérienne, Institut Pasteur. Funded by the Wellcome Trust
M. tuberculosis CDC 1551		National Center for Biotechnology Information, National Institute of Health, Bethesda, Maryland, USA

1.8 KEY RESEARCH ISSUES

What, then, are the important areas where research needs to be targeted in order to protect public health in the longer term? Given the apparent associations between exposure to environmental mycobacteria and lack of responsiveness to BCG vaccination there needs to be an approach to the development of an improved TB vaccine, preferably one that offers protection against the important environmental mycobacteria as well as greater protection against TB.

There is a limited understanding of the social, environmental and personal risk factors responsible for initiating disease caused by most of the environmental mycobacteria and this is aggravated by limited use of typing and environmental testing in investigating individual cases of infection. Greater effort needs to be put into sensitive typing methods and their use in elucidating the epidemiology of these diseases. Sequencing the genomes of further environmental mycobacteria will undoubtedly benefit our long-term understanding of these organisms.

The challenge of the next decade will be to convert our improved knowledge of the organism, immunology, disease and risk factors into a strategy to reduce the morbidity and mortality caused by these organisms that is applicable worldwide. This will require targeted responses to key individual mycobacterial pathogens such as *M. ulcerans* and coordination of these responses with WHO Programmes concerned with TB.

2

Natural ecology and survival in water of mycobacteria of potential public health significance

J.O. Falkinham, G. Nichols, J. Bartram, A. Dufour and F. Portaels

Mycobacteria can be recovered from a wide variety of environmental niches and MAC has been recovered from both fresh water (ponds, lakes, rivers, bogs and swamps), brackish, sea water and wastewater (Martin *et al.* 1987; Falkinham 1996; Torkko *et al.* 2000, 2001), sometimes in high numbers (Kirschner *et al.* 1999). MAC has been recovered from drinking-water systems before and after treatment, from the distribution system and from raw source waters (Falkinham *et al.* 2001). Mycobacterial numbers were higher in the distribution system samples (average 25 000-fold) than in those collected just after treatment, suggesting that they grow in distribution. The increase in mycobacterial numbers correlated with AOC and biodegradable organic carbon levels. MAC

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are relatively resistant to chlorine, monochloramine, chlorine dioxide and ozone (Taylor *et al.* 2000). Soil is also a significant reservoir. Environmental growth of mycobacteria may be enhanced in low pH soils (Iivanainen *et al.* 1999a). Mycobacterial contamination, including MAC, has been demonstrated in mouldy buildings (Huttunen *et al.* 2000).

2.1 THE ECOLOGY OF ENVIRONMENTAL MYCOBACTERIA

2.1.1 Protozoa, helminths and insects

Some environmental mycobacteria have been shown to grow within amoebae (Cirillo *et al.* 1997; Steinert *et al.* 1998; Miltner & Bermudez 2000; Skriwan *et al.* 2002) and it is suggested that this may provide a haven when environmental conditions deteriorate (Steinert *et al.* 1998; Miltner & Bermudez 2000). Strahl *et al.* (2001) have shown that environmental mycobacteria can be phagocytised and grow within the ciliate *Tetrahymena.* This association with amoebae and ciliates may enhance entry, growth and virulence (Cirillo *et al.* 1997). An epizootic of *M. avium* in flamingos was coincident with an algal bloom in the water (Kock *et al.* 1999). It also provides a parasitic cycle within the environment that may partly explain the opportunistic pathogenicity of human infection.

While environmental transmission within amoebae is a possibility, it remains unclear whether amoebae, or any other protozoa, play a role in the pathogenesis or epidemiology of any mycobacterial diseases. In addition, the demonstration of *M. ulcerans* in the salivary glands of aquatic insects (Portaels *et al.* 1999; Marsollier *et al.* 2002) and the demonstration of MAP in trichostrongylid nematode larvae (Lloyd *et al.* 2001; Whittington *et al.* 2001) and earthworms (Fischer *et al.* 2003) suggest that the ecology of many environmental mycobacteria may be considerably more complicated than we currently appreciate. Moreover, the burden of intestinal helminths in people infected with mycobacteria may affect the course of disease through stimulation of the host Th2 response (Diniz *et al.* 2001).

2.1.2 Infections in birds and animals

In contrast to *M. avium* infection in wild and domestic birds, *M. avium* infection in mammals occurs only sporadically and is rarely transmissible (Thorel *et al.* 2001). Infection is usually chronic and generalized disease is uncommon, but disseminated disease has been reported in captive hoofed animals and immunosuppressed dogs and cats. The majority of *M. avium* and *M. intracellulare* infections in livestock are detected at slaughter and lesions are

mostly restricted to lymph nodes close to the alimentary tract. The zoonotic potential of MAC infections is poorly understood.

MAC causes infections in a wide range of animals including water buffalo (Freitas *et al.* 2001), cattle (Bollo *et al.* 1998), pigs (Morse & Hird 1984; O'Grady *et al.* 2000; Pavlik *et al.* 2000; Ramasoota *et al.* 2001), deer (Robinson *et al.* 1989; Fawcett *et al.* 1995; Hunter 1996; O'Grady *et al.* 2000) and horses (Sills *et al.* 1990; Helie & Higgins 1996; Leifsson *et al.* 1997). MAC causes infections in cats (Kaufman *et al.* 1995) and dogs (Shackelford & Reed 1989; Miller *et al.* 1995; Horn *et al.* 2000), armadillos (Dhople *et al.* 1992) and cynomolgus and rhesus macaques (Fleischman *et al.* 1982; Bellinger & Bullock 1988; Goodwin *et al.* 1988). MAC disease is more common in farmers (Falkinham 1996) possibly as a result of contact with animals or their products.

M. avium is a significant cause of disease in endangered marsupial species held in captivity (Mann *et al.* 1982; Schoon *et al.* 1993; Montali *et al.* 1998; Buddle & Young 2000). Experimental infections in ferrets indicate that *M. bovis* is more pathogenic than *M. avium* (Cross *et al.* 2000).

Avian mycobacteriosis affects companion, captive exotic, wild and domestic birds and is most commonly caused by *M. avium* and *M. genavense*. Lesions are commonly found in the liver and gastrointestinal tract, but can affect other organs (Tell *et al.* 2001). MAC causes infections in chickens (Odiawo & Mukurira 1988), white carneaux pigeons (*Columbia livia*) (Pond & Rush 1981), commercial emus (*Dromaius novaehollandiae*) (Shane *et al.* 1993) and farmed rheas (*Rhea americana*) (Sanford *et al.* 1994).

MAC infections in birds appear not to be the source of most human infections (Martin & Schimmel 2000; Pavlik *et al.* 2000a), although MAC lymphadenitis was reported in two children who lived in close proximity to a pigeon loft (Cumberworth & Robinson 1995).

2.1.3 Infections in fish

Mycobacteria can cause disease in fish (Astrofsky *et al.* 2000; Heckert *et al.* 2001). A prospective cohort study of the rate of disseminated infection due to NTM (predominantly MAC) among Finnish AIDS patients found urban residence ($p = 0.04$) and eating raw fish ($p = 0.04$) as independent risk factors (Ristola *et al.* 1999). A study of MAC infection in AIDS patients in developed and developing countries found that among American and Finnish patients occupational exposure to soil and water was protective; whereas, swimming in an indoor pool and regular consumption of raw or partially cooked fish/shellfish were associated with an increased risk of disseminated MAC (Fordham *et al.* 1996c).

2.2 PHYSIOLOGIC CHARACTERISTICS OF *M. AVIUM* RELEVANT TO ITS ECOLOGY AND DISTRIBUTION

The physiology of *M. avium, M. intracellulare* and other mycobacteria determines their presence and number in different environmental habitats. Although *M. avium* is found in waters and soils throughout the world (including North America, Europe, Africa, Australia and Asia) (von Reyn *et al.* 1993), the sites from which it is isolated in highest numbers point to those physiological characteristics that are determinants of its ecology.

2.2.1 Physiologic characteristics of *M. avium* that are determinants of its ecology

2.2.1.1 Growth characteristics

M. avium is a member of the slow-growing mycobacteria. Generation times in rich laboratory medium are usually one day. Slow growth does not reflect a slow metabolism. Rather, slow growth is a consequence of the presence of a single rRNA gene cluster (Bercovier *et al.* 1986), the energy requirements of synthesis of long chain fatty acids (C_{60}-C_{80}), lipids and waxes (Brennan & Nikaido 1995), and the impermeability of the lipid-rich cell wall (Rastogi *et al.* 1981; Brennan & Nikaido 1995). Although slow growth has drawbacks, slow growth also means that *M. avium* dies relatively slowly. As a consequence, *M. avium* can survive starvation and antimicrobial and disinfectant exposure. In fact, *M. avium* may be able to induce protective responses that can act before irreversible processes involving cell division occur.

One contributor to slow growth of *M. avium* and other mycobacteria is the impermeable cell wall (Brennan & Nikaido 1995). However, impermeability also results in the resistance of *M. avium* to antibiotics (Rastogi *et al.* 1981), heavy metals (Falkinham *et al.* 1984; Miyamoto *et al.* 2000) and disinfectants (Safranek *et al.* 1987; Pelletier *et al.* 1988; Best *et al.* 1990; Taylor *et al.* 2000). Resistance to ozone and chlorine-based disinfectants (Taylor *et al.* 2000) is undoubtedly one reason why *M. avium, M. intracellulare* and other mycobacteria grow and persist in drinking-water distribution systems (Covert *et al.* 1999; Falkinham *et al.* 2001). Heavy metal resistance may permit *M. avium* and *M. intracellulare* to populate habitats unavailable to metal-sensitive microorganisms; for example, heavy metal resistance may allow *M. avium* and *M. intracellulare* to attach to metal surfaces and serve as biofilm pioneers. Furthermore, high numbers of *M. avium* are associated with high concentrations of zinc (Kirschner *et al.* 1992) suggesting that galvanized (i.e. Zn-coated) pipe surfaces might be a preferred habitat.

2.2.1.2 *M. avium hydrophobicity*

The presence of fatty acids, lipids and waxes in the cell wall of *M. avium* and other mycobacteria is responsible in part for the extreme hydrophobicity of the cells. Mycobacteria are the most hydrophobic of bacteria (van Oss *et al.* 1975). The high hydrophobicity leads to adsorption to rising air bubbles in water and their enrichment in ejected droplets, their preference to attach to surfaces (e.g. pipes), and to phagocytosis by macrophages (van Oss *et al.* 1975) and protozoa (Strahl *et al.* 2001). High hydrophobicity leads to their concentration at air:water interfaces (Wendt, *et al.* 1980), where organic matter is concentrated (Blanchard & Hoffman 1978) by the same process of preferential adsorption to rising air bubbles.

2.2.1.3 *M. avium response to temperature, oxygen, pH, and salinity*

M. avium can grow over a wide range of temperatures (George *et al.* 1980). Its ability to grow at 45 °C (Mijs *et al.* 2002) is undoubtedly responsible for its presence in hot water systems (du Moulin *et al.* 1988). Not only can *M. avium* grow at 45 °C, but *M. avium* and a number of other environmental mycobacteria are relatively resistant to high temperature (Schulze-Röbbecke & Buchholtz 1992). During the summer, water in the coastal brown-water swamps of the eastern United States is at 45 °C or higher (Parker & Falkinham, unpublished measurement).

M. avium and *M. intracellulare* are capable of growth at reduced oxygen levels. Both species grow rapidly in 12% and 21% oxygen (air) (Lewis & Falkinham, unpublished). Growth occurs at 6% oxygen though at half the rate as in air. The ability to grow at low oxygen concentrations is reflected by the fact that waters and soils yielding highest numbers of *M. avium* and *M. intracellulare* have low oxygen levels (Brooks *et al.* 1984a; Kirschner *et al.* 1992). Neither *M. avium* nor *M. intracellulare* grow anaerobically (Lewis, personal communication). In contrast to members of the *M. tuberculosis* complex, *M. avium* can survive rapid shifts to anaerobiosis (Lewis & Falkinham, unpublished).

M. avium and *M. intracellulare* have acidic optima for growth. The pH range for growth of the two species is wide, but highest rates of growth occur within the pH 5-6 range (Portaels & Pattyn 1982; George & Falkinham 1986). Furthermore, *M. avium* is resistant to acid and the acidic conditions of the human stomach (Bodmer *et al.* 2000). Growth and tolerance of low pH provides an explanation for the high numbers of *M. avium* and *M. intracellulare* in soils and waters of peat-rich boreal forest soils and acid, brown-water swamps.

M. avium and *M. intracellulare* grow in fresh and brackish waters (George *et al.* 1980); indeed, growth in natural waters containing 1% NaCl (brackish) is

faster than growth in natural fresh waters. The ability to grow in brackish water explains the high numbers of *M. avium* and *M. intracellulare* in the tidal waters of large estuaries like the Chesapeake Bay of the eastern United States and in the Gulf of Mexico. It also suggests that *M. avium* and *M. intracellulare* are capable of shifting from an Na^+ rich environment (e.g. estuary) to a K^+ rich environment (within macrophage or cells of protozoa or amoebae) without loss of viability.

2.2.1.4 M. avium metabolism

M. avium can grow in natural waters containing low dissolved carbon (George *et al.* 1980) and in drinking-water distribution systems (Falkinham *et al.* 2001). It should be rightly considered an oligotroph. The growth of *M. avium* and *M. intracellulare* is stimulated by humic and fulvic acids (Kirschner *et al.* 1999). Numbers of *M. avium* and *M. intracellulare* correlate with humic and fulvic acid concentrations (Kirschner *et al.* 1999). Humic and fulvic acids are the principal organic compounds in waters draining from peat-rich boreal forest soils (Iivanainen *et al.* 1997a) and acid, brown-water swamps (Kirschner *et al.* 1999).

2.2.2 *M. avium* physiologic ecology

The widespread presence of *M. avium* in waters, soils, and other environments is due to its ability to exploit niches that are unoccupied by other, faster growing microorganisms. Clearly, an acidic pH optimum, ability to grow under reduced oxygen concentrations and stimulation of growth by humic and fulvic acids results in the high numbers of *M. avium* in two acidic, humic-rich environments: waters and soils from peat-rich boreal forest soils and acid brown-water swamps. Because these waters are used as sources for drinking-water, *M. avium* can be introduced into drinking-water systems. The very high resistance of *M. avium* to ozone and chlorine-based disinfectants allows the organism to persist and grow in drinking-water systems.

Disinfection of water can lead to selection of *M. avium, M. intracellulare* and other mycobacteria. In the absence of disinfection *M. avium* cannot compete effectively for limited nutrients. However, disinfection kills competitors permitting growth of *M. avium* on the available nutrients. This phenomenon is probably responsible for the growth of *M. avium* in drinking-water distribution systems (Falkinham *et al.* 2001) and its presence in hot tubs and spas (Embil *et al.* 1997).

The high hydrophobicity of *M. avium* leads to its adherence to surfaces. That, coupled with its resistance to heavy metals, means that it may be a pioneer of biofilm formation on metals. The ability of *M. avium* to grow at low oxygen levels means that in spite of the reduced oxygen concentration in biofilms (Stewart 1994) *M. avium* can grow.

High hydrophobicity also results in the adsorption of *M. avium* to air bubbles in water and the resulting concentration at the air:water interface. Concentration of *M. avium* and *M. intracellulare* at the air:water interface places it in an environment rich in organic matter where there are few competitors. Adsorption to bubbles leads to concentration in droplets ejected from water to air. Significant numbers of *M. avium, M. intracellulare* and other hydrophobic mycobacteria can be transferred from water to air by that mechanism.

2.3 HETEROGENEITY OF ENVIRONMENTAL ISOLATES OF *M. AVIUM*

2.3.1 Impact of heterogeneity on identifying sources of human infection

Surveys have demonstrated a great deal of heterogeneity amongst environmental isolates of *M. avium* and *M. intracellulare* (Frothingham & Wilson 1993, 1994). As a consequence of the high frequency of *M. avium* infection in AIDS patients (Horsburgh 1991) there was a great deal of interest in identifying the source of *M. avium*. This led to the development of methods for fingerprinting *M. avium*, culminating in the identification of *M. avium* strains from water samples with the same DNA fingerprint as those from AIDS patients who had been exposed to the water (von Reyn *et al.* 1994). It is important to understand that different typing methods will yield different results based on the level of discrimination. A marker may not be useful for fingerprinting and identifying sources of human infection but may be quite useful in placing isolates within epidemiologically important groups. For example, IS*901* is useful for distinguishing *M. avium* groups and may be a marker for a unique *M. avium* subspecies (Thorel *et al.* 1990).

The results of DNA fingerprinting methods have also led to proposals for revision of the taxonomy of the *M. avium* group (Thorel *et al.* 1991; Mijs *et al.* 2002). The lack of knowledge of *M. avium* characteristics that are associated with infection coupled with the fluid state of *M. avium* taxonomy and the heterogeneity of environmental isolates of *M. avium* means that any conclusions concerning identification of sources of human infection are tentative and provisional at this time.

2.3.2 *M. avium* fingerprinting methods

Fingerprinting methods can be used to identify an isolate from the environment as a member of the same clone as that recovered from a patient. Markers for fingerprinting should be present in all strains and in multiple copies to ensure a sufficient number of types. Because all isolates contain DNA and the presence

of DNA is unaffected by growth conditions (unlike phenotypic markers), DNA-based fingerprinting methods are preferred. Markers that are either too stable or too unstable are not suitable. However, the marker should demonstrate polymorphism in populations.

Sequences recognized by restriction endonucleases that make few cuts in DNA have served as markers suitable for fingerprinting *M. avium* (Arbeit *et al.* 1993; Slutsky *et al.* 1994). The large DNA fragments resulting from digestion by such restriction endonucleases are separated by PFGE. This technique was used to identify *M. avium* isolates from AIDS patients and water to which the patients were exposed (von Reyn *et al.* 1994).

IS*1245* is also valuable for DNA fingerprinting *M. avium*: it is present in multiple copies (Roiz *et al.* 1995); the fingerprint patterns in individual strains are stable (Bauer & Andersen 1999); and there is polymorphism in populations. A standard method for IS*1245* fingerprinting has been published (van Soolingen *et al.* 1998). It is not clear whether IS*1245* fingerprinting alone will be sufficient to provide unambiguous evidence of identity of patient and environmental *M. avium* isolates. Results of IS*1245* fingerprinting have identified clusters of types, but there has not been a comprehensive study comparing isolates from humans (e.g. AIDS patients) with environmental isolates that are linked to the patients through exposure to the environmental sample. For example, one study identified a unique cluster of "bird" types (Ritacco *et al.* 1998) and another identified an "AIDS-associated IS*1245* pattern" (Lair *et al.* 1998). These studies, coupled with IS*901* typing and grouping *M. avium* strains on the basis of the sequence of the rRNA ITS region (Frothingham & Wilson 1993, 1994), may lead to identification of types more likely to be associated with human and animal infection.

It is clear that *M. avium* taxonomy and fingerprinting is in a state of flux. What is needed is a comprehensive study of patient and epidemiologically linked environmental isolates in which every possible marker of utility is examined. Such a study will require recovery of many isolates from both patient and environmental samples because of the heterogeneity of *M. avium* isolates in environmental samples and polyclonal infection in patients.

2.4 CHANGES IN THE OCCURRENCE IN MYCOBACTERIAL SPECIES

2.4.1 Shift of *M. scrofulaceum* to *M. avium* in cervical lymphadenitis in children

There has been a dramatic change in the causative agent of mycobacterial-related cervical lymphadenitis in children in England (Colville 1993), the United States (Wolinsky 1995), and Australia (Dawson, personal communication). Historically, the major mycobacterial species recovered from children with cervical lymphadenitis was *M. scrofulaceum* (Wolinsky 1979). Currently, however, *M. scrofulaceum* is almost never isolated and *M. avium* is isolated (Colville 1993; Wolinsky 1995; Dawson, personal communication). Wolinsky (1995) estimated that the shift from *M. scrofulaceum* to *M. avium* occurred over the period 1975 to 1985. What is interesting about this change is that it occurred over the same period of time in England, Australia and the United States. Consequently, any hypothesis concerning the basis for this change must account for events that occurred in all three nations. Possible hypotheses include the fluoridation of drinking-waters and changes in water treatment.

Because the route of infection in these young children is probably via water, the shift to *M. avium* in cervical lymphadenitis in children suggests that the frequency of *M. scrofulaceum* in the environment has fallen. In a survey of natural waters collected in the eastern United States over the period 1976-1979, *M. scrofulaceum* was present in high numbers (Falkinham *et al.* 1980). In contrast, the same waters sampled from 1995 to the present seldom yield *M. scrofulaceum* (Falkinham, unpublished). This specific example suggests that the distribution and number of other mycobacterial species may also be changing.

2.4.2 Selection of mycobacteria by disinfectants

The widespread implementation of improved methods for disinfection of drinking-water and the presence of disinfectant resistant mycobacteria in source waters leads to selection for *M. avium* and other mycobacteria in drinking-water distribution systems. The use of disinfectants in medicine (Carson *et al.* 1978; Safranek *et al.* 1987), industrial settings (Shelton *et al.* 1999) and home spas and hot tubs (Embil *et al.* 1997; Kamala *et al.* 1997; Khoor *et al.* 1999) also leads to the predominance of mycobacteria in these habitats. Because mycobacteria are not detected routinely in drinking-waters and other samples, the presence of mycobacteria in the human environment

may be underestimated. It is important to point out that many outbreaks of mycobacterial infections associated with exposure to medical solutions (Safranek *et al.* 1987), industrial aerosols (Shelton *et al.* 1999) or hot tubs and spas (Embil *et al.* 1997; Kamala *et al.* 1997) have occurred in spite of disinfection of the possible source. This observation is troubling because it suggests that disinfection can lead to mycobacterial infections.

2.5 KEY RESEARCH ISSUES

In spite of the enormous progress in the understanding of *M. avium* epidemiology, ecology and physiologic ecology, there are still important questions concerning this opportunistic pathogen. Some of the questions involve the methodology used to detect, isolate and enumerate *M. avium* in environmental samples. Others involve questions of defining *M. avium* and its various types. The final issue of importance is the development of effective disinfection strategies for reduction of *M. avium* in the environment. Below is a list of methodological research issues:

- improve recovery or detection of *M. avium* in environmental samples
- define *M. avium* and its various types
- identify markers for *M. avium* virulence
- identify the dose-response to *M. avium* infection in different human hosts
- develop effective *M. avium* disinfection strategies.

Current methods for recovery of *M. avium* from environmental samples are limited by losses due to transfer, adherence and decontamination. Another problem that impacts on recovery and enumeration of *M. avium* and other mycobacteria is the fact that colony counts are usually 10-fold lower than counts of cells, even in laboratory medium. This suggests current methods for enumeration of mycobacterial cells as colonies underestimate numbers. Further, recovery methods suffer from the need for relatively long-term incubation. Although PCR-based methods offer the promise of rapid and sensitive detection of *M. avium* and other mycobacteria, they are limited by difficulties in lysing mycobacterial cells and the lack of sensitivity of PCR-based detection compared to colony-formation based detection. Developing a quantitative PCR-based detection system is a further difficult step to achieve.

The current status of *M. avium* taxonomy is in a state of flux (Thorel *et al.* 1991; Mijs *et al.* 2002). The species *M. avium* and *M. intracellulare* must be distinguished from one another. These relatives have different epidemiological and ecological features. *M. avium* predominates in AIDS patients and children with cervical lymphadenitis whereas both are found at equal frequencies in non-AIDS patients with pulmonary disease (Drake *et al.* 1988; Guthertz *et al.*

1989; Colville 1993, Wolinsky 1995). Furthermore, there has been no study comparing the utility of different typing methods (e.g. IS*901*, IS*1245*, PFGE) for discriminating between different *M. avium* isolates from patients and from epidemiologically matched environmental samples. Such a study might identify virulence markers of *M. avium*. Such knowledge would simplify and reduce the cost of efforts to identify sources of *M. avium* in humans and animals. Currently, every mycobacterium is recovered, identified and enumerated.

It is important to develop alternative strategies for reduction of numbers of *M. avium*, *M. intracellulare* and other mycobacteria in the environment. Current disinfection strategies for drinking-water appear to select for mycobacteria and their growth. One strategy for reduction of *M. avium* is reduction of particulates (i.e. turbidity) in raw and treated water (Falkinham *et al.* 2001). Filtration can be used, but *M. avium* and other mycobacteria can grow on filters and the filters can, in turn, serve as sources for mycobacteria by elution (Ridgway *et al.* 1984; Rodgers *et al.* 1999). Another approach would be to identify novel disinfectants that are active against *M. avium, M. intracellulare* and other mycobacteria. Identification of factors leading to disinfectant resistance of *M. avium* would contribute to this goal.

3

Environmental sources of *Mycobacterium avium* linked to routes of exposure

J.O. Falkinham

M. avium has been recovered from almost every environmental compartment that has been investigated including those that bring the organism into contact with susceptible species such as humans, animals, birds, and fish. The sources of *M. avium* in the environment are:

- natural water
- drinking-water
- biofilms
- aerosols
- soils
- foods
- plants and plant products
- fish.

Although a number of independent studies have shown that patient and environmental isolates of *M. avium* are either identical or similar, based on

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patterns of genetic (von Reyn *et al.* 1994; Mansfield & Lackner 1997) and physiologic (Fry *et al.* 1986) markers, it is not clear that all possible sources of *M. avium* in the environment have been identified, let alone the most important reservoirs. The diversity of sources and the heterogeneity of *M. avium* types isolated from the environment have complicated our search for the source of infection. Lack of knowledge of virulence markers has added further difficulty to the identification of the sources of *M. avium* infection. In addition, in many studies *M. avium* has not been distinguished from *M. intracellulare*. Although both are related, they are distinct species and appear to inhabit different but overlapping environments. This chapter presents a review of the environmental sources of *M. avium* and *M. intracellulare* and observations on the physiological determinants of their ecology.

3.1 ENVIRONMENTAL SAMPLE TYPES YIELDING *M. AVIUM*

3.1.1 *M. avium* in water

3.1.1.1 Methods of isolation

Isolation of *M. avium* from water requires consideration of the methods for concentration and disinfection. Filtration and centrifugation can be used to concentrate mycobacteria in water. If the water sample is suspected of containing microorganisms capable of overgrowing *M. avium* colonies, the sample can be disinfected. Disinfection relies upon the relative resistance of mycobacteria to disinfectants and detergents. If filtration is used for concentration, the sample should be disinfected before filtration (Glover *et al.* 1994). Following disinfection and filtration the filter is placed on a medium suitable for growth of mycobacteria (e.g. Middlebrook 7H10 agar). If centrifugation is used for concentration, the pelleted cells can be suspended in a small volume of water. Water is suitable for suspension of mycobacteria because the cells are resistant to lysis. Following concentration the sample can be decontaminated. Decontaminating agents include 1% NaOH, 1% Oxalic acid, 1% HCl (Brooks *et al.* 1984a), and 0.04% CPC (du Moulin & Stottmeier 1978). In addition to killing other microorganisms, the decontaminating agents also kill mycobacteria but to a lesser extent (e.g. 5% survival, Brooks *et al.* 1984). This means that only a minority of the total population of waterborne *M. avium* cells have been recovered and enumerated in the many reports of *M. avium* in water. Different combinations of concentration and disinfection for isolation of mycobacteria have been reviewed (Brooks *et al.* 1984; Schulze-Röbbecke *et al.* 1991; Kamala *et al.* 1994; Iivanainen *et al.* 1997; Neumann *et al.* 1997).

Unfortunately, there is no consensus for the method yielding the highest number of mycobacteria. For drinking-water, decontamination may not be required (Falkinham *et al.* 2001). Because of the hydrophobicity of mycobacteria it is possible that significant numbers of cells are lost during transfer.

A further discussion of analytical methods used for the detection of pathogenic waterborne mycobacteria can be found in Chapter 5.

3.1.1.2 M. avium in natural waters

A variety of natural waters throughout the world, including lakes, rivers, ponds and streams, have been shown to contain a resident *M. avium* population (Falkinham *et al.* 1980; du Moulin & Stottmeier 1986; Kirschner *et al.* 1992; von Reyn *et al.* 1993a; Covert *et al.* 1999). Whereas brackish water (1% NaCl) yields appreciable numbers of *M. avium,* seawater (> 3% NaCl) seldom has any mycobacteria (Falkinham *et al.* 1980). *M. avium* can grow in natural waters but cannot grow in waters of high salinity (George *et al.* 1980). Groundwater seldom contains *M. avium* (Martin *et al.* 1987; Falkinham *et al.* 2001). *M. avium* numbers are highest in waters of low oxygen and high organic matter content (Brooks *et al.* 1984; Kirschner *et al.* 1992). High numbers of *M. avium* are recovered from acid, brown-water swamps of the south-eastern coast of the United States (Kirschner *et al.* 1992) and acidic, brown-waters draining from peat-rich boreal forest soils in Finland (Iivanainen *et al.* 1999a).

3.1.1.3 M. avium in drinking-waters

Drinking-water has been shown to have a resident *M. avium* population (du Moulin *et al.* 1988; von Reyn *et al.* 1993, 1994; Glover *et al.* 1994; Peters *et al.* 1995; Covert *et al.* 1999; Ristola *et al.* 1999; Falkinham *et al.* 2001). A single *M. avium* clone was isolated repeatedly from a hospital water system over a period of 18 months demonstrating that *M. avium* is not a contaminant but is a normal inhabitant of drinking-water (von Reyn *et al.* 1994). Numbers of *M. avium* in recirculating hot water systems are increased relative to numbers in the input water (du Moulin *et al.* 1988) suggesting that *M. avium* is replicating in such systems in agreement with studies in natural water (George *et al.* 1980).

The number and frequency of isolation of *M. avium* varies quite widely between drinking-water systems (Falkinham *et al.* 2001). *M. avium* numbers in raw water sources for drinking-water systems correlates with the concentration of particulate matter suggesting that *M. avium* is associated with particulates (Falkinham *et al.* 2001). Thus, one approach for reducing the number of *M. avium* in a drinking-water system is to remove particulates (e.g. turbidity) from the raw waters during treatment. Numbers of *M. avium* in drinking-water distribution systems are higher in samples collected from the mid- and end-

points of the system compared to numbers in water collected immediately after treatment (Falkinham *et al.* 2001). This suggests that mycobacterial growth occurs in the distribution system. The presence of *M. avium* in treated water is consistent with its high resistance to ozone and chlorine-based disinfectants (Taylor *et al.* 2000).

3.1.1.4 Other water samples

Due to the widespread presence of *M. avium* in drinking-water, recreational and other waters that have their origin in drinking-water yield mycobacteria. Spas and hot tubs have been shown to yield *M. avium* that have been associated with infection in people using the spas (Embil *et al.* 1997; Kahana *et al.* 1997; Khoor *et al.* 2001). Swimming pools yield *M. avium* (Havelaar *et al.* 1985; Emde *et al.* 1992) and long-term exposure to aerosols results in a granulomatous pneumonitis in lifeguards (Rose *et al.* 1998).

3.1.1.5 M. avium in biofilms

M. avium, M. intracellulare and other mycobacteria have been shown to be present in biofilms (Schulze-Röbbecke & Fischeder 1989; Iivanainen *et al.* 1999a; Falkinham *et al.* 2001). In a study of eight drinking-water distribution systems across the United States the average number of *M. avium* in biofilms was 0.3 cfu/cm^2 and the number of *M. intracellulare* was 600 cfu/cm^2 for all surfaces (Falkinham *et al.* 2001). If one considers the size of pipes used to distribute drinking-water and the length of the pipes in a system (e.g. 75 to 7100 miles) the contribution of biofilm to the microbial flora of the drinking-water is substantial.

The presence of *M. avium* in biofilms in drinking-water systems might explain why two groundwater-fed drinking-water systems did not have *M. avium* in their raw source water, but did have *M. avium* in the distribution system (Falkinham *et al.* 2001). The frequency of recovery of *M. avium* in water samples from the same systems was higher than that of *M. intracellulare* (Falkinham *et al.* 2001), suggesting that one preferred habitat of *M. intracellulare* is within a biofilm.

The type of surface apparently had an effect on mycobacterial biofilm numbers. Numbers of *M. intracellulare* were 4400 cfu/cm^2 on brass or bronze surfaces compared to 70 cfu/cm^2 on plastic (Falkinham *et al.* 2001). The values for cfu/cm^2 in the drinking-water distribution systems (Falkinham *et al.* 2001) were 10-fold lower than the numbers for hot water silicon tube biofilms (Schulze-Röbbecke & Fischeder 1989). The presence of hot water would increase the growth of mycobacteria on any surface (George *et al.* 1980).

3.1.2 *M. avium* in soils

3.1.2.1 Methods of isolation

Isolation of *M. avium* from soil samples involves three steps: elution from particulate matter, concentration, and disinfection. Current methods for recovering *M. avium* from soil are inefficient; for example, Brooks and co-workers could only recover 5% of *M.avium* cells added to soil samples (Brooks *et al.* 1984). Decontamination is then required to reduce the high numbers of microorganisms whose colonies overgrow those of mycobacteria. Because decontamination reduces *M. avium* numbers by 95% and only 5% of *M. avium* cells can be separated from particulate matter (Brooks *et al.* 1984), only 0.25% of total mycobacteria in soils are recovered as cfu. As is the case for water, a variety of reports have compared different methods of isolation of mycobacteria from soils (Brooks *et al.* 1984; Portaels *et al.* 1988; Kamala *et al.* 1994; Iivanainen 1996). It has been shown that exposure of soils to polysaccharidases increases the numbers of isolates and species of mycobacteria in soils (Thorel *et al.* 1991).

3.1.2.2 M. avium in soils and peat

A wide variety of soils, sediments and peat have been reported to contain *M. avium*. The soils include those on river banks in the south-eastern United States (Brooks *et al.* 1984a), in swamps in the coastal south-eastern United States (Kirschner *et al.* 1992) and peat-rich boreal forest soils in Finland (Iivanainen *et al.* 1997a), both of which yield high numbers of *M. avium*. Characteristics that correlate with high numbers of *M. avium* include low pH, high organic matter and low oxygen (Brooks *et al.* 1984; Kirschner *et al.* 1992; Iivanainen *et al.* 1997a). The high numbers of *M. avium* reported in drinking-waters in Finland (Ristola *et al.* 1999) and in the north-eastern United States are likely due to the use of source waters rich in mycobacteria.

Yajko and co-workers reported that 55 % of soil samples from potted plants of AIDS patients contained *M. avium* which is consistent with the high numbers of *M. avium* and other mycobacteria in peat-rich boreal forest soils (Yajko *et al.* 1995). Furthermore, we have recently discovered that commercial samples of peat and potting soils sold in the United States are rich in *M. avium* and other mycobacteria (Falkinham, unpublished).

3.1.3 *M. avium* in aerosols, ejected droplets and dust

3.1.3.1 Methods of isolation

M. avium and other mycobacteria in aerosols and dusts can be recovered and enumerated. A simple method is to use the Andersen 6-Stage Cascade Sampler (Andersen 1958). This sampler separates particulates in the air on the basis of size by impacting on the surface of agar medium. Whether the particle collected is from soil (e.g. dust) or from water (e.g. droplet) cannot be determined. Airborne cells are recovered as colonies. Thus the instrument provides a measure of the number of airborne cells and the associated particle size. Particles recovered on the bottom two stages are of a size that can enter the alveoli of the human lung (Andersen 1958). Because the particles are separated by size, most fungal spores are trapped on those stages collecting larger particles. Thus decontamination may not be required unless the air or dust sample contains a substantial number of *Bacillus* spores. Malachite green at a concentration of 0.05% in Middlebrook 7H10 agar medium does not inhibit growth of mycobacteria, but is effective at preventing colony formation of other bacteria and fungi (Jones & Falkinham, in preparation).

M. avium and other mycobacteria can also be recovered from droplets that are ejected from the surface of water. Air bubbles rising through a water column collect particles, chemicals and microorganisms (Blanchard & Szydek 1970, 1978, 1982; Blanchard *et al.* 1981). Hydrophobic interaction drives the adsorption to bubbles and results in bubbles reaching the surface enriched in organic chemicals and hydrophobic microbial cells (Blanchard & Hoffman 1978; Weber *et al.* 1983). Because mycobacteria are the most hydrophobic of microorganisms (van Oss *et al.* 1975), they are enriched in the bubbles. When the bubbles reach the surface they burst and form a crater that results in the ejection of one to several droplets 8-10 cm above the water surface (Blanchard & Szydek 1978). The droplets can be collected on inverted agar medium and the droplet size calculated from the diameter of the craters formed by the droplets on an inverted Petri dish coated with MgO (Blanchard *et al.* 1981). The ejected droplets are enriched in mycobacteria (Parker *et al.* 1983). Indeed, for *M. avium*, the concentration of cfu in the ejected droplets divided by the concentration in the bulk suspension (enrichment factor) can be as high as 10 000 (Parker *et al.* 1983).

3.1.3.2 M. avium in ejected droplets

M. avium can be recovered as cfu from droplets ejected from natural waters (Wendt *et al.* 1980). For these measurements, inverted Petri dishes were held 10 cm above a river surface for 60 minutes. Immediately after collection, the

liquid on the plates was spread and the plates incubated until mycobacteria colonies were recovered (Wendt *et al.* 1980). In a second study both water and ejected droplets were collected at the same site on the James River in Richmond, Virginia. The number of *M. avium* and *M. intracellulare* in ejected droplets was 2715 per ml and the number in water was 0.19 per ml (Falkinham *et al.* 1990). This data demonstrates that there is enrichment of *M. avium* and *M. intracellulare* in natural ejected droplets (~14 000-fold) resulting in considerable transfer of mycobacteria from water to air.

Laboratory experiments showed that *M. avium* and *M. intracellulare*, but not the relatively hydrophilic *M. scrofulaceum*, were preferentially aerosolized (Parker *et al.* 1983). Further studies demonstrated a correlation between enrichment factor and hydrophobicity (Falkinham 1989). Because the bubble burst jet drop mechanism of aerosol transfer of microorganisms occurs in every natural body of water, mycobacteria can be efficiently detected, enumerated and identified with few problems of contamination.

3.1.3.3 M. avium in aerosols

M. avium and other mycobacteria can be recovered from aerosols using the Andersen Cascade sampler (Falkinham *et al.* 1990). Between July 1979 and July 1980, twice monthly aerosol samples were collected at a park by the James River in Richmond, Virginia. The site was far from any agricultural activity. Numbers of *M. avium* and *M. intracellulare* averaged 25 cfu/m^3 of air and 1.4 cfu/m^3 associated with particles able to reach the alveoli (Falkinham *et al.* 1990). Based on an estimate that a human inhales 6 m^3 air per day, a person at that site would inhale 150 *M. avium* and *M. intracellulare* per day (8 reaching the alveoli). The highest number was 250 cfu/m^3 air. The numbers of *M. avium* and *M. intracellulare* were not correlated with the numbers in river water at the same site (Falkinham *et al.* 1990).

3.1.4 *M. avium* in phagocytic protozoa and amoebae

3.1.4.1 Intracellular growth and enumeration

M. avium, like other mycobacteria, are intracellular pathogens able to survive and grow in infected animal macrophages. They are also capable of survival and growth in phagocytic protozoa (*Tetrahymena pyriformis*) and amoebae (*Acanthamoeba polyphaga* and *Acanthamoeba castellanii*). In laboratory experiments it was shown that *M. avium* is readily phagocytozed by protozoa and amoebae (Strahl *et al.* 2001). Five days after infection of protozoa or amoebae, the number of intracellular *M. avium* cells increases to 5-10 per infected *T. pyriformis* cell (Strahl *et al.* 2001) or 10-15 per infected

A. castellanii cell (Cirillo *et al.* 1997). *M. avium* survives cyst formation and germination of *T. pyriformis* (Strahl *et al.* 2001) and *A. polyphaga* (Steinert *et al.* 1998). Thus protozoa and amoebae increase numbers of *M. avium* and other mycobacteria in habitats where both reside. One troubling aspect of intracellular growth of *M. avium* in protozoa and amoebae is that the resulting *M. avium* cells are more virulent in mice (Cirillo *et al.* 1997) and chickens (Falkinham, unpublished).

Recovery of intracellular *M. avium* from protozoa or amoebae can be performed using lysis with 1% SDS (Cirillo *et al.* 1997). The detergent does not reduce colony formation by *M. avium* or other mycobacteria (Cirillo *et al.* 1997). Protozoa and amoebae can be recovered from water samples by centrifugation (1000 x g for 10 minutes), and mycobacteria recovered after lysis or simply by spreading the low speed pellet on Middlebrook 7H10 agar. Eventually the protozoa or amoebae lyse. Individual protozoa or amoebae can be isolated using micropipettes and grown in medium to determine numbers of intracellular mycobacteria.

There are no published reports of any systematic study of numbers of *M. avium* and other mycobacteria in protozoa or amoebae. Protozoa and amoebae offer an environmental sample likely enriched for *M. avium* and mycobacteria. In one study, we compared the recovery of mycobacteria from water with recovery from *T. pyriformis* that had been added to the water sample and incubated one week. More species and higher numbers of *Mycobacterium* were recovered from the protozoa (Falkinham, unpublished). An investigation of intracellular mycobacteria in protozoa and amoebae is important because those environmental habitats yielding high numbers of *M. avium* also harbour high numbers of protozoa and amoebae.

3.1.5 Other sources of *M. avium*

M. avium and other mycobacteria have been isolated from foods (Yajko *et al.* 1995; Yoder *et al.* 1999; Argueta *et al.* 2000), fish (Mediel *et al.* 2000; Rhodes *et al.* 2001) and tobacco products (Eaton *et al.* 1995) all of which come into contact with humans and thus can serve as a source of infection. In one study, 25 of 121 (21%) food samples yielded *M. avium* (Argueta *et al.* 2000). Isolation from tobacco products was attempted because of the report that smokers who suffered from pulmonary alveolar proteinosis were also infected with *M. avium* whereas non-smokers were not infected (Witty *et al.* 1994).

3.2 IMPACT OF UNIDENTIFIED MYCOBACTERIAL ISOLATES

Depending upon the study, a significant proportion of mycobacteria recovered from different environments do not belong to established mycobacterial species. For example, in a study of brook waters in Finland, 15% of isolates belonged to established species and that percentage was raised to only 28% if isolates that were "like" established species were included (Iivanainen *et al.* 1993). In studies of drinking-water the percentage of isolates that did not belong to established species varied from 15-50% (Covert *et al.* 1999; Falkinham *et al.* 2001; Le Dantec *et al.* 2002). Because different computer programs for calculating similarity index values for isolates from the same 16SrRNA gene sequence data yield different values (Drancourt *et al.* 2000), the program used has a direct impact on the diversity of mycobacterial species isolated. The method of recovery directly influences the spectrum of *Mycobacterium* species isolated from environmental samples. Decontamination reduces the number and range of species and colony variants differ in their susceptibility to disinfection (Brooks *et al.* 1984). In addition, the diversity of species recovered from a sample is related to the total number of isolates recovered. For example, polyclonal infection in AIDS patients can only be detected when a sufficient number of isolates are recovered (Slutsky *et al.* 1994). The same holds for diversity in environmental samples.

The impact of these factors means that we are isolating, identifying and characterizing only a minority of cells that are present in the environment. That in turn tempers the impact of any conclusions that can be made concerning the ecology of *M. avium, M. intracellulare* or other mycobacteria.

3.3 ENVIRONMENTS WITH HIGH NUMBERS OF *M. AVIUM*

Because *M. avium* is an opportunistic human pathogen it is important to point out those environmental sources that have high numbers. Boreal, peat-rich forest soils and waters yield high numbers of *M. avium* and other mycobacteria (Iivanainen *et al.* 1997a). Such forests are found in Finland, across northern Europe, the northern United States, and Canada. The presence of high numbers of *M. avium* in Finnish drinking-water was associated with a high frequency of *M. avium* infection in Finnish AIDS patients (Ristola *et al.* 1999). Waters and soils of the acid, brown-water swamps of the south-eastern coastal United States also yield high numbers of *M. avium* (Kirschner *et al.* 1992). Although located in different parts of the world, both share low pH, low oxygen and presence of high concentrations of humic and fulvic acids.

Peat and potting soils yield high numbers of *M. avium* (Yajko *et al.* 1995). This is not unexpected because peat-rich boreal forest soils have high numbers of *M. avium* and other mycobacteria. Commercial peat-rich potting soils and samples of soil in pots with plants have numbers of *M. avium* approaching 10^6 per gram (Falkinham, unpublished).

Recirculating hot water systems, spas, and hot tubs yield high numbers of *M. avium*. The presence of *M. avium* in spas and hot tubs has been associated with *M. avium* infection in humans in a number of independent studies (Embil *et al.* 1997; Kahana *et al.* 1997). Recirculating hot water systems in hospitals were shown to have higher numbers of *M. avium* than the source water for the system (du Moulin *et al.* 1988).

Biofilms contain *M. avium, M. intracellulare* and other mycobacteria. Biofilms in drinking-water distribution systems appear to be a preferred habitat for *M. intracellulare* (600 cfu/cm^2) compared to *M. avium* (0.3 cfu/cm^2) (Falkinham *et al.* 2001). Shower heads collect a biofilm and are good sites to sample for *M. avium*. The ability of *M. avium, M. intracellulare* and other mycobacteria to form biofilms means that they can populate in-line water filters used for water purification (Ridgway *et al.* 1984). In fact, it has been shown that *M. avium* was capable of populating and growing to 100 000 cfu in an inline filter (Rodgers *et al.* 1999). This observation suggests another method for collection of samples to detect or monitor *M. avium* numbers: place in-line filters in the water distribution system and sample them at intervals.

3.4 ROUTES OF EXPOSURE

Routes of *M. avium* exposure include ingestion, inhalation and surface or traumatic exposure. Because of the widespread distribution of *M. avium* in many environments, human and mycobacterial habitats overlap leading to exposure.

3.4.1 Ingestion

There is long-term evidence that ingestion is a route of *M. avium* infection. Cervical lymphadenitis caused by *M. avium* is found in children from six months to two years old (Wolinsky 1995). In addition, *M. avium* infection in simian immunodeficiency virus infected macaques was traced to the water drunk by the monkeys (Mansfield & Lackner 1997). Ingestion of mycobacteria could also occur through eating fish or other foods that are colonized (infected) with *M. avium* or other mycobacteria.

3.4.2 Inhalation

The results of studies of *M. tuberculosis* transmission and evidence of pulmonary infection have been used to postulate an aerosol route of infection for *M. avium, M. intracellulare* and other mycobacteria. Demonstration that *M. avium* and *M. intracellulare* cells are readily aerosolized from water, are highly hydrophobic and can be recovered from aerosols, certainly support that route of infection. However, there has been no demonstration of aerosol transmission of *M. avium* infection to laboratory animals, possibly because of the low infectivity of *M. avium*. Both water droplets and particulates in the atmosphere are likely vehicles of *M. avium* transmission. Not only are *M. avium* cells enriched in water droplets ejected from surfaces (Parker *et al.* 1983), but *M. avium* numbers are associated with particulate matter in water (Falkinham *et al.* 2001). Droplets enriched for *M. avium* (and other mycobacteria) would be generated from any body of water and are formed in large numbers in domestic showers. Because hot water systems can be enriched for mycobacteria, showers may be rich sources for mycobacterial-enriched aerosols. Dust particles may also serve as vectors for transmission of *M. avium*. Discovery that peat soils are rich in *M. avium* and other mycobacteria led us to measure the mycobacteria in aerosols generated by dropping 100 gm of peat or potting soil 30 cm. Using the 6-Stage Andersen Cascade Sampler, we have found that the resulting aerosolized particles contain mycobacteria in substantial numbers. In addition, some of the particles associated with mycobacteria were within the size range that could enter human alveoli (Falkinham, unpublished).

3.4.3 Trauma

Trauma, resulting from either surface abrasions or injury or during surgical procedures, may also lead to infection by *M. avium* and other mycobacteria. *Mycobacterium marinum* granulomas on the hands have been found in individuals who handle fish and have skin abrasions (Wolinsky 1979). The presence of substantial numbers of *M. avium* in water may also result in human exposure. The disinfectant resistance of *M. avium* and other mycobacteria can lead to their persistence in solutions used in surgeries. A nosocomial *M. chelonae* infection was associated with the use of a gentian violet-containing skin-marking solution (Safranek *et al.* 1987).

3.4.4 Biofilms

Biofilms not only represent a mechanism for the persistence and growth of *M. avium* in tubes, but they also represent a reservoir for cells. For example, a persistent *M. avium* infection was shown to be associated with biofilm

formation in a catheter (Schelonka *et al.* 1994). Not only are the biofilms sources for more cells, but *M. avium* and *M. intracellulare* cells in biofilms are more resistant to antibiotics (Steed & Falkinham, in preparation). Furthermore, *M. avium* and *M. intracellulare* cells grown in biofilms but exposed to antibiotics in suspension are more resistant to antibiotics (Steed & Falkinham, in preparation), suggesting that biofilm growth alone renders *M. avium* more resistant to antibiotics.

3.5 OVERLAP OF HUMAN AND *M. AVIUM* ENVIRONMENTS

There are a number of factors that suggest that the incidence of disease caused by *M. avium, M. intracellulare* and other mycobacteria will continue to increase. In part, this will be a consequence of the existence of overlaps of human and mycobacterial environments. For example, it is possible that widespread use of chlorine and other disinfectants to improve water quality have led to increases in mycobacterial numbers. Because *M. avium* is so resistant to disinfection, competing microorganisms are killed by disinfectants leaving mycobacteria free to proliferate in the absence of competition. Distribution systems and hot water systems provide environments for the growth of mycobacteria and thus increase numbers to which humans and animals are exposed. The use of potting soils rich in peat leads to exposure of gardeners (professional and amateur) to particles containing high numbers of *M. avium* and other mycobacteria. Thus some human activities may increase the risk of exposure of individuals to mycobacteria. Those overlaps, coupled with an ageing human population containing more individuals with immunodeficiency through therapy or infection, suggest that infection by *M. avium*, *M. intracellulare* and other mycobacteria may become more prevalent.

3.6 KEY RESEARCH ISSUES

In spite of the enormous progress in the understanding of *M. avium* epidemiology, ecology, and physiologic ecology, there are still important questions concerning this opportunistic pathogen. Some of the questions involve the methodology used to detect, isolate, and enumerate *M. avium* in environmental samples. Others involve questions of defining *M. avium* and its various types. The final issue of importance is the development of effective disinfection strategies for reduction of *M. avium* in the environment. Below is a list of methodological research issues.

- Improve recovery or detection of *M. avium* in environmental samples
- Define *M. avium* and its various types

- Identify markers for *M. avium* virulence
- Identify the dose-response to *M. avium* infection in different human hosts
- Develop effective *M. avium* disinfection strategies

Current methods for recovery of *M. avium* from environmental samples are limited by losses due to transfer, adherence, and decontamination. Another problem that impacts on recovery and enumeration of *M. avium* and other mycobacteria is the fact that colony counts are usually 10-fold lower than counts of cells, even in laboratory medium. This suggests current methods for enumeration of colonies underestimate numbers. Furthermore, recovery methods suffer from the need for relatively long term incubation. Although, PCR-based methods offer the promise of rapid and sensitive detection of *M. avium* and other mycobacteria, they are limited by difficulties in lysing mycobacterial cells and the lack of sensitivity of PCR-based detection compared to colony-formation based detection. Developing a quantitative PCR-based detection system is a further difficult step to achieve.

The current status of *M. avium* taxonomy is in a state of flux (Mijs *et al.* 2002). First, the species *M. avium* and *M. intracellulare* must be distinguished from one another. The relatives have different epidemiological and ecological patterns. *M. avium* predominates in AIDS patients and children with cervical lymphadenitis, whereas both are found at equal frequencies in non-AIDS patients with pulmonary disease (Drake *et al.* 1988; Guthertz *et al.* 1989; Colville 1993; Wolinsky 1995). Further, there has been no study comparing the utility of different typing methods (e.g., IS*901*, IS*1245*, PFGE) for discriminating between different *M. avium* isolates from patients and from epidemiologically matched environmental samples. Such a study might identify virulence markers of *M. avium*. Such knowledge would simplify and reduce the cost of efforts to identify sources of *M. avium* in humans and animals. Currently, every mycobacterium is recovered, identified, and enumerated.

It is important to develop alternative strategies for reduction of numbers of *M. avium, M. intracellulare*, and other mycobacteria in the environment. Current disinfection strategies for drinking water appear to select for mycobacteria and their growth. One strategy for reduction of *M. avium* is reduction of particulates (i.e., turbidity) in raw and treated water (Falkinham *et al.* 2001). Filtration can be used, but it is important to recall that *M. avium* and other mycobacteria can grow on filters and the filters can, in turn, serve as sources for mycobacteria by elution (Ridgway *et al.* 1984; Rodgers *et al.* 1999). Another approach would be to identify novel disinfectants that are active against *M. avium, M. intracellulare*, and other mycobacteria. Identification of factors leading to disinfectant-resistance of *M. avium* would contribute to this goal.

4

Biology of waterborne pathogenic mycobacteria

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4.1 INTRODUCTION

4.1.1 Taxonomy and terminology

The waterborne mycobacteria are members of a large and very significant family of human pathogens. *Mycobacterium* is the single genus in the family Mycobacteriaceae, order Actinomycetales. Over 70 *Mycobacterium* species have been defined, at least 30 of which cause disease in humans or animals. *Mycobacterium* species fall into two groups: the slow growers and the rapid growers. This descriptive division predates modern genotype-based taxonomic methods; however, it has been shown to be consistent with genotypic taxonomy (Rogall *et al.* 1990). Most pathogenic mycobacteria are slow growers, the most notable exception being the rapid-growing *M. fortuitum* complex. Mycobacteria

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are easily identifiable by the mycolic acids in their cell envelopes. Rich in other lipids as well, mycobacterial cell envelopes can account for up to 40% of total cell weight. These structures are responsible for the ability of the bacteria to resist decolourization by weak acids after staining, hence the diagnostic term "acid-fast bacilli".

The *M. tuberculosis* complex, a cluster of closely related species that includes the causative agent of TB, ranks with HIV and malaria parasites as among the most significant microbial pathogens of humans. The genus also includes *M. leprae,* the causative agent of Hansen disease (leprosy). Neither *M. tuberculosis* nor *M. leprae* are encountered in water. A variety of other *Mycobacterium* species, some of which are very common in water, occasionally infect humans. When isolated from patients' samples, these organisms are sometimes referred to as "atypical" or "NTM"; however, the term "environmental mycobacteria" is more informative.

From the standpoint of human health, the most significant of the environmental mycobacteria are the MAC and *M. ulcerans.* MAC is considered to include MAA, MAP, *M. avium* subspecies *silvaticum* and *M. intracellulare.* These divisions are well defined based on phenotypic as well as genotypic (rRNA) criteria (Boddinghaus *et al.* 1990; Frothingham & Wilson 1994). Recently, a proposal was made to confine the designation MAA to a cluster of closely related strains that are commonly isolated from birds and rarely from humans (avian strains) and assign a new name, *M. avium* subspecies *hominissuis,* to the more diverse group of strains that are more commonly isolated from mammalian (including human) environments (Mijs *et al.* 2002). This proposal was based upon genotypic and phenotypic analysis of a large number of strains. However, at the time of this writing it is still a proposal, not a universally accepted standard. Therefore, the standard terminology is used here.

Many human and environmental isolates of MAC do not fall neatly within any of the designations listed above. Such isolates can exhibit genotypic features of two or more different MAC species, and are usually given designations such as "MAC_x". The existence of such intermediate types may help explain the common view of MAC as a continuum of more or less indistinguishable species and subspecies. However, as emphasized elsewhere in this book, the pathogens we know as MAA, MAP and *M. intracellulare* are distinct in many significant ways, including environmental niches, host preferences and clinical manifestations. Recognition of these distinctions is important to the understanding of the biology of MAC.

M. ulcerans is the causative agent of Buruli ulcer, a debilitating disease characterized by large necrotic skin ulcers that is currently widespread throughout West and Central Africa (refer to Chapter 8). *M. ulcerans* is closely related to the fish pathogen *M. marinum.* Analysis of their full 16SrRNA

sequences show > 99.8% identity and comparisons of other gene sequences show a similar high level of sequence conservation (Stinear *et al.* 2000b). Yet despite this high level of genetic relatedness *M. ulcerans* and *M. marinum* are phenotypically distinct. Notably, *M. ulcerans* is slow growing with a doubling time of 36 hours *in vitro*, whereas *M. marinum* is a robust environmental organism that doubles every 6 hours *in vitro* and is readily isolated from diverse aquatic environments. It is extremely difficult to isolate *M. ulcerans* from the environment, although the pathogen may occupy niche environments such as the salivary glands of particular aquatic insects (Masollier *et al.* 2002). *M. marinum* causes a tuberculoid disease in fish and other poikilotherms and a relatively minor skin infection in humans. As with other mycobacterial pathogens, *M. marinum* can replicate within the host macrophage and provokes the formation of a granuloma by the host. In contrast, *M. ulcerans* is not known to cause disease in fish and in humans it produces large necrotic skin lesions caused by massive necrosis of subcutaneous fat. Histopathology shows a marked absence of a host inflammatory immune response and massive numbers of bacilli are found extracellularly. This unusual pathology has been linked to the presence of a macrolide toxin produced by *M. ulcerans* called mycolactone (George *et al.* 1999). *M. marinum* does not produce mycolactone.

In addition to the use of rRNA-based systematics, the species and subspecies of environmental mycobacteria have also been classified by a number of phenotypic characteristics including Runyon Group, pigment production, serotype and host range. However, genotypic classifications based upon rRNA structural gene and spacer sequences, species-specific insertion elements and other genetic markers described in later sections, are considered by most to be more reliable markers. They are becoming more practical with the increasingly widespread availability of commercial DNA probes (Accuprobe, Genprobe, Inc., San Diego, California), PCR and DNA sequencing capabilities.

4.1.2 Evolution and diversity of MAC

Epidemiological evidence indicates that humans are infected by MAC that is living in the environment, not via person-to-person transmission. Although viable MAC cells can occasionally be cultured from the stools of AIDS patients, there is little evidence to suggest that infective populations of the bacteria commonly make their way back to the environment. Therefore, the populations of MAA and *M. intracellulare* cells to which humans are exposed are not likely to have human environments in their recent evolutionary histories. Their pathogenic capabilities were selected by environmental challenges encountered outside of human hosts. Unfortunately, very little is known about the lives of MAC cells in the environment.

It is possible that extracellular environments select for traits in MAC that are coincidentally advantageous in human infections. For example, resistance to environmental toxins might also confer protection from the killing effects of antibiotics and host immune cells. Alternatively, the bacteria may routinely encounter and defend themselves against the phagocytic cells of animal hosts. MAP is well known to infect livestock while MAA and *M. intracellulare* infect a variety of mammals and birds in serovar-specific fashion. Certain MAA strains are commonly excreted in the faeces of birds after which they can persist in the soil for extended periods of time. Although there is little evidence for direct transmission from animals to humans a few serovars and strain types can be recovered from both, consistent with the possibility that virulence mechanisms are maintained in animal reservoirs (Mijs *et al.* 2002; Inderlied *et al.* 1993).

An alternative model is that environmental mycobacteria commonly reside within environmental amoebae, where they encounter an environment similar to that found in human phagocytic cells (Cirillo *et al.* 1997; Steinert *et al.* 1998; Miltner & Bermudez 2000). This is supported by laboratory studies which have shown that MAA cells grown within *Acanthamoeba* are more virulent than extracellularly grown MAA. However, it is not known how often this happens in the environment. Nonhuman reservoirs must be explored if we are to understand how environmental mycobacteria infect humans. Such studies may also lead to improved nonhuman disease models, and refined methods for detecting harmful MAC populations in drinking-water.

Compared to closely related groups such as the *M. tuberculosis* complex, MAC is diverse phenotypically as well as phylogenetically. This diversity is evident between species (e.g. MAA and *M. intracellulare*), between individual isolates within a species and even within a single clinical isolate. Most clinical isolates of MAC form multiple colony morphotypes that vary with regard to infectivity, susceptibility to antibiotics and ability to survive in various environments. MAC also exhibits considerable genetic polymorphism, mediated in part by mobile insertion elements that are abundant in their genomes (Arbeit *et al.* 1993; von Reyn *et al.* 1995; Eckstein *et al.* 2000; Matsiota-Bernard *et al.* 2000; Laurent *et al.* 2002).

The heterogeneity of MAC has important implications with regard to epidemiology and risk assessment. It is possible that some strains found in the environment are especially infectious to humans, while others may be relatively harmless. This would compromise the predictive value of environmental monitoring efforts that assume uniform levels of infectivity. Therefore, a significant priority for MAC research is the identification of genetic or phenotypic markers that can distinguish the most infective strains from those

that are relatively harmless to humans. Such markers could help refine epidemiological analysis and lead to more accurate methods of risk assessment.

4.2 MYCOBACTERIAL GENOMES

Our understanding of the biology of *M. tuberculosis* and *M. leprae* improved dramatically with the determination of their genomic nucleotide sequences. Further rapid progress is being made with studies on genome-wide patterns of gene expression and gene function. Our understanding of MAC and similar environmental pathogens is on the cusp of similar progress, with the impending completion of the genomic nucleotide sequences of MAA, MAP, *M. ulcerans,* and *M. marinum.*

4.2.1 The MAA genome

The genome sequence of MAA strain 104, a clinical isolate originating from an AIDS patient in California, is nearing completion by The Institute for Genomic Research (http://www.tigr.org). The sequence has a high G+C content (68.5%) and is about 5.48 mb long. It has been annotated by M. Behr and co-workers at McGill University, Montreal, Canada (Semret *et al.* submitted). Approximately 4480 coding sequences were identified that are likely to be genes. In contrast, the genome of *M. tuberculosis* is about 4.4 mb long and has 3959 likely genes (Cole *et al.* 1998; http://genolist.pasteur.fr/TubercuList/). Approximately 385 MAA genes have no counterpart in *M. tuberculosis*. Presumably, some of these MAA-specific genes confer the ability to live and grow in the environment, which *M. tuberculosis* lacks. Others code for unique cell surface properties that are discussed below.

When using genome sequence information on MAC it is important to bear in mind that the organism's heterogeneity is such that there is probably no truly representative strain. The choice of MAA strain 104 for genome sequencing was made in the mid-1990s based on several criteria. As an AIDS isolate it was representative of the majority of MAC disease cases at the time. It is virulent in nonhuman disease models and intrinsically multi-drug resistant. It is a stable "red transparent" morphotypic clone, which is somewhat unusual in a virulent clinical isolate (Mukherjee *et al.* 2001). Its relative stability would have simplified the process of sequence determination and assembly at the possible cost of using a strain that is less typical than an isolate that exhibits normal morphotypic switching.

As with *M. tuberculosis* the genome of MAA contains a large number of mobile genetic elements termed IS. These elements are usually small (1 to 2 kb), with little or no genetic information other than that required for transposition

(hopping) from site to site within the genome. The variable patterns of IS insertion can be used to generate strain-specific "bar codes" for molecular epidemiological analysis. These codes are relatively stable in *M. tuberculosis*, but in MAA they exhibit considerable diversity even within a single clinical isolate (Laurent *et al.* 2002). IS are thought to be important drivers of genetic diversification in mycobacteria. When an IS hops into a gene that gene is usually disrupted and its function is lost. Moreover, genetic recombination between neighbouring IS can result in large-scale deletions and rearrangements of genomic DNA. This phenomenon has been shown to play an important role in the generation of spontaneous "rough" colony type variants of MAA (Eckstein *et al.* 2000).

The positions of insertion elements IS*1245* and IS*999* (Laurent *et al.* 2002) within the MAA strain 104 genome are shown in Figure 4.1. IS*999* insertion sites in three other strains are also shown. Regions of the genome with high IS concentrations are likely to contain DNA sequences that are not required for life in at least some environments. An example of such a region is the ssGPL gene cluster which is deleted in rough colony type variants. These IS-rich regions are potential hot spots for loss or rearrangement over the course of MAA evolution.

A microarray of the MAA genome has been prepared by M. Behr and co-workers (Semret *et al.*, submitted manuscript). A microarray is a wafer dotted with small nucleotide probes, each specific for an individual MAC gene. Hybridization of a DNA or RNA sample to the array, followed by sophisticated bioinformatic analysis, reveals the presence or absence of genes or gene products in the sample. This tool will allow us to rapidly assess and quantify the genomic diversity found among large numbers of clinical and environmental isolates. Comparison of gene deletion patterns by "chromosome alignment" may lead to the identification of genomic signatures characteristic of virulent strains, a useful outcome for risk assessment. Microarray analysis of conditional gene expression (RNA samples) during intracellular growth might also reveal virulence genes.

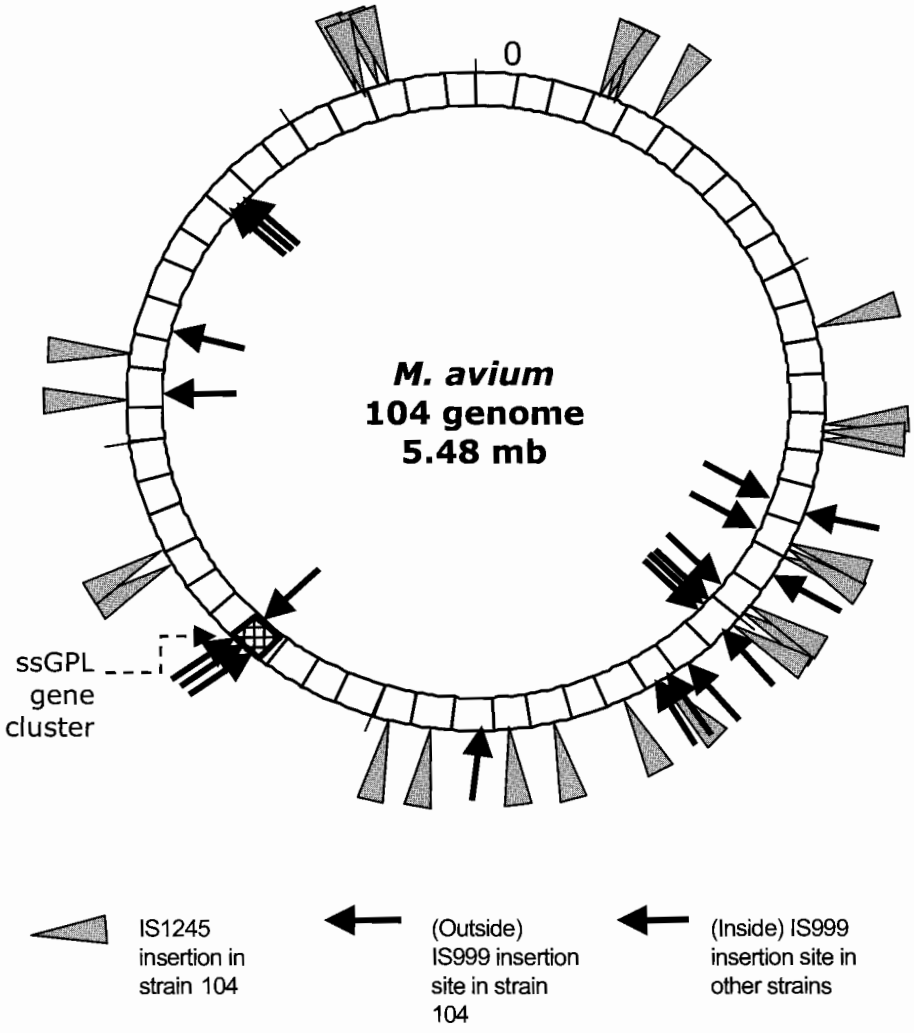

Figure 4.1 IS1245 and IS999 insertion sites in MAA strains 104, 102, HMC02 and HMC10. Insertion sites of IS1245 and IS999 in strain 104 were mapped by computer analysis of the preliminary 5.48 mb genome sequence generated by the Institute for Genomic Research (http://www.tigr.org). Positions of these sites are indicated on the outside of the circle. Insertion sites of IS999 in strains 102, HMC02 and HMC10 were mapped by inverted PCR and sequencing as described (Laurent *et al.* 2002). This strategy yielded positions of the subset of IS999 insertions that could be amplified in this fashion. This depended on the random occurrence of specific restriction endonuclease sites in the genomic regions bordering the insertions. Therefore, the data represent a random sample of IS999 insertion sites in these strains.

4.2.2 The MAP genome

A collaboration to sequence the genome of the virulent bovine MAP isolate K-10 is nearing completion (http://www.cbc.umn.edu/ResearchProjects/AGAC/Mptb/Mptbhome.html). The genome, at 4.8 mb, is 0.7 mb shorter than the MAA strain 104 genome but remarkably similar in sequence and organization (98% overall homology). The G+C% content at 69% is also comparable to MAA.

A comparative alignment of preliminary drafts of the MAP and MAA genomes has provided some insights into the biological distinctions between the two organisms. MAA has about 700 kb of sequence not found in MAP, while MAP has about 140 kb of sequence, containing 60-70 probable genes, not found in MAA. The MAP specific regions are often located at the same genomic positions as MAA specific regions. These contain all previously identified MAP specific genes (Poupart *et al.* 1993; Ellingson *et al.* 1998; Tizard *et al.* 1998; Bannantine *et al.* 2002; Nielsen & Ahrens 2002) and are organized within six large regions ranging from 10 to 60 kb, and several minor regions of 1 to 5 kb (Bull, unpublished). Many of the MAP specific regions contain, or are flanked by, remnants of bacteriophage (viral) sequences, suggesting that they may have been acquired from other bacterial species via viral cross-infection. MAP appears to have evolved from an MAA-like progenitor by a combination of gene deletion and acquisition (Brennan & Nikaido 1995; Tizard *et al.* 1998). This model is supported by more recent microarray analysis of multiple MAA and MAP strains (Semret *et al*, submitted).

Functional genomic analysis of MAP specific regions will prove useful to understanding the unique biology of MAP. Computer analysis of these regions has already identified several genes with high degrees of homology to genes involved in pathogenicity by other bacteria. A major 60 kb MAP specific region encodes several gene sets (operons) including membrane transport systems and a novel putative mycobactin-like peptide synthesis pathway that may help to sequester important bacterial growth factors such as Fe-III and cobalt (Stratmann *et al.* in press). Systems such as these that scavenge trace nutrients are often important for the survival of bacteria in intracellular environments. A cluster of MAP specific genes homologous to *mce* genes is present. This is in addition to the common *mce* genes found in many other mycobacterial species which may be involved with specific host entry and survival.

Other MAP specific regions carry putative catalase, peroxidase and nitrile hydrolase genes associated with protection from free-radical mediated intracellular killing. One of these regions (previously designated Locus 6) is deleted in some attenuated (non-virulent) MAP vaccine strains (Hermon-Taylor & Bull 2002). Additional MAP specific genes that may play roles in virulence are described in section 4.4.3. The possibility that MAP carries virulence genes not found in MAA is consistent with the distinctive ecological niche that MAP occupies, namely that of a chronic persistent pathogen of animals.

The MAP genome also contains multiple insertion elements including IS*1311* (Marsh *et al.* 1999), IS*Mav*2 (Strommenger *et al.* 2001) and IS*900* (Green *et al.* 1989). IS*900* is unique to MAP (Hermon-Taylor *et al.* 2000; Bull *et al.* 2003a) and is involved in most of the known diversifications between MAP strains. There is a high degree of clonality (genetic homogeneity) between different MAP strains which may be due to the unusually slow growth rate of MAP (22-26 hrs compared with MAA 10-12 hrs) (Harris & Barletta 2001) and its relatively recent dissemination throughout the world (Moreira *et al.* 1999; Pavlik *at al.,* 1999). Nonetheless, over 30 MAP types with variations in the genomic insertion positions of IS*900* are known (Bull *et al.* 2000).

4.2.3 Genomes of other environmental mycobacteria

In addition to MAA and MAP there are two other genome projects that are nearing completion: *M. ulcerans* at the Pasteur Institute (http://genopole.pasteur.fr/Mulc/BuruList.html and *M. marinum* at the Sanger Centre (http://www.sanger.ac.uk/Projects/M_marinum). The *M. marinum* genome has been estimated to be about 6.5 mb long, consistent with extensive metabolic and environmental versatility. Relative to *M. ulcerans*, *M. marinum* is a genetically heterogeneous species (Stinear *et al.* 2000b; Ucko *et al.* 2002). The *M. ulcerans* genome is estimated to be smaller, at around 5.8 mb, but a more accurate size assessment will come with further sequence assembly. The *M. ulcerans* genome is extraordinarily rich in IS with two different elements (IS*2404* and IS*2606*) accounting for 10% of the total genome. The biological consequences of the presence of these high copy number IS are as yet unknown but it seems possible that they may contribute to the relatively fastidious nature of *M. ulcerans*.

Recently a very large plasmid in *M. ulcerans* was found to encode all the genes necessary for the synthesis of mycolactone (Stinear *et al.* submitted). *M. ulcerans* may be a clonal derivative of *M. marinum* that arose by the acquisition of a plasmid from another microorganism combined with the loss of genetic information on the chromosome. The role that mycolactone plays in the natural ecology of *M. ulcerans* remains to be determined, but it seems unlikely that its primary role is pathogenesis. This molecule may confer a fitness advantage for the survival of *M. ulcerans* in a niche environment such as the salivary glands of aquatic insects. The development of *M. ulcerans* from an *M. marinum*-like ancestor may prove to be a good illustration of how significant human pathogens evolve from environmental forebears.

Currently there is no effort underway to sequence the genome of *M. intracellulare*. Given the increasing recognition of *M. intracellulare* as a species with distinct (from MAA) pathobiological characteristics, we believe that this pathogen should be the next focus of genomic analysis.

4.3 BACTERIAL PHYSIOLOGY

4.3.1 The cell envelope and its role in virulence and antimicrobial resistance

Perhaps the most distinctive feature of mycobacterial physiology is the cell envelope. The ability of pathogenic mycobacteria to survive in hostile environments such as the phagocytic vesicles of immune cells may be related in part to cell wall impermeability. The same is probably true of the pathogen's intrinsic resistance to diverse antimicrobial agents (Inderlied *et al.* 1993; Jarlier *et al.* 1994; Portillo-Gomez *et al.* 1995; Heifets 1996; Cangelosi *et al.* 2001).

The MAC cell wall is a complex array of hydrocarbon chains perforated by porins through which nutrients and other compounds pass into and out of the cell. The electron-dense peptidoglycan layer is surrounded by a hydrophobic arabinogalactan-peptidoglycan-mycolic acid layer characteristic of all mycobacteria. This layer is surrounded by a second electron-dense layer made up, in part, of ssGPL found only in MAC (Belisle *et al.* 1991, 1993; Inderlied *et al.* 1993; Wayne *et al.* 1993; Belisle & Brennan 1994). The ssGPL consists of a core nonspecific GPL modified by serovar-specific oligosaccharide side chains. The core non-specific GPL which is common to many environmental mycobacteria has a tetrapeptide structure linked to a 6-deoxy-L-talose, which in MAC is further modified with variable oligosaccharide structures to form the ssGPL.

The synthesis of ssGPL is one the distinguishing characteristics of MAC relative to *M. tuberculosis*. Genes coding for ssGPL synthesis are clustered in a region spanning approximately 50 kb of the MAC genome (Eckstein *et al.* 2000; Laurent *et al.* 2003). The ssGPL is one of the major antigens in host environments, and is responsible for the "watery" (hydrophilic) appearance of MAC colonies *in vitro*. The pathobiological role of ssGPL has not been determined. However, these molecules have been reported to accumulate in the phagosome during intracellular growth contributing to the formation of a capsule around the bacteria (Tereletsky & Barrow 1983; Rulong *et al.* 1991). Stable rough colony type variants that lack GPL frequently arise during extracellular growth, whereas strains cultivated from patient samples are invariably smooth.

4.3.2 Morphotypic switches

Most MAC isolates form multiple colony types (morphotypes) that vary with regard to infectivity, susceptibility to antibiotics and ability to grow in various environments. The transition from the smooth colony type to rough is irreversible. Rough mutants fall into two categories: those that lack all traces of GPL and those that produce a lipopeptide core of GPL that is not glycosylated. Both categories result from spontaneous deletions within the ssGPL cluster (Belisle *et al.* 1991, 1993). These

deletions are mediated by homologous recombination between neighbouring insertion elements (Eckstein *et al.* 2000).

Additional morphotypic switches in MAC are less well understood. The transparent-to-opaque switch is reversible at frequencies ranging from 10^{-4} to 10^{-5} per generation (Woodley & David 1976; Inderlied *et al.* 1993; Prinzis *et al.* 1994). Transparent variants are more virulent and more drug resistant than their opaque counterparts. Opaque variants grow more quickly on laboratory media but are rarely isolated from environmental or clinical samples. That may be an artefact of decontamination protocols applied to clinical and environmental samples, including sodium hydroxide and CPC, which are toxic to opaque variants. However, MAC colonies grown directly from the blood of AIDS patients without decontamination are transparent suggesting that this morphotype is the true form in which the pathogens exist *in vivo* (Meylan *et al.* 1990).

A separate switch, termed red-white, becomes visible when clinical isolates are grown on agar media containing the lipoprotein stain CR (Cangelosi *et al.* 1999, 2001; Mukherjee *et al.* 2001; Laurent *et al.* 2003). The red to white and opaque to transparent switches are independent of each other, such that red opaque, red transparent, white opaque and white transparent forms can be isolated from most strains. Relative to red variants, white variants are more resistant to multiple antibiotics *in vitro*, more common in patient samples and more virulent in disease models. When stable red variants are inoculated into disease models (mice and human macrophages) they switch en masse to the white morphotype. This occurs over the course of one or two generation times consistent with a switch at the level of gene expression rather than selection of a pre-existing white subpopulation. However, red transparent variants are occasionally recovered from patient samples, and the genome sequence strain MAA 104 is a stable red variant.

A gene, *crs*, has been identified that is required for the synthesis of the major CR binding site, possibly a cell wall glycolipid (Laurent *et al.* 2003). We have proposed that white variants, but not red variants, express surface components that mask underlying CR binding sites. These components, or components that are co-expressed in white variants, may also be responsible for the reduced antibiotic susceptibility associated with the white morphotype (Cangelosi *et al.* 2001). Consistent with this model, mutational loss of *crs* results in reduced CR binding and a white morphotype, but does not increase multi-drug resistance. Recently, a polyketide synthase (lipid biosynthetic) gene, *pks12,* and a second surface-associated gene of unknown function, Maa2520, were found by mutational analysis to be required for the multi-drug resistant white morphotype (Cangelosi et al., submitted manuscript). However, mutational analysis ruled out a role for ssGPL in multi-drug resistance (Laurent et al., 2003).

Like other MAC strains, MAP also forms smooth and rough colony type variants on agar media. Colonies of MAP grow more slowly than those of MAA and *M. intracellulare*, and only on media supplemented with the siderophore mycobactin.

An intriguing physiological feature of MAP is its ability to persist intracellularly in a cell wall deficient form, similar to a spheroplast (Chiodini *et al.* 1986; Wall *et al.* 1993). This form is non-acid fast by the ZN method. It is presumed to result from an induced cessation or radical alteration of cell wall mycolic acid production, and it may be part of a mechanism for avoiding host immune surveillance. It is not observed in other MAC. The cell wall deficient form has been found intracellularly in macrophages associated with the gut wall of up to 92% of patients with CD and 26% of controls (Sechi *et al.* 2001; Naser *et al.* 2002; Bull *et al.* 2003). Reversion to the conventional acid-fast vegetative phenotype after prolonged culture (up to two years) is possible (Chiodini *et al.* 1984; Schwartze *et al.* 2000) showing that this morphotypic switch is not permanent. Cell wall deficient forms have also been shown to persist intracellularly for more than a year when "fed" to amoebae. It is not certain, however, if MAP cells divide whilst in this chronic persistent state.

4.3.3 Metabolism and catabolism

The mycobacteria are aerobic organisms. Many environmental species such as *M. fortuitum* are fast-growing with *in vitro* doubling times in the range of 2 hours, whereas MAC is slow growing with doubling times exceeding 15 hours. Consistent with its slow growth, MAC has only a single copy of the *rrn* (ribosomal RNA) operon and a correspondingly low ribosome copy number.

MAC can utilize a variety of carbon and energy sources. Growth is stimulated by glycerol and fatty acids. The availability of fatty acids and the ability of the pathogens to utilize them are thought to be important factors in the colonization of host tissues. As with *M. tuberculosis*, optimal growth of MAC *in vitro* is observed on relatively elaborate media such as the Middlebrook series, supplemented with glycerol, oleic acid and low purity (96%) BSA. The function of the BSA supplement is not known, but it is thought to detoxify harmful by-products of fatty acid metabolism. In contrast to some pathogenic mycobacteria, MAC grows well on minimal media such as Sauton's, which consists of nothing more than glycerol, citric acid, L-asparagine as a nitrogen source and trace salts of potassium, magnesium, iron and zinc. Thus the organisms have comprehensive biosynthetic capabilities, consistent with their ability to colonize dilute environments.

There are some interesting exceptions to the rule of nutritional independence in MAC. In contrast to virulent "white" variants, spontaneously occurring non-virulent "red" variants of MAA require the presence of BSA for growth *in vitro* (Millones & Cangelosi, unpublished results). This observation might help explain the relatively poor survival of this morphotype in certain environments, including host cells and tissues (Mukherjee *et al.* 2001). In contrast to MAA, MAP strains require growth media supplemented with the siderophore mycobactin, a distinction that has been useful for bacteriological identification.

4.4 BIOLOGY OF MAC IN HOST ENVIRONMENTS

4.4.1 Entry and survival in host cells

MAA is thought to enter the human body through the bronchial or intestinal mucosa. The bacteria are ingested by host macrophages primarily (but probably not exclusively) via complement receptors and complement component C3-mediated phagocytosis (Swarz *et al.* 1988; Bermudez *et al.* 1990; Schlesinger *et al.* 1990, 1991). Complement-mediated phagocytosis does not trigger an oxidative burst within the macrophage thus facilitating mycobacterial survival after ingestion. Once inside the non-activated macrophage, MAA prevents fusion between the phagosome in which it resides and the lysosome thereby escaping the normal lytic functions of the macrophage (Frehel *et al.* 1986, 1991; Crowle *et al.* 1991; de Chastellier *et al.* 1993; Sturgill-Koszycki *et al.* 1994). MAA grows and multiplies within the phagosomal compartment until the macrophage lyses. If the released bacilli are phagocytosed by other non-activated macrophages, the cycle of growth, multiplication, release and phagocytosis continues unabated until the infected host succumbs or mounts an immune response. However, if MAA is ingested by activated macrophages, phagosome-lysosome fusion is not impeded and the bacilli are killed. Induction of a cell-mediated immune response is necessary for controlling the infection and in severely immunocompromised individuals (e.g. AIDS patients) this type of response does not function effectively and the infection is not controlled.

4.4.2 MAA genes involved in intracellular life

Understanding the metabolic activities of MAA during growth in macrophages should provide clues as to how the bacilli survive in this hostile environment. One approach has been to analyse global patterns of gene expression during intracellular growth. Plum and Clark-Curtiss (1994) were the first to use a cDNA subtractive approach to identify MAA genes that were up-regulated for expression or uniquely expressed in bacteria growing within primary human macrophages. A gene designated macrophage-induced gene (Mig) was identified. The Mig protein is an acyl-Coenzyme A synthetase that uses saturated medium-chain fatty acids, unsaturated long-chain fatty acids and some aromatic carbon acids as substrates (Morsczek *et al.* 2001). Mig protein may be involved in the metabolism of fatty acids for synthesis of mycobacterial lipids during intracellular growth.

More recently, Hou *et al.* (2002) used the SCOTS technique to identify 46 genes that are up-regulated or uniquely expressed by MAA during growth in human macrophages. This analysis provided some insights into *M. avium* metabolism during intracellular growth. For example, both the tricarboxylic acid cycle and the glyoxalate shunt appeared to function during intracellular growth. The tricarboxylic acid cycle is the central metabolic pathway responsible for generation of CO_2, adenosine

triphosphate, reduced nucleotides and precursors of several amino acids. The glyoxalate shunt functions to prevent loss of carbon molecules by bypassing the steps in which CO_2 is generated. In other bacteria the kinds of carbon sources available affect the operation of these cycles with the glyoxalate shunt becoming operative when fatty acids are used as carbon sources. The glyoxalate shunt is not used exclusively but operates simultaneously with the tricarboxylic acid cycle (Cronan & LaPorte 1996).

Expression of genes encoding several enzymes involved in biosynthetic pathways for amino acids and mycolic acids also appeared to be up-regulated during intracellular growth of MAA (Hou *et al.* 2002). In addition, genes encoding enzymes involved in mycobactin biosynthesis were up-regulated (Hou *et al.* 2002). Mycobactins are siderophores produced by mycobacteria to enable them to obtain iron, an essential nutrient for all organisms, but one which is not usually readily available to intracellular organisms. Another up-regulated gene was a homologue of the *M. tuberculosis narK3* gene (Hou *et al.* 2002). This gene codes for a nitrite extrusion protein. Excess nitrite is toxic to some mycobacteria.

Several genes that code for proteins involved in regulation of gene expression were also up-regulated for expression during growth in macrophages. Finally, a number of genes encoding homologues to *M. tuberculosis* proteins that may be important in mycobacterial pathogenesis were also observed to be up-regulated in MAA during growth in macrophages. These included genes belonging to two of the *mce* operons and two genes encoding PPE proteins (Hou *et al.* 2002). Certain *mce* proteins have been implicated in entry and intracellular survival (Arruda *et al.* 1993; Graham & Clark-Curtiss 1999), while PPE proteins have been postulated to participate in antigenic variation (Cole *et al.* 1998).

Additional global gene expression analyses have targeted proteins. Honer zu Bentrup *et al.* (1999) and Sturgill-Koszycki *et al.* (1994) employed two-dimensional gel electrophoresis to identify a protein, isocitrate lyase that is up-regulated for expression in MAA grown in mouse macrophages. Additional experiments are necessary to definitively prove that up-regulated proteins are essential for intracellular life. Moreover, there probably are additional proteins important to intracellular life which remain to be identified. Nevertheless, valuable information regarding MAA metabolism during intracellular growth has been obtained. Identification of additional up-regulated genes will further enhance our understanding of MAA physiology in this environmental niche.

Do all MAA strains express the same genes after phagocytosis by human macrophages? The MAA strain used for the SCOTS analyses was a serotype 4 strain isolated from an HIV-infected human. Do MAA strains that are able to infect humans express genes that are not expressed in strains that are not pathogenic in humans? Answers to these questions may provide better ways to assess the risks of infection by MAC-contaminated water supplies.

4.4.3 MAP genes involved in intracellular life

Accumulating evidence supports a role for unique cell wall structures in intracellular survival and virulence of MAP. A gene designated *des*A1, which codes for a desaturase associated with mycobacterial cell wall synthesis (Jackson *et al.* 1997), is intact in 90% of human isolates but is truncated by the insertion of an IS*900* element in all animal and environmental isolates (Bull *et al.* 2000). Some animal MAP isolates, when exposed to long-term intracellular persistence in amoebae, can promote the auto-excision of IS*900* from inside the *des*A1 gene thereby restoring its function (Bull, unpublished). The activation of genes such as *des*A1 in intracellular environments may therefore be important factors in MAP pathogenesis.

Differential expression analysis using a subgenome microarray on intracellular and extracellular cultured MAP, has highlighted a set of genes, the GS cassette, which make and modify fucose (Bull *et al.* 2000a) and are up-regulated on cell entry. The cassette is found on a 9 kb MAP specific region bounded by a repeat sequence. It has a relatively low G+C% content, suggesting that was acquired horizontally from another bacterial species. The GS cassette is found in *M. avium* subspecies *silvaticum;* however, in that organism the putative fucose acetylation gene is truncated by the insertion of IS*1612*, an element not found in MAP (Bull *et al.* 2000b). Acetylated fucose as a terminal sugar of cell wall ssGPL is the defining moiety of serotype 2 in MAA. Homologues of the GS cassette are also present in *Mycobacterium bovis* but are deleted in BCG vaccine strains (Behr *et al.* 1999). Work is in progress to determine the significance of these observations relative to MAP cell wall synthesis and virulence.

4.5 KEY RESEARCH ISSUES

This chapter presented a broad overview of what is known, and not known, about the biology of MAC and similar environmental mycobacteria in host and external environments. The following issues were singled out as priorities for future research.

4.5.1 Diversity

This issue is especially important with regard to MAA and *M. intracellulare*. These species are versatile and heterogeneous and it is possible that some strains found in the environment are infectious while others may be relatively harmless. Risk assessment may be ineffective if it is based on the assumption that all environmental strains are uniformly infectious. Strain-to-strain diversity can and should be quantified using available disease models combined with molecular epidemiological and genomic tools such as microarrays. The results could significantly improve the way we approach MAC epidemiology and risk assessment.

4.5.2 Molecular markers of virulence

If environmental MAC isolates are heterogeneous it may be possible to identify markers of pathogenic strains. If readily detectable such markers could help refine epidemiological analysis and lead to more accurate methods of risk assessment. Public health efforts to control MAC exposure will thereby benefit from molecular analysis of MAC pathogenicity.

4.5.3 Taxonomy

The pathogens we know as MAA, MAP and *M. intracellulare* are distinct in many significant ways, including environmental niches, host preferences and clinical manifestations. Recognition of these distinctions is essential to the understanding of the biology of MAC.

4.5.4 Evolutionary context

Populations of environmental mycobacteria to which humans are exposed do not appear to have human environments in their recent evolutionary histories. Their pathogenic capabilities were selected by environmental challenges encountered outside of human hosts. The ecology of these organisms in their natural habitats must be better understood if we hope to fully understand their pathogenic capabilities.

4.5.5 Genomic analysis

The impending completion of the genome sequences of several environmental mycobacteria, combined with tools for global analysis of gene expression such as microarrays and SCOTS, will greatly improve our understanding of these organisms and our ability to address the biological issues listed above. Genomic analysis should be expanded to include *M. intracellulare* and additional environmental mycobacteria. Comparative genomic analysis of mycobacteria will continue to bolster our understanding of how environmental microorganisms become human pathogens.

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5

Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria

T. Stinear, T. Ford and V. Vincent

5.1 INTRODUCTION

This chapter reviews the state-of-the-art methods for the detection of pathogenic mycobacteria in the environment. It provides a concise review of the literature surrounding this aspect of mycobacteriology.

Some unusual features define the genus *Mycobacterium*. These include a waxy cell wall made up of long-chain mycolic acids and DNA with a high G+C content. However, these shared characteristics (discussed in Chapter 4) belie a high degree of inter-species heterogeneity. Widely varying growth rates, specific growth requirements and differing natural ecologies are three examples of this heterogeneity (see Chapter 2). It has important consequences for the detection of mycobacterial pathogens in the

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environment because it means that general mycobacterial methods must often be tailored to detect specific pathogens. In mycobacteriology there are no standard methods.

Mycobacterial diagnostics have benefited enormously from the many recent advances in biotechnology. These advances have had the greatest impact at the level of isolate identification and characterization. Techniques such as IMS, PCR and automated DNA sequencing are now beginning to form the foundation for modern mycobacterial analytical methods. However, to a large extent one of the most important steps in analysis - the isolation in culture of mycobacteria from the environment - has not significantly changed since the 1950s and 1960s when methods for chemical treatment of samples to remove background microorganisms were first implemented. This lack of progress is unfortunate as there are serious issues surrounding the speed and sensitivity of these culture methods. Furthermore, the power of the molecular tools now available for modern diagnostics is largely dependent on the availability of isolates in pure culture. One could pessimistically summarize this situation as being akin to a present day Formula One racing car powered by a 19th century steam engine.

Nevertheless, by using these flawed techniques over 70 mycobacterial species have been formally recognized and the study of these isolates by molecular genetic methods has shed considerable light on the population structure of the genus mycobacteria and is dramatically improving our understanding of the relationship between pathogenic and non-pathogenic mycobacteria. An important consequence of this work is the discovery of genetic markers of virulence. These markers are discussed later in this chapter (and in Chapter 4) and they are the basis for modern molecular detection methods.

The search for genetic markers has been greatly assisted with the advent of whole-genome sequencing projects (i.e. determining the complete DNA sequence of an organism). As of September 2002, four mycobacterial genomes have been fully sequenced (a cumulative total of 17 000 000 bp) and there are a further seven currently in progress (http://wit.integratedgenomics.com/GOLD/). These projects are a fundamental resource for unlocking the pathways to pathogenesis among the mycobacteria and in so doing they are revolutionizing mycobacteriology. The full diagnostic potential afforded by whole genome sequences is still yet to be realized but significant advances in this area have already begun. Comparative genomic analysis of the different members within the MTB complex has permitted identification of a panel of DNA markers that unequivocally differentiate each sub-species within this very homogeneous complex (Brosch *et al.* 2002). Comparisons between complete mycobacterial genome sequences have also highlighted the mechanisms and the significant extent to which some members of the genus can evolve. DNA insertions, deletions and point mutations are important drivers of genome change. IS in particular mediate many changes within mycobacterial genomes and some of these changes can

have profound effects on virulence (see McAdam (2000) for a review of mycobacterial IS). IS are short stretches of non-essential DNA, around 1.5kb in size, often repeated many times throughout a genome. They encode a transposase, an enzyme that permits the sequence to copy itself. In this respect IS are said to be mobile DNA elements. IS have been shown to move between different bacteria and thus they are a means for the exchange of DNA between bacteria. But despite this potential to jump across so-called species boundaries some mycobacterial IS have proved to be useful species and sub-species markers. Examples include IS*1245* and IS*1311* for MAC, IS*900* for MAP and IS*2404* for *M. ulcerans*. As IS copy themselves within the genome they can produce a unique and heritable distribution pattern for a particular strain. Over time this can lead to inter-strain IS variation and it is this variation that has been extensively exploited for the development of molecular fingerprinting methods. These methods are discussed in section 5.2.4.

Our improved understanding of both mycobacterial genetics and ecology has had obvious implications for those undertaking surveillance studies for these bacteria. Analysis of only grab samples of water is unlikely to be sufficient to reflect the mycobacterial composition of the greater environment from which the water came, nor will such analyses reflect the inherent risk of mycobacterial infection from that environment. A holistic approach to environmental surveillance is required that draws on a detailed knowledge of the biology and ecology of the mycobacteria.

Their ubiquitous presence means it is a relatively simple affair to find environmental mycobacteria per se but it is the specific detection of a particular mycobacterial pathogen that can require a comprehensive understanding of its biology and ecology, with subsequent modification or development of the detection methodology. All this knowledge of the organism must be supported by good epidemiological data. The recent finding of *M. ulcerans* in the salivary glands of aquatic insects is a useful example of the application of these principles. (Refer to Chapter 8 for a discussion of Buruli Ulcer). For 50 years the environmental source of *M. ulcerans* was unknown despite very good epidemiological evidence linking it to aquatic environments (Barker 1973). In 1997 an *M. ulcerans* specific IS was identified, named IS*2404*, and a highly sensitive PCR test was developed based on this sequence (Ross *et al.* 1997a). This test circumvented the need to culture *M. ulcerans* as a means of demonstrating its presence in the environment. The new PCR was applied to water and plant samples collected from *M. ulcerans* endemic and non-endemic areas. PCR-positive samples were obtained only in the endemic regions (Roberts & Hirst 1997; Ross *et al.* 1997; Stinear *et al.* 2000). This was the first direct evidence of *M. ulcerans* in the environment. Subsequent studies using the same DNA marker found *M. ulcerans* PCR-positive samples in aquatic insects collected in West Africa (Portaels *et al.* 1999). Most recently, in a series of experiments that modelled and tracked the fate of *M. ulcerans* in aquatic insects, Marsollier and co-workers succeeded

in culturing *M. ulcerans* from the salivary glands of aquatic insects captured from endemic areas in the Ivory Coast (Marsollier *et al.* 2002).

In summary, mycobacteria are not all the same and the efficiency of the many methods used for their detection is highly variable. A good knowledge of the biology and ecology of a target organism using the best available technology is essential to maximize the chance of detection.

5.2 THE ANALYSIS PROCESS

From the perspective of the water industry there are two general requirements of a microbiological analytical method, namely to be able to provide data for routine surveillance (e.g. as part of risk assessments or catchment management programmes) and to be able to respond quickly to a public health emergency. Each scenario will require a different approach to analysis.

Analysis is a multi-step process and the reader is referred to *Handbook of Water Analysis and Standard Methods* for a more general introduction to this topic. A schematic of the analysis pathway for environmental mycobacteria is given in Figure 5.1. It has been depicted as five distinct phases: (i) sample collection; (ii) sample storage; (iii) sample preparation; (iv) detection; (v) result interpretation.

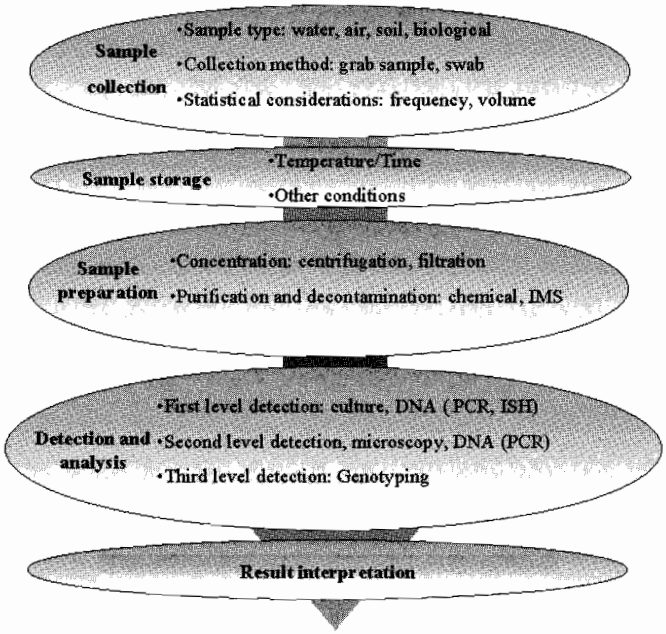

Fig. 5.1 Flow chart of the analysis process

The following sections review the various methods that have been developed for mycobacterial analysis of the environment. A summary of methods has been made from a representative selection of recent reports that have described the isolation of a range of both slow and rapid growing mycobacteria from various environmental sources (Table 5.1). The summary has been constructed following the criteria outlined in Figure 5.1.

Table 5.1 Methods recently used for the isolation of mycobacteria from the environment

Sample type	Method Summary	Reference
Water (raw and treated): surface and groundwater sources. Biofilms: pipes, water meters	Sampling: 300 ml water samples, 4 cm^2 biofilm samples. Concentration: centrifugation. Decontamination: CPC, 0.005%, 30 min. Isolation: 7H10 agar (OADC), 37 °C. Detection: representational colony selection, ZN staining, sub-culture to purity, 16SrRNA PCR and sequencing	(Falkinham *et al.* 2001)
Water (raw and treated): surface and groundwater sources	Sampling: 1000 ml water samples. Concentration: filtration. Decontamination: SDS/NaOH. Isolation: Lowenstein-Jensen, 37 °C. Detection: ZN staining, sub-culture to purity, 16SrRNA PCR and sequencing, *hsp65* PRA and sequencing	(Le Dantec *et al.* 2002)
Water: hospital and consumer taps	Sampling: 500 ml water samples. Concentration: 0.45 µm filtration. Decontamination: CPC, 0.005% 15 min. Isolation: BACTEC and Lowenstein-Jensen, 37 °C. Detection: AFB staining, sub-culture, biochemical ID,	(Peters *et al.* 1995)
Water: various sources	Sampling: 25 ml water samples. Concentration: centrifugation. Decontamination: none used. Isolation: Tsukamura minimal-Tween80-cycloheximide agar, 37 °C. Detection: AFB staining, sub-culture to purity, DNA probes for MAC.	(von Reyn *et al.* 1993)
Water (raw and treated): cold taps, hot taps, ice, showers, bottled water	Sampling: 500 ml samples. Concentration: 0.45 µm filtration. Decontamination: CPC. Isolation: 7H10 agar (cycloheximide), 37 °C, 5% CO_2 Detection: colony morphology, sub-culture, 16SrRNA PCR and sequencing	(Covert *et al.* 1999)
Swimming pools: water, biofilms	Sampling: 500 ml samples, swabs (20 cm^2). Concentration: 0.45 µm filtration. Decontamination: CPC 0.04%, 30 min. Isolation: 7H10 agar, 30 °C, 5% CO_2 Detection: colony morph, sub-culture, biochemical ID	(Leoni *et al.* 1999)
Water (raw and treated)	Concentration: 0.45 µm filtration. Decontamination: CPC 0.05% - CPC 0.005% 30 min. Isolation: Lowenstein-Jensen, 30 °C. Detection: sub-culture based on colony type on 7H10 agar, TLC and *hsp65* PRA.	(Neumann *et al.* 1997)
Hospital tap water	Sampling: 200 ml samples. Concentration: centrifugation. Decontamination: not specified. Isolation: Lowenstein-Jensen, 37 °C. Detection: AFB, sub-culture, *hsp65* PRA	(Chang *et al.* 2002)
Surface water, vegetation, soil	Concentration: centrifugation, filtration. Decontamination: IMS, CPC 0.005% 30 min, Isolation: Brown & Buckle agar, 30 °C, microaerophilic. Detection: subculture, AFB, 16SrRNA PCR sequencing	(Stinear *et al.* 2000)

5.2.1 Sampling

The objective of sampling is to obtain a sample from a particular environment that can be said to represent that environment. Given the stochastic nature of bacterial populations how can this be achieved? There is no simple answer to this question. Sampling demands at least that the researcher clearly define the question(s) being asked in any investigation. Consideration must be given to both spatial and temporal aspects of a particular environment. Thus the issues of sampling frequency and sample type will vary with each situation. Sampling an aquatic environment requires collection of more than just bulk water samples. The habitats of mycobacteria are diverse (refer to Chapter 3 for a review of the different sources of mycobacteria). As an example of this diversity, a recent survey of several drinking-water distribution systems has shown that pipe biofilms are probably the most significant sources of mycobacteria within a drinking-water supply. Conservative concentration estimates of 4300 slow growing mycobacteria per cm^2 were reported (Falkinham *et al.* 2001). This study and others (Schulze-Röbbecke & Fischeder 1989), suggests that efforts to examine the risk posed by mycobacteria in drinking-water supplies should focus more heavily on pipe biofilms than bulk water samples. Biofilms can be sampled either by collecting swabs from which mycobacteria may be recovered by culture or DNA methods. Alternatively, the biofilm can be kept intact and a portion embedded. This portion can then be thin-sectioned and the mycobacteria analysed *in situ* using techniques discussed in section 5.2.4.1. Given the ubiquity of slow growing mycobacteria in both potable and hot water systems, research is increasingly focused on monitoring growth of these organisms using recirculating or by-pass systems. This parallels other trends in drinking-water microbiology research, where it is realized that accurate simulations of drinking-water systems are necessary to examine pathogen survival and proliferation in biofilms and to implement control methods.

Considerable research efforts are in place to optimize these simulations. The most accurate simulations are those involving removable coupons that are placed in by-pass systems plumbed directly into a hot or cold water supply. The primary method of sampling is through removal of coupons and either direct DNA extraction or embedding followed by cryosectioning and imaging with fluorescent labelled antibodies.

Developed at Montana State University, the cryosectioning technique has been widely applied to imaging mixed species biofilms (Yu *et al.* 1994; Murga *et al.* 1995). Its application to dual species biofilms (*Pseudomonas aeruginosa* and *M. avium*) grown in cold water recirculating systems has

shown preferential survival of *M. avium* at the metal coupon-biofilm interface (Ford 1999). Research is now focused on optimized ISH methods to visualize mycobacterial species in biofilms (refer to section 5.2.4.1).

Methods have also been described for sampling aerosols (Wendt *et al.* 1980). The following list is a summary of potential mycobacterial environments with references to reports concerning sampling each of them. In short, everything must be considered as a potential mycobacterial habitat:

- aerosols (Wendt *et al.* 1980)
- air/water interface (Falkinham 2003)
- biofilms (Schulze-Röbbecke & Fischeder 1989)
- insect populations (Marsollier *et al.* 2002)
- animal populations (Fischer *et al.* 2000)
- vegetation (Stinear *et al.* 2000)
- soil (Iivanainen *et al.* 1999b)
- food (Argueta *et al.* 2000)
- water (refer to Table 5.1)

5.2.2 Sample storage

Several studies have been performed to examine the effects of different storage conditions on the viability of mycobacteria collected from the environment. Iivanainen *et al.* (1995) demonstrated that long-term storage at –80 °C of crude cell-concentrates obtained from water samples without the addition of cryoprotectant reduced levels of background bacteria and enhanced the recovery of mycobacteria. However, it is important to understand something of the physiology of individual mycobacteria as some species show significant changes to their cell wall or lose viability as a consequence of freezing (Thoen *et al.* 1977; Silva *et al.* 1989).

5.2.3 Sample preparation

This step incorporates sample concentration, decontamination and purification. It is usual to analyse sample volumes up to one millilitre. If the concentration of mycobacteria in a given sample is expected to be low, as is often the case with treated water samples, then it may be necessary to analyse up to 1000 ml sample volumes. Either filtration through 0.45 μm membranes or centrifugation methods are employed to concentrate mycobacterial cells in samples of this volume prior to analysis.

Sample decontamination methods have been designed to remove or reduce the non-mycobacterial microbial flora in a sample. These techniques utilize the relative resistance of mycobacteria to chemical treatments such as

combinations of acid/alkali or detergents. Several studies have been conducted to try and determine the optimum decontamination method. There is no clear consensus as to the optimum method; however, treatment with the detergent CPC (du Moulin & Stottmeier 1978; Fischeder *et al.* 1991) has repeatedly been shown to promote the recovery of a wide range of mycobacteria with low rates of background contamination (Table 5.1). The comparative efficacy of agents such as CPC and NaOH is greatly influenced by the contact time and concentrations used (Hunter *et al.* 2001). Thus it may be necessary for investigators to test empirically the relative resistance of different mycobacteria under authentic test conditions before commencing any environmental analyses (i.e. conduct seeding experiments to determine the best decontamination regime in a given sample matrix for a given group of mycobacteria).

Sometimes it is insufficient just to reduce the background microflora. To enable the detection of mycobacterial pathogens such as MAP and *M. ulcerans* in the environment it has been necessary to develop methods that very specifically enrich for target cells. A recent development has been IMS. This technique uses 1-2 μm paramagnetic beads coated with anti-mycobacterial antibodies. These antibody-coated beads are incubated with a sample concentrate, typically in a 1.5 ml tube. The beads specifically bind mycobacterial cells. A magnet is then applied to the tube and a complex of magnetic beads and mycobacterial cells form on the side of the tube. This complex can then be decontaminated by a low concentration CPC treatment and then used as the inoculum in mycobacterial culture media or subjected to a diagnostic PCR. This method has been used successfully to isolate *M. ulcerans* in culture from the environment (Marsollier *et al.* 2002). It has also been used to detect *M. tuberculosis, M. avium* and MAP (Li *et al.* 1996; Mazurek *et al.* 1996; Grant *et al.* 1998; Mason *et al.* 2001). The general approach is summarized in Figure 5.2. There are variations on this method; for example, "indirect capture" where the antibodies are first incubated with the sample before the addition of the magnetic beads. The success of IMS is dependent on the specificity of the anti-mycobacterial antibodies used to coat the magnetic beads. Improvements here will see the more widespread application of IMS in mycobacterial diagnostics.

Future decontamination protocols may focus on the use of free-living aquatic amoebae as a means for purifying and isolating mycobacteria as they are natural hosts for many mycobacteria (Steinert *et al.* 1998).

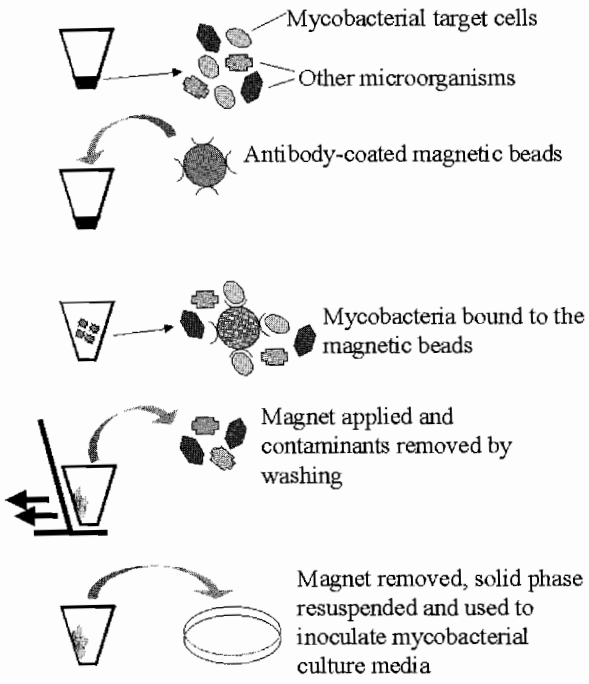

Fig. 5.2 The basic principle of IMS

5.2.4 Detection

Detection methods can be subdivided into three categories. First level detection refers to those methods involved with primary isolation; second level detection refers to methods that provide genus and species identification; and third level detection refers to methods used for sub-species discrimination and molecular epidemiology.

5.2.4.1 Detection (first level)

There are three general approaches for the detection of mycobacteria in the environment.

The first approach is to identify the mycobacteria in a sample by culture enrichment. That is, take a sample, perform some pre-treatments (such as those described above) to try and remove other microorganisms and then enrich for

the mycobacteria using artificial media. Mycobacterial-like colonies can then be confirmed using a number of techniques. These types of methods are said to be quantitative because the output is cfu per volume analysed.

There have been several comparisons of different media for the recovery of mycobacteria from the environment. The conclusions from different studies are difficult to compare and often contradictory. An extensive and recent review of the literature in this field concluded that, depending on the mycobacterial species under investigation, multiple types of media should be used and incubation conditions should be varied, such as medium pH, temperature and atmospheric CO_2 concentration (Hunter *et al.* 2001). In general, combinations of egg-based (e.g. Lowenstein-Jensen agar slopes) and defined salt media (Middlebrook 7H10 agar with OADC supplement), with lower incubation temperatures (around 32 °C) seem to promote the recovery of the widest range of mycobacteria. More information regarding different mycobacterial culture media can be found in Brown & McNeil (2003). To suppress fungal overgrowth and other microbial overgrowth, agents such as cycloheximide or malachite green are often added to the media. Antibiotics, such as a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA supplement), are frequently added to liquid media. It is regularly used in clinical mycobacteriology in conjunction with the BACTEC medium (Becton Dickinson, Sparks, Maryland, USA). For the recovery of some mycobacterial species it is necessary to use media containing specific growth factors such as mycobactin, a siderophore required for the growth of MAP.

It is well recognized that culture enrichment methods for mycobacteria are inefficient and are hindered by many factors. The differences in growth rates between mycobacteria in artificial media are very large. Rapid growers such as *M. smegmatis* double in 2 hours compared to slow growers such as *M. ulcerans* that double in 48 hours or, at the extreme of the spectrum of growth rates, *M. leprae* which does not grow at all in axenic culture. Thus a major issue for culture methods is the overgrowth of slowly growing mycobacteria by faster growing mycobacteria or by other microorganisms. Chemical decontamination methods were developed to reduce overgrowth problems but these methods are necessarily harsh and there are always a percentage of mycobacterial cells destroyed during the decontamination process. Mycobacterial reduction levels of 1-2 log are not uncommon (Dundee *et al.* 2001; Le Dantec *et al.* 2002a). There are also no selective media for mycobacteria in the same way that there are for members of the enterobacteriacae. There have been some modifications made to try and improve selectivity for particular species such as MAC (George & Falkinham 1986) but several method comparisons have suggested that less selective methods perform better (Neumann *et al.* 1997). In addition to the problems of overgrowth there are always the issues of mycobacteria in a viable but non-culturable state (Kazda 2000). Furthermore, colony confirmation is both subjective and laborious. So even though one obtains a numerical result there

are so many accumulated uncertainties that it is difficult to define these methods as quantitative. Despite these problems a wide range of known (*M. gordonae, M. chelonae, M. gastri, M. kansasii, M. fortuitum, M. avium, M. flavescens, M. malmoense, M. xenopi, M. mucogenicum, M. intracellulare, M. peregrinum, M. scrofulaceum, M. shimoidei, M. szulgai, M. haemophilum, M. terrae, M. abscessus, M. aurum, M. phlei, M. marinum, M. sphagni, M. farcinogenes, M. nonchromogenicum* and *M. hodleri*) and many unidentified mycobacterial species have been recovered by the techniques reviewed in Table 5.1, suggesting that these methods have some merit.

An alternative approach, which overcomes these problems of culture isolation, is to use direct DNA detection methods. These are relatively rapid techniques that indicate the presence of mycobacteria by detecting signature mycobacterial DNA sequences without a prior culture enrichment step. Result turnaround times can be hours instead of weeks or months as is often the case with culture methods. The general approach is to take a sample, extract all DNA present in that sample and then identify in that pool of DNA, specific mycobacterial sequences. However, the concentration of mycobacterial DNA in environmental samples is too low to be detected directly, so PCR has been used to amplify target mycobacterial DNA sequences to detectable levels (Wang *et al.* 1996; Stinear *et al.* 2000; Marsollier *et al.* 2002).

A critical point in this type of analysis is the primary DNA extraction step. The mycobacterial cell wall is a lipid-rich envelope that resists common bacterial cell lysis treatments such as NaOH/SDS combinations. Optimal protocols for extraction of mycobacterial nucleic acids have been developed recently and shown to permit the detection of less than 10 bacterial cells in complex sample matrices such as soil and faeces (Stinear *et al.* 2000; Bull *et al.* 2003a). These methods use high-speed cell disintegrators to disrupt mycobacterial cells and liberate the nucleic acids. Stinear *et al.* (2001) have published a detailed protocol for the detection of mycobacterial DNA in plant, water and soil samples.

Several years ago PCR screening of environmental samples was dogged by problems of poor sensitivity due to inhibition of the Taq polymerase by ubiquitous environmental compounds, such as humic and fulvic acids. PCR inhibition is no longer the significant issue it was as there are several technologies now available to remove these contaminants (Rochelle 2001). However, direct DNA detection methods have their limitations. No isolate is recovered and one can only detect short stretches of DNA, thus the amount of genetic information obtained is limited. There is also the issue of detecting dead cells or remnant DNA sequences. Direct DNA detection does have the advantage of giving an absolute indication of the presence or absence of mycobacteria in a given sample and can be quantitative when used in conjunction with a most probable number format (Stinear *et al.* 2000) or with quantitative PCR (MacGregor *et al.* 1999).

The third approach to first level detection is ISH. A sample is immobilized (such as a biofilm sample) and then probed for mycobacteria using either antibodies (Naser *et al.* 2002), or short DNA or RNA sequences (oligonucleotide probes) that have been labelled fluorescently or by some other means. The samples are then visualized by epifluoresence microscopy. By careful design of the probes one can detect at the genus level or at the species level. These methods have the advantage of allowing one to visualize the mycobacteria *in situ*. These techniques work very well for other bacteria and show great promise for mycobacteria but they are still under extensive development. ISH with oligonucleotide probes for the detection of mycobacteria show poor detection sensitivity (Stender *et al.* 1999a). This is thought to be due to problems with the poor penetrability of the hydrophilic probes through the lipid-rich mycobacterial cell wall. Some recent studies have shown successful ISH detection of mycobacteria with peptide nucleic acid probes (hydrophobic, DNA analogue probes) (Stender *et al.* 1999; Zerbi *et al.* 2001).

5.2.4.2 Detection (second level)

This is the primary identification step and ten years ago this category would have included combinations of techniques such as microscopy, biochemical ID methods or cell wall lipid analysis by HPLC. Nowadays, these techniques, with the exception of microscopy, have been almost completely replaced by molecular genetic methods. Unlike the other techniques, genetic methods are independent of growth rate and independent of phenotype. Assuming appropriate DNA sequences are analysed then one can identify all known mycobacterial species and readily discern the presence of new ones. Thus, genetic methods offer greater speed, accuracy and precision. A basic flow chart of this process is given in Figure 5.3, linking PCR-based identification of mycobacterial DNA sequences with the culture enrichment and the direct DNA isolation methods. After obtaining pure culture isolates by culture enrichment, each colony is subjected to ZN stain, which is a stain for cell wall acid fastness. This is a rapid and cost-effective screen with a high negative predictive value that eliminates many non-mycobacteria. ZN-positive isolates can then be screened for mycobacterial DNA sequences by PCR. For direct DNA detection methods, the purified DNA is subjected directly to PCR. It is common at this stage to use mycobacterial genus-specific primers based on the 16SrRNA gene (Boddinghaus *et al.* 1990a) or on the *hsp65* gene (Plikaytis *et al.* 1992). The type of PCR performed is dependent on the goals of the investigation. It may be a PCR designed to detect a particular pathogen, such IS*900* to detect MAP, or a genus-level PCR to detect all mycobacteria, or a combination of PCR tests.

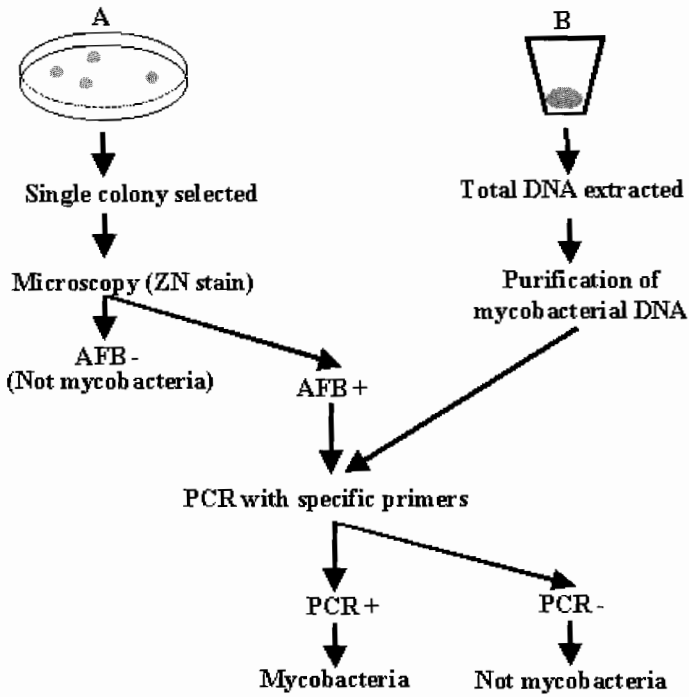

Fig. 5.3 Flow chart for the second level detection of mycobacteria following (A) culture isolation or (B) direct DNA detection

5.2.4.2.1 Genus and species identification

The 16SrRNA gene is the most widely used target for genus and species level identification. The complete DNA sequence of this gene (approximately 1500 bp) has been extensively studied and compared among many organisms. Sequences have been identified that are conserved among all mycobacteria but differ to those in other bacteria. These sequences have been used for genus level identification; usually by amplification of a 1030 bp product (Boddinghaus *et al.* 1990a). The detection of this product indicates the presence of mycobacteria. The region of the 16SrRNA gene amplified encompasses two hypervariable regions, A and B. By subjecting the 1030 bp PCR to nucleotide sequence analysis and then comparing the resulting sequences of the two regions to those obtained from reference strains, an isolate can be readily classified or associated with a species or species complex. There is now a large worldwide, searchable database of mycobacterial 16SrRNA gene sequences (http://www.ncbi.nlm.nih.gov). This

repository of sequences forms the basis for mycobacterial systematics. However, the mycobacteria are unusual in that, compared to other bacteria, there is a very high degree of sequence conservation of the 16SrRNA gene. Indeed, there are several examples of distinct species such as *M. gastri* and *M. kansasii*, and *M. ulcerans* and *M. marinum* that share identical 16SrRNA sequences through their A and B hypervariable regions. One very important consequence of this is that it is rarely possible to identify a mycobacterial isolate based on the 16SrRNA sequence alone, as sequence identity - whilst indicating an obviously close evolutionary relationship - does not infer species identity. Other conserved genes such as *hsp*65 (Plikaytis *et al.* 1992), *rpo*B (Lee *et al.* 2000), *dna*J (Inyaku *et al.* 1993), *sod*A (Zolg, 1994), *gyrB* (Kasai *et al.* 2000) or intergenic regions such as the 16S-23S ITS (Roth *et al.* 1998) have been used as alternative diagnostic targets in an attempt to find sequences with greater polymorphism than the 16SrRNA gene.

The *hsp65* gene has proved to be a very useful target in this respect because, among many mycobacterial species and species complexes, it has a shown a greater amount of sequence diversity than 16SrRNA sequences (Devallois *et al.* 1997). A very widely used test is *hsp65* PCR restriction enzyme analysis (*hsp65* PRA). The principle of this test is quite simple. A 439 bp fragment is amplified from the *hsp65* gene. The DNA sequence of the PCR product is then interrogated by restriction enzyme digestion with two enzymes, *Hae*III and *Bst*EII and the products of the digestion reaction are separated and visualized by gel electrophoresis. The pattern of bands obtained is then compared with the banding patterns obtained from reference strains. A website has been established that contains a database of *hsp65* PRA patterns to assist with species identification (http://www.hospvd.ch/prasite). Many mycobacteria, including *M. avium*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. absecuss*, *M. perigrinum* and *M. chelonae*, can be recognized by their unique banding pattern. In some cases there is sufficient diversity within this region to identify multiple alleles within a species (da Silva Rocha *et al.* 2002). PRA is a rapid and relatively inexpensive diagnostic tool. However, not all mycobacteria can be distinguished by this technique such as the *M. tuberculosis* complex, or *M. marinum* and *M. ulcerans*.

Modern automated DNA sequencing methods have made it possible to rapidly determine the nucleotide sequence of short stretches of DNA. One can rapidly sequence the 439 bp PCR product from the *hsp65* gene, even for a large number of isolates. This approach reveals all the potential nucleotide polymorphisms within this region and thus offers an even higher level of discrimination than PRA (Ringuet *et al.* 1999). In a recent study of 273 clinical and environmental MAC isolates, *hsp65* sequencing revealed 18 different *hsp65* alleles and 54 different polymorphic sites (Smole *et al.* 2002).

Apart from demonstrating the extraordinary genetic diversity among MAC isolates, this study also clearly demonstrated the power of DNA sequencing to unambiguously define the true relationship between isolates.

Single gene analyses such as these have their limitations, as the level of sequence conservation between some species is so high that there is insufficient intra-gene variation to provide discrimination. Again, as for *hsp65* PRA, *M. marinum* and *M. ulcerans* are examples of this category as a recent study of a large number of *M. marinum* fish isolates demonstrated (Ucko *et al.* 2002). MLST is a technique that analyses the nucleotide sequence of several genes. This resolves many of the issues surrounding discrimination between species. MLST is discussed in more detail in the following section, 5.2.4.3.5.

5.2.4.2.2 Commercial tests for genus and species identification

Some of the DNA sequences described above have been used to develop commercial tests for rapid, species-level identification of mycobacteria. The INNO-LiPA Mycobacteria kit (Innogenetics, Ghent, Belgium) is a reverse line-blot hybridization assay that uses a panel of different probes, based on species-specific sequence variations in the 16S-23S ITS region, to identify several different mycobacteria (Suffys *et al.* 2001). The test is in a convenient format but there are several instances where the probes cross-hybridize with other species because of insufficient sequence variation or polymorphism within the ITS. Other commercial DNA-based detection kits include Accuprobe, a DNA probe system based on the detection of 16SrRNA molecules. The tests are performed directly on isolates obtained from either solid or liquid media. The test is simple and rapid but, as the probes are based on highly conserved sequences, cross-hybridization does occur and can give false positive results.

5.2.4.2.3 Pathogen-specific PCR tests

Some DNA sequences, such as certain IS, have been shown to be markers for pathogenic strains or at least potentially pathogenic strains. The DNA targets DT1 and DT6 are often used to detect MAC as these sequences have been shown to be present in all members of the complex (Thierry *et al.* 1993) but absent from other species. A DNA fragment cloned in the plasmid p6123 has been shown to be a specific marker for *M. kansasii* (Yang *et al.* 1993; Picardeau *et al.* 1997a). IS*2404* is a high copy number IS in *M. ulcerans* that is a highly specific and sensitive target for this species (Ross *et al.* 1997a). Similarly IS*900* has been shown to be a very useful marker for the detection of MAP (Hermon-Taylor *et al.* 1990). Table 5.2 contains the primer sequences for these different PCR tests.

Table 5.2 Primer sequences for mycobacterial pathogen-specific PCR tests

Species	Target	Primer	Sequence 5' – 3'	Size	Reference
M. ulcerans	IS*2404*	MU5	AGCGACCCCAGTGGATTGGT	492 bp	(Stinear *et al* 1999)
		MU6	CGGTGATCAAGCGTTCACGA		
MAC	DT1	AV6	ATGGCCGGGAGACGATCTATGC GGCGTAC	666 bp	(Devallois *et al.* 1997a)
		AV7	CGTTCGATCGCAGTTTGTGCAG CGCGTACA		
MAC	DT6	IN38	GAACGCCCGTTGGCTGGCCATT CACGAAGGAG	187 bp	(Devallois *et al.* 1997a)
		IN41	GCGCAACACGGTCGGACAGGC CTTCCTCGA		
MAP (nested PCR)	IS*900*	TJ1	GCTGATCGCCTTGCTCAT		(Bull *et al.* 2003a)
		TJ2	CGGGAGTTTGGTAGCCAGTA		
2nd round for TJ1/TJ2		TJ3	CAGCGGCTGCTTTATATTCC	294 bp	
		TJ4	GGCACGGCTCTTGTTGTAGT		
M. kansasii	p6123	K1	GTGCCACACCGACGTTGC	268 bp	(Picardeau *et al.* 1997a)
		K2	GGTAGTGGGCTCGGATATGGA		

5.2.4.3 Detection (third level)

This section is concerned with the methods that are used to analyse strain variation. These methods are an important component of the techniques used to investigate the epidemiology of mycobacterial diseases. A significant research effort has been put into this area as people try to address the key question: "is this strain the pathogenic strain?" Traditional techniques such as biotyping, serotyping and phagetyping have now been replaced by DNA methods. The aim of these methods is to try and find regions of DNA difference between strains. For mycobacteria the following approaches have been used successfully.

5.2.4.3.1. IS restriction fragment length polymorphism

As discussed earlier, IS are short stretches of DNA that have the ability to copy themselves in a random or semi-random fashion. This means that they are often present in multiple copies in a bacterial chromosome and the pattern of IS distribution can vary significantly from one strain to the next (see Chapter 4). By using restriction enzymes to specifically cut the bacterial chromosome into fragments, size-separating those fragments by gel electrophoresis, and then probing the fragments with a labelled copy of the IS, it is possible to obtain an IS banding pattern or fingerprint for that strain. This technique is used for several environmental mycobacteria. Normally, MAC isolates can be readily discriminated by RFLP

analysis using IS*1245* and IS*1311* (Guerrero *et al.* 1995), and standardized protocols have been developed (van Soolingen 1998). *M. xenopi* isolates have been typed using IS*1395*-RFLP but this species displays limited polymorphism (Picardeau *et al.* 1996). Significant polymorphism was discovered among *M. gordonae* strains typed using IS*1511*/IS*1512*-RFLP (Picardeau *et al.* 1997).

5.2.4.3.2 Pulsed-field gel electrophoresis

PFGE is a technique that permits very large DNA fragments to be separated and visualized. Bacterial DNA specially prepared in agarose plugs to prevent shearing is digested with rare-cutting restriction enzymes. This results in a pool of 10-20 DNA fragments that are then separated using a special electrophoresis apparatus that size-separates DNA in an alternating electrical field. It is widely used in bacterial molecular epidemiology and especially in mycobacteriology. It does not require any prior knowledge of a particular strain, such as the presence of repetitive IS. It has been used to type many species of mycobacteria. The preparation of the agarose-embedded DNA can be problematic. Clumping of cells and subsequent difficulties in standardizing cell lysis efficiencies can lead to dramatic differences in DNA yield and quality between preparations. Recent advances have been made to try and tackle this problem (Hughes *et al.* 2001) by culturing the bacteria under constant, stirred agitation. A significant drawback of PFGE is that while it can reliably demonstrate strain relatedness, the converse is not true: large chromosome rearrangements can readily occur and produce very different PFGE profiles between otherwise highly related strains.

5.2.4.3.3 Inter-insertion sequence polymerase chain reaction

Some mycobacterial species, such as MAC, contain multiple copies of different IS. Different strains have varying distances between the IS copies, depending on the pattern of distribution of each IS within that strain. Inter-IS PCR uses PCR to amplify between adjacent copies of different IS. Outward facing primers are designed to each IS type and a PCR is performed on genomic DNA extracted from the isolate. The resulting amplified DNA fragments are separated and visualized by gel electrophoresis. This method is rapid and simple to perform. It is used for genotype analysis of MAC by targeting IS*1245* and IS*1311* (Picardeau & Vincent 1996) and for *M. ulcerans* by targeting IS*2404* and IS*2606* (Stinear *et al.* 2000a). It also has the advantage of not requiring a high concentration or high quality DNA. Using this technique, it has been possible to genotype strains of *M. ulcerans* directly from tissue specimens (Stinear *et al.* 2000a).

5.2.4.3.4 Random amplified polymorphic DNA (RAPD)

This technique uses short oligonucleotides of random sequence in a low stringency PCR reaction to produce a strain-specific pattern of PCR fragments after gel electrophoresis. It is a rapid test that, like PFGE, requires no prior knowledge of the strain. RAPD has been used relatively widely and has shown utility in outbreak investigations (Zhang *et al.* 2002). There are issues surrounding the reproducibility of this method but attempts have been made to try and standardize the procedure (Ramasoota *et al.* 2001).

5.2.4.3.5 Multi-locus sequence typing

All the techniques discussed thus far in this section have one major limitation in common. They all rely on producing DNA bands on electrophoretic gels and then comparing the bands obtained with one strain with those obtained from another. Strain relatedness is inferred by noting the number of shared bands; the more shared bands, the more similar the strains and vice-versa. Electrophoresis is a difficult method to standardize as it is influenced by many parameters, such as sample quality, agarose quality, agarose concentration, buffer composition, running times, apparatus, voltage, etc. Even within the same laboratory it is difficult to compare the banding patterns obtained from one experiment with those obtained from the next. Genotyping methods are now moving away from these approaches and new techniques are being developed that offer high levels of discrimination, reproducibility and portability between laboratories.

MLST is a recently developed technique, widely used now for bacterial molecular epidemiological and population genetics studies (Clarke 2002). The technique is analogous to Multi-Locus Enzyme Electrophoresis except that the nucleotide sequences for the genes of housekeeping enzymes are determined rather than looking for differences in the electrophoretic mobility of the enzymes themselves. This technique identifies unique combinations of alleles. A strain displaying a unique allele combination is assigned a sequence type (analogous to a genotype).

The method is quite straightforward. As described for 16SrRNA and *hsp65* sequencing, DNA is extracted from a strain and then PCR is used to amplify specific gene sequences of approximately 500 bp. It is usual to select seven or more distinct loci. The more loci that are analysed, the greater the level of discrimination. The products are then subjected to nucleotide sequencing and then sequence comparisons are made using combinations of alignment and phylogenetic software. MLST has many advantages over other typing methods; in particular it provides unambiguous data that is readily standardized, easily transferred and compared between laboratories. MLST has not yet been widely used for the analysis of mycobacteria but one recent study, examining the relationship between *M. ulcerans* and *M. marinum*, demonstrated the power of this approach. MLST analysis at 7 loci

of 20 strains of *M. ulcerans* and 22 strains of *M. marinum* provided a means to discriminate not only between the species but also within the species (Stinear *et al.* 2000b). Future research must be directed toward developing a pan-mycobacterial MLST scheme. Such a scheme would be the basis for examining the fundamental relationship between all members of the genus.

5.3 KEY RESEARCH ISSUES

The mass of information and information potential presented by the availability of whole genome sequences for the mycobacteria means that we will soon have access to very intimate details regarding the biology of the genus. Mycobacterial diagnosticians must be prepared to use this data to develop specific tests and testing strategies for specific mycobacteria. Information gleaned from genome analyses about the metabolic pathways of a particular species could be used to design better, more selective media or isolation conditions; for example, culturing under anaerobic conditions on a defined substrate medium. New technology such as high-throughput DNA sequencing will continue to promote rapid changes in laboratory method manuals. But traditional microbiology still has its place and must not be overlooked by the glamour of molecular methods. Research focused on environmental modelling of mycobacterial habitats has been shown to be a potent tool to understand the ecology of mycobacterial pathogens. The continuation of these types of studies, supported by the best available diagnostic technology and epidemiology will be the key to understanding and controlling the spread of PEM.

6

The *Mycobacterium avium* subspecies *paratuberculosis* problem and its relation to the causation of Crohn disease

J. Hermon-Taylor and F.A.K. El-Zaatari

6.1 *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

MAP is a member of the *M. avium* complex. A genotypic definition of MAP and its distinction from other MAC is given in Chapter 4. MAP is, however, also defined phenotypically by its specific ability to cause chronic inflammation of the intestine in many species. MAP is a small mycobacterium of about 0.5 μm by 1-2 μm and is an obligate intracellular pathogen. Bovine strains of MAP, which can usually be isolated in laboratory culture, grow much more

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slowly than other MAC. They may take an initial 16 weeks to produce visible colonies on primary cultures but can take much longer. MAP also requires exogenous mycobactin an iron-transport protein for *in vitro* growth. On solid media such as Middlebrook 7H11, MAP colonies appear rough and translucent; on Herrold's media containing egg yolk they are smooth and opaque (Fig 6.1A). As the cultures become older and the medium dries the colonies take on a crumbly appearance. Culture conditions have a substantial effect on MAP phenotype and resistance (Sung & Collins 2003). In liquid media MAP grows in characteristic tight clumps (Fig 6.1B). In laboratory culture most of the microbial cells stain red by the ZN reagent (Fig 6.1C). However, this classical mycobacterial image is not the only form these pathogens can adopt. MAP is phenotypically versatile and can switch to a tough ZN-negative form in which it is invisible by ordinary light microscopy in infected tissues. Furthermore, as with some other mycobacteria, MAP can shut down into latency in which state it differs both functionally and in its physical properties from activated MAP cells, especially in its resistance to lysis and the subtleties of its interaction with the immune system. MAP is historically difficult to isolate, and strains from sheep or humans may require months or years of incubation before their gradual emergence becomes visible. Many strains of MAP cannot be grown at all. Conventional laboratory culture is not therefore, a consistently reliable method for detecting or assessing the viability of these difficult pathogens.

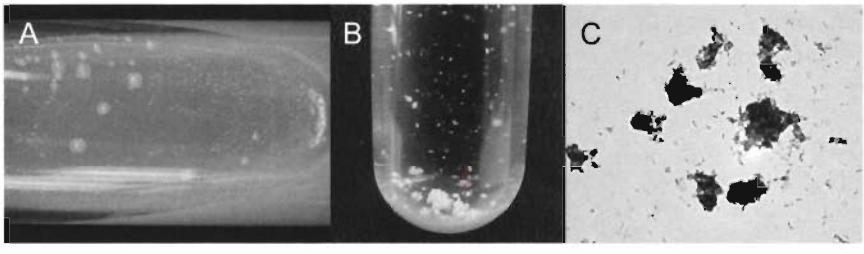

Fig. 6.1 A: Smooth colonies of a bovine strain of MAP after 10 weeks of culture on a Herrold's media containing egg yolk slope in a sealed tube. B: Bovine MAP after 10 weeks of culture in MGIT liquid medium (Becton Dickinson) showing characteristic clumping. C: Microscopic appearance of a bovine MAP strain from liquid medium showing the red acid alcohol-fast ZN staining typical of mycobacteria in bacillary form. (See also colour plate section between pages 82 and 83).

6.2 MAP INFECTION AND JOHNE DISEASE IN DOMESTIC LIVESTOCK

MAP was first identified in Germany in 1895 at the Veterinary Pathology Institute in Dresden by Professor Johne and Dr Frothingham (Johne & Frothingham 1895).

The organism was causing chronic inflammation of the intestine in a cow. The condition became known as Johne Disease. Detailed descriptions of the clinicopathological features of JD and of MAP infection in animals are presented in several reviews (Doyle 1956; Buergelt *et al.* 1978; Riemann & Abbas 1983; Chiodini *et al.* 1984a; Cocito *et al.* 1994; Clarke 1997; Beth Harris & Barletta 2001; Manning & Collins 2001).

Clinical JD in dairy cattle usually presents with loss of condition, a reduction in milk yield, weight loss and diarrhoea. Diarrhoea is not, however, a constant feature particularly in small ruminants such as sheep and goats. There is no treatment and the disease is invariably fatal. JD is not just a disease of ruminants. Many species including monogastrics such as dogs, pigs, horses, chickens and primates are affected. MAP shows a distinct tissue tropism and causes chronic inflammation of the intestine even if administered subcutaneously or intravenously. The regions of the gastro-intestinal tract usually affected are the terminal ileum and colon with segmental lesions as well as rectal involvement. The gut wall is thickened, the mucosa is swollen with occasional ulcers and the regional mesenteric lymph nodes are enlarged. Microscopically, MAP disease in animals shows a broad range of histopathological characteristics in the gut wall, from pluribacillary disease with abundant ZN-positive acid-fast bacilli in intestinal macrophages, to an extreme paucimicrobial form with no visible acid-fast organisms and florid chronic granulomatous inflammation (Fig 6.2). The pluribacillary to paucimicrobial range of MAP disease in animals thus closely resembles the range in the appearances of leprosy in humans: the lepromatous to tuberculoid forms. The pluribacillary picture is the common form seen in naturally as well as in experimentally infected animals.

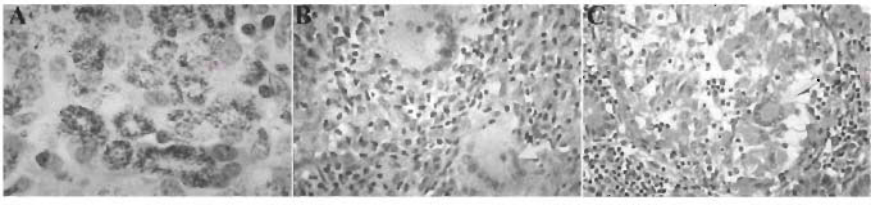

Fig. 6.2 A: Microscopic appearance of the gut wall in pluribacillary JD showing macrophages containing abundant ZN-positive MAP organisms in their classical mycobacterial phenotype. B: The contrasted appearance of the gut wall in paucimicrobial JD showing no ZN-staining MAP and florid granulomatous inflammatory disease with prominent giant cells (arrow). C: The chronic granulomatous inflammation of the gut wall of CD in humans showing giant cell (arrow). (See also colour plate section between pages 82 and 83).

Animals that are naturally or experimentally infected with MAP develop an enteric neuritis with inflammatory cells surrounding autonomic nerve fibres in the gut wall (Gwordz *et al.* 2001). The paucimicrobial form of JD closely resembles CD in humans. MAP in JD is a systemic infection; the organisms traffic widely in macrophages and parasitize the reproductive organs of both males and females. Granulomatous lesions are seen microscopically in the spleen and liver as well as widely in lymphoid tissue.

In a herd with one or two clinically diseased animals, up to 50% of the other apparently healthy animals will be subclinically infected. Infection is transmitted from cow to calf in colostrum and in milk, and from animal to animal in crowded, contaminated farm environments. MAP can persist in the intestinal tract of subclinically infected animals for years without causing clinical disease. The emergence of clinical JD can be triggered by physical or psychological stress such as calving or overcrowding. Animals are most susceptible when infected at an early age, but there is a long lead-time of months or years before clinical disease, if it is going to develop, eventually emerges. There are marked genetic influences in the susceptibility of animals to MAP infection and JD (Koets *et al.* 2000). Guinea pigs and rats are particularly resistant whereas young deer and goats are highly susceptible. Within bovines, Channel Island cattle, Limousin and specialist breeds such as Welsh Blacks are particularly susceptible.

The principal diagnostic tests for MAP infection in animals are individual or pooled faecal culture, ELISA and IFNγ release from activated white cells in response to MAP antigens. PCR diagnostics have also been introduced. Faecal culture remains the gold standard and its performance has been improved by the commercial availability of BACTEC and MGIT media (Becton Dickinson) and the application of IS*900* PCR to the culture (Eamens *et al.* 2000; Kalis *et al.* 2000; Whittington *et al.* 2000). To date, commercially available ELISA kits lack the sensitivity and specificity to diagnose subclinical MAP infection at an early stage. The performance of these ELISA tests may improve through the progressive introduction of MAP antigens of greater specificity. ELISA is, however, a cheap and convenient screening test. Their practical usefulness can be enhanced mathematically and by the derivation of likelihood ratios (Beyerbach *et al.* 2001; Collins 2002). IFNγ tests of cell-mediated immunity to MAP antigens detect sub clinical MAP infection at an earlier stage than ELISAs.

The use in recent years of all these diagnostic procedures has revealed the widespread nature of MAP infection in domestic livestock throughout Western Europe and North America. Results from Austria, Denmark, Belgium, the Netherlands and the United Kingdom have ranged from individual animal infection rates of 1.9-9% and herd prevalences (1 to 2 test-positive animals per herd) in the range 0.8-86%. The highest herd prevalences have been reported in the Netherlands and Denmark (Cetinkaya *et al.* 1996; Gasteiner *et al.* 1999, 2000;

Boelaert *et al.* 2000; Jakobsen *et al.* 2000; Muskens *et al.* 2000; Nielsen *et al.* 2000). Results from dairy cattle in the USA and Canada have been similar showing individual animal infection rates in the range 1.8-7.29% and herd prevalences in the range 16.7-54% (McNab *et al.* 1991; Collins *et al.* 1994; Wells *et al.* 1996; Johnson-Ifearulundu & Kaneene 1999; Van Leeuwen *et al.* 2001). Infection rates reported in beef herds have been much lower (Dargatz *et al.* 2001; Waldner *et al.* 2002).

6.3 DIFFERENT STRAINS OF MAP

With the opportunity to amplify in domestic livestock exposed to increasingly intensive farming practises over the course of more than a century, MAP has almost certainly done what other pathogens have done and has undergone an adaptive radiation (Colwell 1996). More than 30 different MAP strains have been identified using methods such as restriction endonuclease analysis, IS*900* RFLP, and PFGE (Collins *et al.* 1990; Whipple *et al.* 1990; Bauerfeind *et al.* 1996; Feizabadi *et al.* 1997; Pavlik *et al.* 1999; Cousins *et al.* 2000; Stevenson *et al.* 2002). Typing of over a thousand MAP isolates obtained from all over the world has demonstrated differences between ovine strains (S-type or type I) and bovine strains (C-type or type II), suggesting an adaptation to their respective preferred hosts. Although phenotypic and genotypic differences are found between ovine strains and between bovine strains, they nonetheless share substantial intra-species commonality. The major differences are inter-species. Studies in Iceland and the Netherlands have shown that sheep strains of MAP can infect cattle, and cattle strains of MAP can give rise to long-standing subclinical infection in sheep grazing the same pastures (Fridriksdottir *et al.* 2000; Muskens *et al.* 2001). Bovine strains, however, have a much broader host range. IS*900* RFLP typing of MAP isolates from humans with CD has so far demonstrated that they are all based on the cattle C-type, type II background (François *et al.* 1997; Pavlik *et al.* 1999; Whittington *et al.* 2000a).

A unique 12 bp tandem repeat sequence present in sheep strains and absent from bovine strains enables these strains to be distinguished by a single specific PCR (Collins *et al.* 2002). The use of representational difference analysis has further identified an 11 bp fragment present in sheep strains which was absent from bovine strains tested (Dohmann *et al.* 2003). Differences in MAP strains from cattle and sheep have been demonstrated between Argentina and Europe (Moreira *et al.* 1999), and between Australia and Iceland (Whittington *et al.* 2001b). Typing of IS*1311* polymorphisms from MAP isolates obtained from nine bison in Montana, USA showed consistent variation at base position 223 compared with 13 C-type isolates from cattle and goats in the United States. The finding that bison strains of MAP (designated B-strain) differed from ambient

cattle strains suggested that the epidemiology of paratuberculosis in bison in Montana may be distinct from that found in farmed livestock in other regions (Whittington *et al.* 2001a). Taken together, the findings are consistent with predictable geographical differences in MAP isolates between continents and different regions. Diversification of MAP strains is a continuing dynamic process and human MAP strains with type-specific features can be expected.

RFLP and PFGE are methods which limit the typing of MAP to those strains which can be cultured. Given the very slow growth, and in some cases unculturable nature of these organisms, PCR-based typing procedures for these difficult pathogens are highly desirable. The methods developed so far, and discussed in Chapter 5, include random amplified polymorphic DNA patterns (Scheibl & Gerlach 1997; Pillai *et al.* 2001) and a multiplex PCR typing procedure which utilizes a common IS*900* primer together with a locus-specific primer (Bull *et al.* 2000). PCR typing of *M. tuberculosis* based upon mycobacterial interspersed repetitive units (Supply *et al* 2000) has been adapted for MAP (Bull *et al.* 2003a). PCR typing based upon six mycobacterial interspersed repetitive units loci, distinguishes MAP from other MAC. This maybe useful in demonstrating that a liquid culture of MAP isolated from a sample does not also contain other MAC organisms.

6.4 MAP IN WILDLIFE AND IN THE ENVIRONMENT

Clinically and subclinically infected farm animals, particularly those with the common pluribacillary form of disease shown in Figure 6.2 (A), can shed huge numbers of MAP onto pastures. MAP infection and JD are endemic in Western Europe and North America. Taking north-east Scotland as an example, studies beginning in 1994 demonstrated MAP infection in 8-53% of wild rabbits culled from farms reporting clinical JD in cattle and sheep. MAP infection was also found in a smaller proportion of rabbits obtained from farms without clinical JD (Greig *et al.* 1999). Typing of the isolates demonstrated that they were all of the bovine type. MAP-infected wild rabbits shed the pathogen in their faecal pellets which are consumed by grazing cattle. Experimental paratuberculosis has been demonstrated in calves following infection with a rabbit MAP isolate (Beard *et al.* 2001a; Daniels *et al.* 2001). Thus a cycle of infection is established comprising MAP amplified in domestic animals, environmental contamination, infection of rabbits and re-infection of domestic livestock. In the same group of studies, MAP infection was also found in a high proportion of rabbit predators such as stoat, weasel and fox, as well as in the carrion birds crow, rook and jackdaw. Rat, wood mouse, hare and badger were found to harbour MAP (Beard *et al.* 2001). MAP infection in wildlife has been extensively reported in other regions as exemplified by MAP-infected red deer and ibex from the European

Alps, bison and elk in North America, and wild ruminants as well as insects and earthworms in the Czech Republic (Buergelt *et al.* 2000; Ferroglio *et al.* 2000; Nebbia *et al.* 2000; Pavlik *et al.* 2000; Fischer *et al* 2001, 2003). MAP in domestic animals and wildlife thus constitutes a reservoir of these pathogens capable of being disseminated over substantial distances.

The survival of *Mycobacterium bovis* in the environment is thought to be limited to hours or days. By contrast, the physically more robust MAP is known to survive for months and perhaps years since no upper limit on the environmental survival and persistence has been established. Geographical regions characterized by acid soils rich in humic and fulvic acids, boreal forests and areas with a high rainfall and water table, may favour the accumulation of MAP in the environment (Kopecky 1977; Kazda *et al.* 1990; Johnson-Ifearulundu & Kaneene 1997; Iivanainen *et al.* 1999b; Kirschner *et al.* 1999; Reviriego *et al.* 2000). Although investigations of MAP in the environment and in surface waters are currently in progress, there are to date no published studies to give us a detailed understanding of the ecology and fate of MAP in the environment and the potential cycling of these pathogens through human populations. From what is known about other MAC and organisms such as *Legionella* sp. it is highly likely that environmental MAP are taken up into protozoa (Barker & Brown 1994; Falkinham 1996; Ford 1999; Hermon-Taylor *et al.* 2000). Intracellular adaptation of MAP within protozoa in the environment and in biofilm communities may profoundly influence microbial survival, phenotype and virulence. *M. avium* grown in vacuoles in *Acanthamoeba castellanii* has been shown to develop an increased capacity to infect other amoebae, macrophages and human colonic epithelial cells, as well as an enhanced virulence in a beige mouse model of infection (Cirillo *et al.* 1997). *M. avium* can survive for long periods within encysted forms of *Acanthamoeba polyphaga* (Steinert *et al.* 1998). The environmental exposure of MAP and its cycling through unicellular organisms, as well as through animal and human populations, has the potential to have a profound effect on the evolution of these organisms and the development of strains with enhanced pathogenicity. Much research remains to be done in this area.

6.5 TRANSMISSION OF MAP FROM ANIMALS TO HUMANS

It is unlikely that crowded human populations sharing the same geographical regions as their widely MAP-infected domestic animals would be excluded from any exposure to these robust and versatile pathogens.

6.5.1 In food

It has long been known, and has more recently been confirmed, that infected animals secrete MAP in their milk (Doyle 1954; Taylor *et al* 1981; Sweeney *et al.* 1992; Streeter *et al.* 1995). Faecal contamination in the milking parlour is another source of MAP in milk. MAP is more thermotolerant than *M. bovis*. Studies carried out in several laboratories have sought to determine whether exposure to 72 °C for 15 seconds, conditions commonly used in commercial pasteurization, would ensure the destruction of all viable MAP (Chiodini & Hermon-Taylor 1993; Grant *et al.* 1996, 1999, 2002a; Meylan *et al.* 1996; Stabel *et al.* 1997; Keswani & Frank 1998; Sung & Collins 1998; Pearce *et al* 2001; Gao *et al.* 2002). Despite some potential limitations in these studies, the substantial balance of experimental evidence strongly predicted that pasteurization at 72°C for 15 or 25 seconds, while reducing the number of viable organisms, would not ensure the destruction of all MAP.

Field studies in the United Kingdom using IS*900* PCR to screen retail pasteurised cows' milk for MAP, while unable to distinguish between live and dead organisms, indicated a high risk of the transmission of MAP to humans by this route (Millar *et al.* 1996). Further work from the Department of Food Science, Queen's University Belfast, using optimized decontamination protocols and immunomagnetic capture, found that 11.8% of 567 samples of retail pasteurized cows' milk in the UK tested MAP-positive by PCR, and that 1.8% of samples were MAP-positive by culture (Dundee *et al.* 2001; Grant *et al.* 2002). For Britain alone therefore, it is known that people are from time to time drinking live MAP in the milk supply. The finding in Switzerland that 19.7% of 1384 samples of bulk-tank milk tested IS*900* PCR positive emphasizes the risk that this may be happening elsewhere (Corti & Stephan 2002). More data are required from other countries where MAP infection in dairy herds is endemic. Exploitation of specific peptide-mediated capture of MAP from milk will advance the sensitivity of detection (Stratmann *et al.* 2002). Quantitative RT-PCR (reverse transcription PCR) and sensitive methods including culture of MAP within cell lines, and the use of susceptible C57/BL6 or immune deficient mice, may improve on conventional culture in their ability to reveal residual viable MAP and assist in the selection of new industrially applicable processes to eliminate these pathogens from the food chain. Procedures already tested on milk include filtration, cold shock, hydrostatic pressure and pulsed electric fields (Miller *et al.* 2000; O'Reilly *et al.* 2000; Rowan *et al.* 2001). Specialist cheeses derived from raw milk need to come from certified MAP-free animals.

In many countries existing legislation still permits clinically diseased JD cattle or sheep, in which MAP are widely present in liver, lymph nodes and other tissue, to be sent for slaughter and the meat and offal passed for human

consumption. These animals contain huge numbers of MAP with a high risk of dissemination in the abattoir environment, and surface contamination of other meat being processed. Vegetables are at risk where MAP-infected slurry is applied to market gardens or agricultural land as a fertilizer.

6.5.2 In water supplies and aerosols

Although work is currently in progress there are, at the end of 2003, no detailed published studies using molecular and other methods of established validity which reliably inform us about MAP contamination of waters close to population centres, or of those sourced for domestic supply. However, the information available for other robust zoonotic pathogens that can survive in the environment (Szewzyk *et al.* 2000; Le Dantec *et al.* 2002a) would suggest that there is a high risk that MAP may from time to time be transmitted to people in drinking-water or by aerosols (Hermon-Taylor *et al.* 2000). MAP in lakes and rivers contaminated by run-off from heavily grazed pastures will be present in planktonic form, within protozoa or, more likely, both. If these adhere to particles of suspended solid, then the MAP content of native water abstracted for domestic supply will be depleted by subsequent treatments, such as counter-current dissolved air floatation filtration. CT values for the effect of chlorine on MAP have been estimated to be up to 580 to 2300 times greater than those for *E. coli* (Taylor *et al.* 2000; Whan *et al.* 2001). MAP getting through the stage in water treatment plants of removal of suspended solids is therefore unlikely to be destroyed by subsequent chlorination. These pathogens arriving at domestic outlets in high dilution may accumulate in biofilms present in household cold and hot water storage and delivery systems. If research tells us that this is indeed happening, we may need to consider exploiting the susceptibility of MAP to UV irradiation (Miyamoto *et al.* 2000) using additional industrially applicable treatments in flow-through units.

While we wait for reliable scientific data, it is worth revisiting two published studies where exposure to waters whose catchments included heavily grazed pastures was associated with conspicuous clusters of CD. The first of these involved the village of Blockley, a rural community of about 2000 people in Gloucestershire, England in which 12 people developed CD between 1960 and 1983, an increase of observed over expected (for that time) of 6.7-fold and equating with a CD incidence of $28/10^5$ per year (Allan *et al.* 1986). The village, which had its own water supply from local springs, lay in a hollow surrounded by upland pastures grazed by cattle in which clinical JD was evident (R.N. Allan, personal communication, 1992). The second CD cluster occurred in the town of Mankato, Minnesota, USA and involved the occurrence of 7 cases of CD amongst 285 graduates of the Mankato West High School class of 1980.

All seven had been swimming in local ponds and lakes. The school also lay close to the Minnesota River, just downstream from the entry of the Blue Earth River whose catchment included rich agricultural grazing land. High faecal coliform counts in the Blue Earth River, monitored over the period, indicated extensive contamination with faecal run-off. Seventy-five percent of the water supply to Mankato was reported to be drawn from beneath the Blue Earth River (Van Kruiningen & Freda 2001). Although not discussed by these authors, it is highly likely that these waters were from time to time heavily contaminated with MAP. There are also data which implicate domestic hot water systems. Two case control epidemiological studies carried out independently in the United Kingdom, each unexpectedly identified the availability of fixed hot water supplies in the early childhood home as a significant risk factor for the subsequent development of CD, but not for ulcerative colitis (Gent *et al.* 1994; Duggan *et al.* 1998).

Mycobacteria are known to occur in aerosols, and to concentrate to high levels in the water droplets (Blanchard & Syzdek 1972; Wendt *et al.* 1980; refer also to Chapter 3). Cardiff is a city on the coastal plain of South Wales in the United Kingdom, beside the sea. North of the city lie the Brecon Hills, steep upland pastures that are grazed by sheep and cattle in whom MAP infection is endemic. Heavy rains from the Atlantic wash off these pastures into spate rivers. One of these rivers, the Taff, runs through the middle of Cardiff. Research carried out in Cardiff during the 1970s demonstrated a highly significant increased incidence of CD ($p < 0.001$), but not of ulcerative colitis, in 11 of the local electoral city wards (Mayberry & Hitchens 1978). Of these high incidence wards, eight directly bordered the river Taff and the three that did not were immediately adjacent to the north and east. This is the direction in which aerosols would be carried by the prevailing south-westerly winds (Hermon-Taylor 1993). Inflammatory involvement of the trachea and bronchi with abnormal lung function tests are demonstrable in a significant proportion of people with CD, and CD in children can present with chronic granulomatous tracheo-bronchitis (Heatley *et al.* 1982; Bonniere *et al.* 1986; Calder *et al.* 1993; Dierkes-Globisch & Mohr 2002; Herrlinger *et al.* 2002). Much research is needed on MAP in the environment, in surface and groundwaters and in aerosols.

6.6 CROHN DISEASE

6.6.1 Definition

CD is a systemic disorder whose principal clinicopathological manifestation is chronic inflammation of the intestine. Any part of the gastrointestinal tract from

mouth to anus may be involved in the chronic granulomatous process, but the terminal ileum and colon are the regions most frequently affected (Fig 6.3).

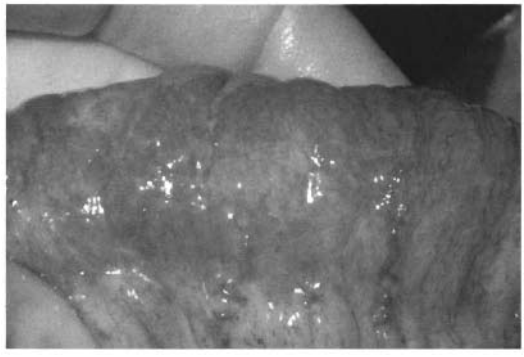

Fig. 6.3 Typical appearance of an inflamed terminal ileum in a person with active CD. (See also colour plate section between pages 82 and 83).

CD usually presents with abdominal pain, feeling unwell, loss of energy and weight, night sweats, mouth ulcers and joint pains. It sometimes presents as an abdominal emergency with peritonitis, perforation of the terminal ileum, or mimicking acute appendicitis. As in animals, onset of clinical disease may be triggered by physical and psychological stress. About 60% of patients have diarrhoea which may contain pus and blood. The tissues around the anus and perineum may become ulcerated or chronically inflamed with sinuses discharging pus and faecal material. In children, growth and sexual maturation is retarded or arrested. The mucosa lining the gut first becomes leaky, then ulcerated with long serpiginous fissures. Mucosa surviving between the ulcers is swollen, inflamed and oedematous and frequently goes on to form inflammatory pseudopolyps. The chronic inflammatory process and inflammatory cell infiltrate extends deep into and often right across the gut wall. Granulomata, consisting principally of clusters of activated macrophages with conspicuous multinucleate giant cells, are seen microscopically in only about half of CD cases. As in naturally and experimentally MAP-infected animals (Gwordz *et al.* 2001), humans with CD demonstrate abnormalities of the enteric nervous system, with neuronal and axonal hyperplasia, axonal damage and periaxonal inflammatory cell cuffing, associated with MHC class II expression on enteric glial cells (Geboes *et al* 1992; Geboes & Collins 1998).

Treatment of CD has been limited to the suppression or modulation of the inflammatory process. This can sometimes achieve and maintain remission over prolonged periods. Relapse frequently occurs and is often triggered by physical and psychological stress. Surgery is required if the disease gets out of control or if

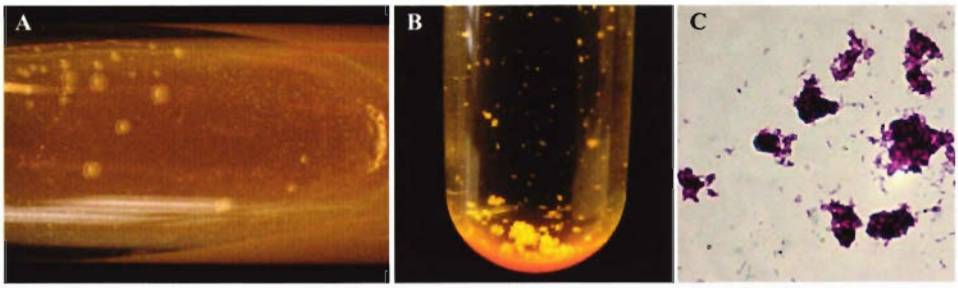

Fig. 6.1 **A**: Smooth colonies of a bovine strain of MAP after 10 weeks of culture on a Herrold's media containing egg yolk slope in a sealed tube. **B**: Bovine MAP after 10 weeks of culture in MGIT liquid medium (Becton Dickinson) showing characteristic clumping. **C**: Microscopic appearance of a bovine MAP strain from liquid medium showing the red acid alcohol-fast ZN staining typical of mycobacteria in bacillary form.

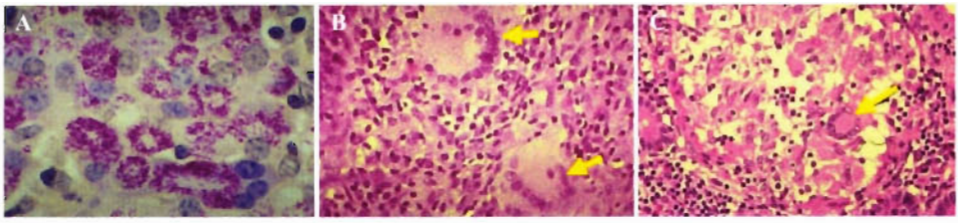

Fig. 6.2 **A**: Microscopic appearance of the gut wall in pluribacillary JD showing macrophages containing abundant ZN-positive MAP organisms in their classical mycobacterial phenotype. **B**: The contrasted appearance of the gut wall in paucimicrobial JD showing no ZN-staining MAP and florid granulomatous inflammatory disease with prominent giant cells (arrow). **C**: The chronic granulomatous inflammation of the gut wall of CD in humans showing giant cell (arrow).

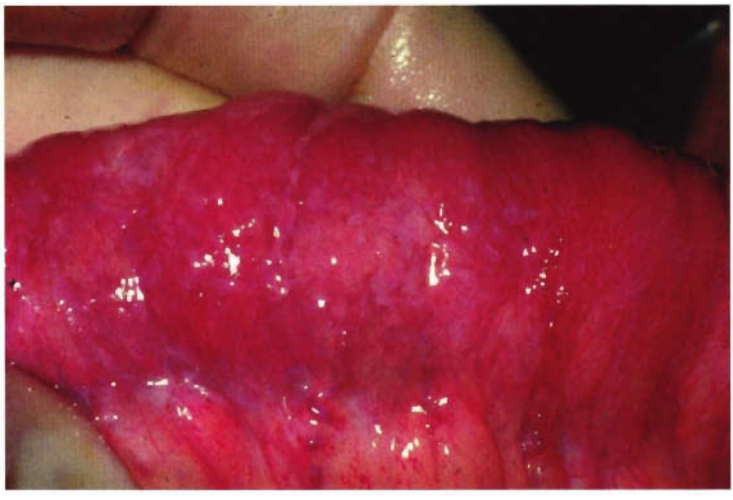

Fig. 6.3 Typical appearance of an inflamed terminal ileum in a person with active CD

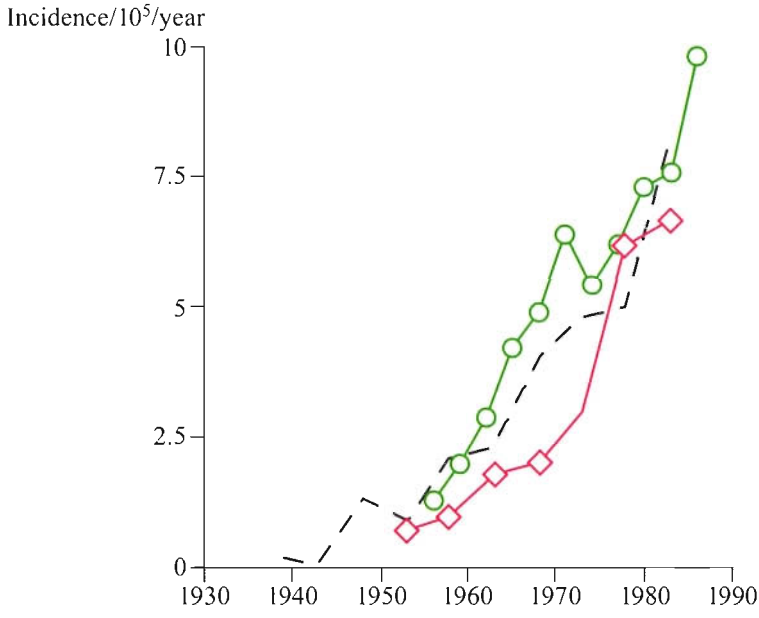

Fig. 6.4 The incidence of CD in three regions of the United Kingdom: South Wales — (Rose *et al.* 1988); the Midlands ◇ (Fellows *et al.* 1990); and north-eastern Scotland ○ (Kyle 1992) over the 50 year period 1940 to 1990

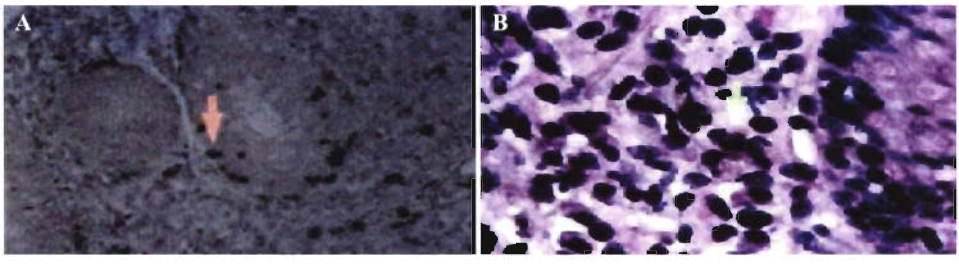

Fig. 6.5 Demonstration of MAP in CD tissues by *in situ* hybridization on paraffin-embedded tissue sections using an IS*900* probe. **A**: *In situ* hybridization with no counter stain showing MAP DNA in the lamina propria and occasionally infiltrating a gland (x40). **B**: Same as in **A**: with H & E as a counter stain showing MAP as brown positive spots within macrophages in the lamina propria (x100).

specific complications develop. These take the form of obstruction of the gut due to stricturing, abdominal abscesses, perforation of the gut or fistulous connections leading to discharge of intestinal content from other organs such the bladder or vagina. About 40% of people with colonic CD will end up having to have their whole colon removed, and an abdominal bag collecting intestinal effluent from an ileostomy. CD characterized by cycles of disease remission followed by activity, with its physical, emotional, sexual, social and family morbidities, involves a lifetime of medical care and huge economic cost (Sandler *et al.* 2002).

6.6.2 Epidemiology, environmental factors, and inherited susceptibility to CD

CD is a 'new' disease first appearing in developed societies in temperate regions with intensive farming. From a low background level of sporadic cases recorded over many years (Combe 1813; Moschcowitz & Wilensky 1923), chronic inflammation of the intestine of the CD type began to emerge perceptibly about a third of the way into the 20th century (Crohn *et al.* 1932). Thereafter, with plateaus at times in some regions, the incidence and prevalence of CD have continued to climb (Fig 6.4).

Comparable increases in the incidence of CD were recorded in North America and continental Europe (Loftus *et al.* 1998; Munkholm *et al.* 1992). In the United Kingdom in recent years, increases in CD have particularly affected children (Cosgrove *et al.* 1996; Armitage *et al.* 2001; Sawczenko *et al.* 2001). Continents in the northern hemisphere demonstrate a north-south gradient in the incidence of CD (Sonnenberg *et al.* 1991; Shivananda *et al.* 1996). In Europe, while CD remains uncommon in Greece (Tsianos *et al.* 1994), there is evidence that a higher incidence is spreading south to the Iberian peninsular (Ruiz 1989; Veloso *et al.* 1989; Cebolla *et al.* 1991; Lopez Miguel *et al.* 1999) and east to European countries such as Hungary and Croatia (Lakatos *et al.* 2002; Mijandrusic Sincic *et al.* 2002). CD also appears to be rising in countries formerly presumed to have a low incidence such as Iran (Merat *et al.* 2002), India (Pai & Khandige 2000) and Brazil (Gaburri *et al.* 1998), as well as in China and Japan which have substantially increased production and consumption of dairy products (Yao *et al.* 2000).

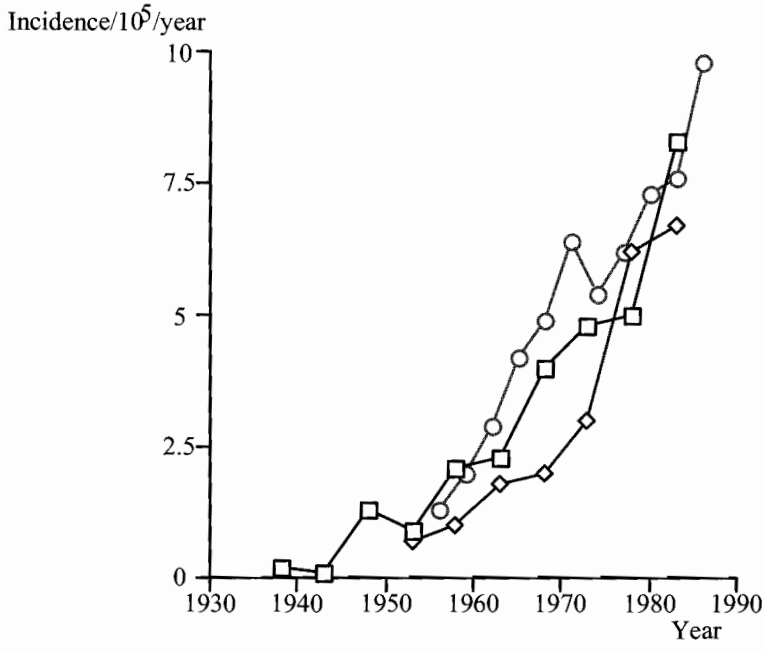

Fig. 6.4 The incidence of CD in three regions of the United Kingdom: South Wales □---□ (Rose *et al.* 1988); the Midlands ◊---◊ (Fellows *et al.* 1990); and north-eastern Scotland ○---○ (Kyle 1992) over the 50 year period 1940 to 1990. (See also colour plate section between pages 82 and 83).

The highest overall incidence (15.6/100 000 per year) and prevalence (198.5/100 000 population) of CD so far reported in the world is in Manitoba, Canada (Bernstein *et al.* 1999; Blanchard *et al.* 2001). Individual incidence rates for CD across the 52 postal regions in these Manitoba studies ranged from 1 to 26/100 000 per year, making the province a fruitful region for further environmental research into CD causation. In the absence for the most part of national population-based data, the overall scale of the CD problem in human populations at the present time can only be estimated. Loftus *et al.* (2002) estimated the number of CD sufferers in the United States to be 600 000 although it could be as high as one million; for western Europe 300 000-500 000; and for Britain about 100 000 (Rubin *et al.* 2000). In Northern Stockholm County, Sweden the incidence of CD in children under 16 increased from 1.7/100 000 year in 1990-92 to 8.4/100 000 per year in 1999-2001 (Hildebrand *et al.* 2003). In Victoria, Australia the CD incidence in children rose from 0.128 to 2.0/100 000 per year over the period 1971-2001 (Phavichitr *et al.* 2003). In each case, this represents an average five-fold increase in CD in children per decade. There is a lack of recent data for the incidence and prevalence of CD in

adults in Australia, New Zealand and South Africa, and a need for the relevant epidemiological research to be carried out.

CD occurring in spouses and their children sharing common environments, and CD increasing to that of the host population in migrants moving from low to high incidence areas, clearly indicate the involvement of one or more environmental factors in CD causation (Montgomery *et al.* 1999; Laharie *et al.* 2001). Exposure to MAP with its ability to cause chronic inflammation of the intestine in so many species including primates is a strong candidate environmental factor. The familial occurrence of CD (Orholm *et al.* 1991; Peeters *et al.* 1996), the higher incidence in some races such as Jewish people (Yang *et al.* 1993a), and the concordance rate of CD of 58% in monozygotic twins and of 0% in dizygotic twins (Orholm *et al.* 2000) also show that a susceptibility to CD can be inherited. Recognition of one molecular basis for this came with the discovery of missence variants and frameshift mutations affecting the *CARD15(NOD2)* gene on human chromosome 16 (Hampe *et al.* 2001; Hugot *et al.* 2001; Ogura *et al.* 2001). This gene encodes a transmembrane receptor for bacterial products like lipopolysaccharide expressed on monocytes, and related to the Apaf-1 family of apoptosis regulators. Several polymorphisms are associated with CD susceptibility (Cuthbert *et al.* 2002; Lesage *et al.* 2002) the strongest being an insertional mutation in exon 10 resulting in truncation of the leucine-rich carboxyterminus of the protein, and a reduction in cellular response to lipopolysaccharide activation. However, while the linkage between *CARD15(NOD2)* mutations and CD has been confirmed for Europeans, North Americans, Australians and Jewish people (Brant *et al.* 1998; Cavanaugh *et al.* 1998; Cavanaugh 2001; Vermeire *et al.* 2002; Zhou *et al.* 2002), it does not occur in Japanese, Korean or Chinese patients with CD (Inoue *et al.* 2002; Yamazaki *et al.* 2002; Croucher *et al.* 2003). An Ala893/Thr polymorphism in the multidrug resistance gene on chromosome 7q is also associated with an increased risk of inflammatory bowel disease (Brant *et al.* 2003), and a number of other loci have been implicated on chromosomes 1, 3, 4, 5, 6, 7, 12 and 14 (Duerr 2002; Watts & Satsangi 2002; Bonen & Cho 2003). These genetic loci may influence susceptibility to CD; they do not cause it.

6.6.3 The isolated case of Iceland

Does the distribution of MAP infection in animals match the distribution of CD in humans? The answer to this on a continental basis is yes. However, the picture is blurred by the putative dispersal of MAP in food products, water and the environment happening across regional, national and international boundaries, as well as by the potential exposure to MAP during international travel. Our understanding is also limited by our lack of knowledge of MAP in

the environment, in different habitats and phenotypes, and also because the necessary epidemiological research to detail the comparative incidence and prevalence of MAP infection in animals and in humans has not been carried out.

It is, however, worth taking a closer look at the isolated community of Iceland, an island of 103 000 km^2 in the north Atlantic. The population was 229 187 in 1980 rising to 266 006 in 1994, with a low migration rate and ethnically homogeneous Nordic population. About 60% of the people live in the capital Reykjavik. There are three hospitals, the main one in Reykjavik, and centralized registration of health information. Farming involves principally the 480 000 Icelandic breed of hill sheep with some dairy and beef cattle.

Prior to 1930 MAP infection and JD in Iceland were virtually unknown. Then in 1933, 20 Karakul sheep were imported from Germany and, after quarantine, were distributed to 14 farms (Fridriksdottir *et al.* 2000). Although apparently healthy, some of the Karakul sheep were subclinically infected with MAP. They transmitted MAP to the Icelandic sheep population though they never developed disease themselves. By 1938 clinical JD appeared in Icelandic sheep on five of the original farms. By about 1945, clinical JD was in the cattle on the same farms, although infection in the cattle was difficult to diagnose as the organisms would not grow in culture; a characteristic of sheep MAP strains. The organism from these cattle was later confirmed as the sheep strain of MAP by IS*1311* restriction endonuclease analysis (Whittington *et al.* 2001b). Slowly the infection spread so that by the late 1950s the disease was epidemic with about 30% of sheep farms affected and huge annual losses. The mean incidence of CD (number of cases/10^5 per year) in the human population was 0.4 from 1950-59, 0.45 from 1960-69, 0.9 from 1970-79, 3.1 from 1980-89 and 5.6 from 1990-94 inclusive, the highest annual figure over this last five-year period being 8.2 in 1992. Young people were particularly affected (Bjornsson 1989; Bjornsson *et al.* 1998; Bjornsson & Johannsson 2000).

Apart from an increase in the sale of cigarettes during World War II, no nutritional or environmental risk factors were found to explain the magnitude of this increase in CD. Although causation is not proven, with the slow growth of MAP, the need for the pathogen to adapt to each new host (Woodhouse *et al.* 2001) and the long lead time to the emergence of clinical disease (if it is going to occur) in both animals and humans, the sequential picture of JD then CD observed in Iceland over 50 years is exactly what would be expected if the major environmental factor causing CD was MAP.

6.7 MAP CAUSING CROHN DISEASE

In 1988 a previously healthy seven year-old-boy living in a village outside Cambridge, England developed NTM cervical lymphadenitis which was later

shown to be caused by MAP (Hermon-Taylor *et al.* 1998). After failing to respond to standard anti-TB treatment, the enlarged lymph glands were removed. Five years later he developed severe CD of the terminal ileum and adjacent colon. This healed completely after a year's treatment with anti-MAP drugs rifabutin and clarithromycin leaving a dense fibrous ileal scar with narrowing of the gut and impending intestinal obstruction. The scar was removed and the continuity of the gut restored. The scar tested strongly positive for MAP by IS*900* PCR. Drug treatment was continued for almost two more years during which he was disease free. About two years after stopping the drugs, the CD recurred in the ileum next to the anastamotic site, despite his having been off all British milk products. His CD again responded to rifabutin and clarithromycin.

The value of this isolated case lies in the way in which it illustrates the relationship between MAP and CD. MAP infection of the cervical lymph glands in this boy was probably acquired from British milk just as with *M. bovis* before pasteurization. The ingested MAP pathogens would also have colonized his gut at the same time, but as in animals, a lead time of several years passed before clinical disease emerged. On this occasion most of the organisms were sensitive and the disease healed on anti-MAP drug treatment, but continuing colonization of the gut with residual MAP probably in a state of latency, such as occurs with TB and *M.avium*, persisted (Bermudez *et al.* 1999; Manabe & Bishai 2000; zu Bentrup & Russell 2001). When the residual MAP reactivated, the disease responded again to the same therapy. At no time was MAP either seen microscopically or isolated in conventional culture from his diseased tissues. Recognition of the true nature of the causation of the lymphadenitis and the subsequent chronic enteric infection depended entirely on the detection of MAP using appropriate molecular methods. This isolated case also shows that MAP infections are extremely difficult to eradicate.

6.7.1 MAP in the inflamed gut of people with Crohn disease

The proposition that MAP (Johne's bacillus) could cause chronic inflammation of the intestine in humans as well as animals, was first published by the Glasgow surgeon T.K. Dalziel in 1913 (Dalziel 1913). The uncertainty, nearly 100 years later, as to whether or not this is true is almost entirely due to the difficulties of reliably detecting this robust, versatile and often unculturable pathogen. A pivotal contribution was made by Dr R. Chiodini in the United States during the mid-1980s when he and his co-workers, using optimized cultures and incubation times of months or years, isolated an unclassified *Mycobacterium* sp. from the inflamed gut of three people with CD (Chiodini *et al.* 1984a, 1986; Chiodini 1989). These isolates caused chronic inflammation of

the intestine when administered to young goats. In half the goats no ZN-positive mycobacteria could be seen microscopically in the inflamed tissues, as with CD (Van Kruningen *et al.* 1986). Other workers were able to isolate spheroplasts and acid-fast bacilli from CD, but in the absence at that time of molecular methods of sufficient specificity and sensitivity, the nature of these could not be precisely demonstrated (Markesich *et al* 1988). Similar contributions were made by other research groups (Hermon-Taylor *et al.* 2000; Chamberlain *et al.* 2001; El-Zaatari *et al.* 2001).

The availability of IS*900* (Green *et al.* 1989) and its use as a probe and a target for PCR, confirmed the CD isolates of Chiodini as MAP, and showed that a substantial proportion of long-term CD cultures contained these pathogens (McFadden *et al.* 1987a; Moss *et al.* 1992; Wall *et al.* 1993). IS*900* PCR together with DNA extraction protocols optimized using fresh surgically removed MAP-positive CD tissues, demonstrated MAP in the inflamed gut of 65% of people with CD and in 12% of uninflamed control gut samples (Sanderson *et al.* 1992). Subsequent PCR studies over the period 1994-99 were conflicting (Hermon-Taylor *et al.* 2000), though work from the University of Bari in Italy showed that people with CD may excrete MAP in their stool (Del Prete *et al.* 1998).

Recent research has established the extraordinary resistance of MAP in human and animal tissues, and in milk and other samples, to chemical as well as enzymic lysis, and the need to incorporate an optimized mechanical disruption step in sample processing to ensure reliable access to MAP DNA for PCR detection (Hermon-Taylor *et al.* 2000; Odumeru *et al.* 2001). Recent years have also brought the commercial availability of improved media for the isolation of MAP such as the MGIT system, the result of some years of developmental work in Becton Dickinson. New methods have been applied to the localization of MAP in CD tissues, such as laser capture microdissection and *in situ* hybridization. Researchers at the University of Central Florida and El Paso Texas cultured MAP in MGIT medium after about a year of incubation from the inflamed gut of six of seven (86%) people with CD (Schwartz *et al.* 2000). They also cultured MAP from the breast milk of two women with CD who had recently given birth, but not from the milk of five women who did not show the disease (Naser *et al.* 2000). Collaborative research at the Baylor College of Medicine, USA and at the University of Oulu, Finland demonstrated MAP for the first time in 6 of 15 (40%) granuloma-positive CD patients and in none of 22 patients without CD using *in situ* hybridization (Hulten *et al.* 2001).

In situ hybridization studies from the Universities of Sassari in Sardinia and Rome demonstrated MAP in 27 of 33 (82%) CD patients with no relationship to the presence of granuloma, and in none of 40 patients without CD (Sechi *et al.* 2001). Research in Ireland using laser capture microdissection and PCR

(without a mechanical disruption step) of sub-epithelial granulomas detected MAP in 6 of 15 (40%) patients with CD and in none of 12 disease controls (Ryan *et al.* 2002). IS*900* PCR (without a mechanical disruption step) was positive for MAP in 15 of 79 (19%) CD patients and 3 of 48 (6%) control patients from the United States and Denmark (Collins *et al.* 2000). Research in London, England using optimized tissue processing (with mechanical disruption) and nested IS*900* PCR, detected MAP in fresh ileocolonoscopic mucosal biopsies (Fig 6.5) in 9 of 34 (26%) of people without clinicopathological CD, and in 34 of 37 (92%) of people with CD (odds ratio 3.47; p = 0.0002) (Bull *et al.* 2003). In this study, identity with IS*900* was verified in every case by amplicon sequencing. The IS*900* multicopy element as defined by its entire DNA sequence is unique for MAP.

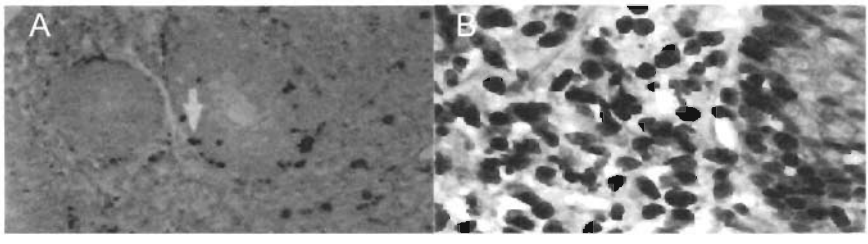

Fig. 6.5 Demonstration of MAP in CD tissues by *in situ* hybridization on paraffin-embedded tissue sections using an IS*900* probe. (A) *In situ* hybridization with no counter stain showing MAP DNA in the lamina propria and occasionally infiltrating a gland (x40). (B) Same as in (A) with H & E as a counter stain showing MAP as brown positive spots within macrophages in the lamina propria (x 100). (See also colour plate section between pages 82 and 83).

Taken together, these studies show that the detection rates for MAP in CD depend critically on the validity of the methods used. When these are optimal almost everybody with CD is found to be infected with MAP. The presence of MAP colonization of the gut in a minority proportion of people without CD is consistent with widespread environmental exposure to these pathogens, as exemplified also in the population biology of *M. tuberculosis*, *S. pneumonae, N. meningitidis,* and *H. pylori.*

6.7.2 Serological recognition of MAP proteins in Crohn disease

Sera from animal healthcare workers exposed to abundant ZN-positive bacillary-form MAP show significantly higher levels of IgG antibody binding to microtitre plates coated with the crude soluble fraction of MAP lysates, than do sera from healthy humans (Chiodini *et al.* 1996). Sera from people with CD in general show no significant difference in antibody binding to such crude MAP

extracts compared with controls (Hermon-Taylor *et al.* 2000). In this situation, such tests report an overall immune responsiveness to common MAC antigens.

The ZN-negative form of MAP in CD minimizes immune recognition. Significant differences in IgG and/or IgA antibody binding are, however, observed in ELISA tests using selected highly purified or recombinant MAP proteins and peptides. This has so far been demonstrated for a 24kDa MAP protein and an 18kDa bacterioferritin (Elsaghier *et al.* 1992), for the MAP-specific C-terminal recombinant peptide fragment of the 34kDa component of the MAP A36 complex (Vannuffel *et al.* 1994), for the recombinant p35 and p36 antigens from MAP (El-Zaatari *et al.* 1999; Naser *et al.* 1999; Naser *et al.* 2000), for the alkylhydroperoxide reductase AhpC and a 14kDa protein secreted by MAP (Olsen *et al.* 2001), and for the mycobacterial protein HupB (Cohavy *et al.* 1999). These serological data strongly support the hypothesis that CD patients are infected with MAP.

6.7.3 Response of Crohn disease to treatment with anti-MAP drugs

Clinical infections caused by MAC organisms are known to be difficult to eradicate by treatment using standard anti-TB therapy. Relapses and the development of microbial drug resistance are common. MAP is generally resistant to natural streptomyces antibiotics and MAP infections in animals have never been convincingly eradicated (Hermon-Taylor *et al.* 2000). Other anti-mycobacterial agents such as isoniazid, ethambutol and pyrazinamide act by blocking the biosynthesis of cell wall components including mycolic acids. MAP in CD is in its ZN-negative form and does not have a conventional mycobacterial cell wall. Treatment of CD with combinations of drugs such as these would not, therefore, be predicted to confer any lasting benefit, and it does not (Hermon-Taylor 1998; Thomas *et al.* 1998). Drugs such as rifabutin and clarithromycin are man-made chemical modifications of natural streptomyces antibiotics with enhanced activity against MAC and MAP. In their inhibition of RNA polymerisation and of microbial protein synthesis at the level of the ribosome, rifabutin and clarithromycin act in synergy and may also be potentiated by the anti-leprosy drug clofazimine (Warek & Falkinham 1996; Ghebremichael *et al.* 1996; Hermon-Taylor 2002). All three agents have the additional advantage of being concentrated within macrophages where MAP in CD occurs. A double blind randomized placebo-controlled trial of rifabutin clarithromycin and clofazimine treatment in CD, based on several centres throughout Australia, is due to report in November 2004 (Selby *et al.* 2001). In the meantime, the results of four open-label clinical studies of the use of rifabutin and clarithromycin, with or without clofazimine, all say essentially the

same thing: that a substantial proportion of people with active CD will get better and their inflamed gut will heal when treated with these anti-MAP agents (Gui *et al.* 1997; Douglass *et al.* 2001; Borody *et al.* 2002; Shafran *et al.* 2002).

Rifampicin and erythromycin, the parent compounds, will kill many ordinary gut bacteria, but they are not active against MAP and do not heal CD. Rifabutin and clarithromycin will also kill many ordinary gut bacteria, but they are usually active against MAP and can heal CD. This reasoning favours the conclusion that when CD heals on rifabutin and clarithromycin treatment, it is because these agents are acting against the underlying causative MAP infection (Hermon-Taylor 2002).

6.7.4 Pathogenic mechanisms of MAP in Crohn disease

Although there have been recent advances (Clark-Curtiss 1998; Brosch *et al.* 2001) we still do not have a complete understanding of the way in which *M. tuberculosis*, *M. leprae* and MAC cause disease. We know little of the specific pathogenic mechanisms of MAP. How can a relatively low copy number of very slowly replicating ZN-negative intracellular MAP, able to minimize immune recognition, cause so much chronic inflammatory disease right across the gut wall in CD? It is most unlikely to be a direct florid response to MAP 'antigens'.

Epidemiological evidence suggests that the increased gut permeability well known to occur in CD is determined by exposure to environmental factors (Soderholm *et al.* 1999). Monocyte dysfunction and impaired immune regulation are also well known in CD (Fiocchi 1998; Monteleone *et al.* 2002; Shanahan 2002). *M. avium* infection perturbs immune function (Holland 2001; Wagner *et al.* 2002). A model for the way in which MAP causes CD, which is consistent with all the clinicopathological and therapeutic data, is one in which parasitization of immunoregulatory cells like macrophages and cells of the lamina propria by MAP, makes the gut mucosa leaky and establishes a variable impaired immune regulation throughout the gut wall and probably elsewhere. The inflammation itself then results from a disordered immune response to entry into the gut wall of food residues and microorganisms from the gut lumen. This would be why immunosuppression or immunomodulation can make CD better, when it would make TB worse. This would be why CD can improve with elemental diets, by reducing the allergic component of the inflammatory response to food residues and altering the intestinal flora. This would be why in colonic disease CD can improve on treatment with drugs such as ciprofloxacin and metronidazole which are active against the invasion by ordinary gut bacteria. It would also be why active CD usually returns when these treatments are stopped, because the underlying causative MAP pathogens are still there.

Other specific disease mechanisms involve MAP-induced damage to enteric glial cells and enteric neurones which may be an early event in MAP infection of the gut (Hermon-Taylor & Bull 2002). Through ligands such as the HupB protein (Cohavy *et al.* 1999; Shimoji *et al.* 1999) which participates with specific terminal trisaccharides in mediating initial Schwann cell adhesion by the leprosy bacillus (Ng *et al.* 2000), MAP shares some of the neuropathic properties of *M. leprae* (Rambukkana *et al.* 1997). Parasitization of the abundant and heterogeneous population of enteric glial cells by MAP would account for the MHC class II expression on enteric glial cells in CD (Geboes *et al.* 1992). It would also participate in establishing the enteric neuritis and the neuronal changes well known in CD, and clearly demonstrated in the gut of MAP-infected animals (Geboes & Collins 1998; Gwozdz *et al.* 2001). Damage to enteric glial cells in a transgenic mouse model has been shown to impair gut mucosal as well as vascular integrity, and to result in inflammatory disease of the small and large intestine with pathological features reminiscent of early CD (Cornet *et al.* 2001; Bush 2002). Abnormalities affecting enteric glial cells and enteric neurones are clearly involved in the pathophysiology of CD (Shanahan 1998; Cabarrocas *et al.* 2003) and it is probable that these are caused by MAP.

6.8 KEY RESEARCH ISSUES

Much research is needed to identify the environmental compartments, habitats and pathways of MAP, as well as the effect on MAP physiology and evolution, of intracellular trafficking through protozoa. Much research is also needed to identify the detailed distribution of MAP infection in animals and humans and to develop a range of preventative and therapeutic vaccines.

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7

Disseminated infection, cervical adenitis and other MAC infections

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7.1 DISSEMINATED MAC INFECTION

7.1.1 Clinical aspects

Disseminated infection with organisms of the MAC was first recognized in immunocompromised patients, including those with hairy cell leukaemia or on steroid therapy (Horsburgh *et al.* 1985). Subsequently, disseminated MAC was identified as a common complication of advanced AIDS (Zakowski *et al.* 1982). Patients with disseminated MAC typically present with a chronic wasting illness characterized by several weeks of fever, night sweats, malaise and weight loss. Abdominal pain and diarrhoea may also be seen (Horsburgh 1991; Benson & Ellner 1993). Laboratory studies demonstrate anaemia in most patients and an elevated alkaline phosphatase in a minority (Havlik *et al.* 1992).

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In AIDS patients, *M. avium* infection is acquired predominantly through the gastrointestinal tract where it is able to translocate the intestinal mucosa, infect and replicate in the submucosal macrophages, and cause bacteremia leading to dissemination of the organism (McGarvey & Bermudez 2001). Pathological studies show widespread distribution of organisms in the liver, spleen, bone marrow and gastrointestinal tract with 10^7-10^{10} cfu/gm of tissue. Renal involvement, possibly associated with nephrocalcinosis (Falkoff *et al.* 1987), and inflammation of the peritoneum have also been reported in AIDS patients with disseminated MAC infection (van der Reijden *et al.* 1989; Perazella *et al.* 1993).

A characteristic feature is the presence of numerous acid-fast staining organisms within foamy macrophages (Wong *et al.* 1985; Torriani *et al.* 1996). The high organism burden is associated with persistent bacteraemia and the diagnosis is typically confirmed by culture of blood or biopsy and staining of bone marrow, liver or small bowel.

Another clinical form of MAC disease has also been recognized in HIV infection. HIV-infected patients with previously unrecognized or subclinical *M. avium* infection may experience an immune reconstitution syndrome within two months of responding to HAART. This is a local inflammatory syndrome distinct from disseminated MAC. Clinical features include culture-positive lymphadenitis or other localized granulomatous disease, sometimes with draining sinuses, and negative blood cultures for mycobacteria (DeSimone *et al.* 2000).

7.1.2 Microbiology

Most blood isolates from HIV patients with disseminated MAC are *M. avium*. Early studies using serotyping methods indicated that a majority of these strains from the United States were serotype 4 or 8, with serotype 6 isolated from patients in Scandinavian countries (Hoffner *et al.* 1990; Tsang *et al.* 1992). More recent molecular studies have shown that *M. avium* strains isolated from blood represent phylogenetic lineages distinct from those among strains isolated from pulmonary sources or the environment (Smole *et al.* 2002). An interesting and unique feature of disseminated *M. avium* infection complicating AIDS is that as many as 25% of patients are infected simultaneously with two or more different strains (von Reyn *et al.* 1995). This implies that patients are either infected from exposure to mixed populations in a single environmental source or from multiple exposures to singular populations in different environmental sources.

7.1.3 Epidemiology and risk factors

Disseminated *M. avium* in HIV-negative people occurs primarily in patients with identifiable defects in cellular immunity. Cases have been described among patients with

familial immunodeficiencies (especially hereditary defects in IFN-gamma receptors), among patients with leukaemia (especially hairy cell leukaemia) and lymphoma, patients on steroid therapy, patients with collagen vascular disease, and among bone marrow and solid organ transplant recipients (Weinstein *et al.* 1981; Horsburgh *et al.* 1985; Bennett *et al.* 1986; Newport *et al.* 1996; Roy & Weisdorf 1997; Barcat *et al.* 1998; Nagy & Rubin 2001). Nonetheless, approximately 40% of HIV-negative patients with disseminated *M. avium* have no identifiable immune defect (Horsburgh *et al.* 1985).

Among AIDS patients, the risk of disseminated *M. avium* is related to the CD4 count and disease occurs predominantly among patients with CD4 counts less than 75-100/mm^3. In a large cohort of AIDS patients the overall risk of disseminated *M. avium* was found to be approximately 20% per year. Stratified by CD4 count the risk is approximately 39% per year with a CD4 count of < 10, 30% for CD4 10-19, 20% for CD4 20-39, 15% for CD4 40-59, 8% for CD4 60-99 and 3% for CD4 100-199 (Nightingale *et al.* 1992).

Epidemiological studies indicate that the risk of disseminated *M. avium* in advanced AIDS is high in developed countries and low or absent in developing countries (e.g. sub-Saharan Africa) (von Reyn *et al.* 1996). It is not known if genetic factors or geographic differences in mycobacterial immunity or pathogenicity of *M. avium* stains may contribute to this difference. MAC are present in the African environment (von Reyn *et al.* 1993) and healthy African populations have significant rates of skin test reactivity to MAC antigens (von Reyn *et al.* 1993) suggesting that exposure to MAC does occur in developing countries. The low rate of disseminated *M. avium* in these settings is probably best explained by a high rate of mycobacterial immunity conferred by cross- protection from prior TB and or BCG immunization (von Reyn *et al.* 2002).

Epidemiological studies of disseminated MAC in AIDS have consistently identified several other risk factors for disease including prior *Pneumocystis carinii* pneumonia, exposure to uncooked seafood and prior endoscopy (von Reyn *et al.* 1996, 2002; Ristola *et al.* 1999). Showering has been identified as protective in two different studies, but this finding remains unexplained (Horsburgh *et al.* 1994; von Reyn *et al.* 2002). Molecular epidemiological studies have implicated recirculating hospital hot water systems as the source of two clusters of cases (von Reyn *et al.* 1994).

Detailed water exposure studies have failed to identify the dominant source of exposure to MAC in HIV infection. A study from Los Angeles identified *M. avium* isolates from hospital water that were identical to sputum isolates from non-AIDS patients, but failed to identify water isolates identical to isolates from AIDS patients (Aronson *et al.* 1999). In a recent study of 359 AIDS patients in the United States and Finland, patient-directed potable water samples were cultured for MAC. Overall, 13% of samples were positive for *M. avium*, principally from home water supplies (von Reyn *et al.* 2002). Recirculating hot water supplies had the highest risk of colonization (Arbeit, unpublished) but there was no association between *M. avium* colonization of a patient's home water supply and the risk of disseminated MAC. Only 1 of 31 patients who

developed disseminated MAC was infected with a strain also present in their home water source (toilet bowl water) (von Reyn *et al.* 2002).

Studies have also explored the possible role of other environmental sources. *M. avium* was isolated from potting soil in the homes of AIDS patients in California but typing methods were only able to show that they were similar but not identical to corresponding clinical isolates (Yajko *et al.* 1995). In an investigation of food products from the homes of AIDS patients 1 of 121 food samples was found to have a PCR pattern identical to a clinical isolate (Yoder *et al.* 1999).

Collectively, these findings have been interpreted to indicate that the sources of disseminated MAC in AIDS are diverse and probably not wholly identifiable or avoidable.

7.1.4 Burden of disease

MAC disease is not reportable in the United States but passive surveillance data on disseminated MAC are available for patients with AIDS. Prior to the advent of effective prophylaxis against disseminated MAC in 1993, cases of disseminated MAC were continuing to increase and outnumbered annual cases of TB in the United States. The peak incidence of MAC in the United States was thought to have occurred in 1994 when it was estimated that there were approximately 37 000 people with disseminated MAC (Horsburgh *et al.* 2001). Cases of disseminated MAC are now much less common and occur principally among people with advanced AIDS who are not under medical care or are non-compliant with HAART or MAC prophylaxis (Kovacs & Masur 2000).

7.1.5 Prevention and treatment

In the pre-HAART era disseminated *M. avium* infection was associated with a mean four to five month reduction in survival (Horsburgh *et al.* 1991; Chin *et al.* 1994). Treatment of disseminated *M. avium* with clarithromycin (or azithromycin) and ethambutol increases the survival time of patients. Treatment must be continued indefinitely in patients with advanced AIDS but may be safely discontinued after 12 months in those who are also treated with HAART and experience immune reconstitution (defined as an increase in CD4 count to > 100/mm^3 for at least six months) (Aberg *et al.* 1998; CDC 2002).

Disseminated *M. avium* can be prevented in AIDS patients with CD4 counts < 50/mm^3 by the administration of antibiotic prophylaxis with clarithromycin or azithromycin (Havlir *et al.* 1996; Benson *et al.* 2000). Treatment of AIDS with HAART also eliminates the risk of disseminated MAC in most patients. Thus patients who have been treated with HAART and have an increase in CD4 count to above 100/mm^3 for more than three months may be safely taken off antibiotic prophylaxis for MAC (CDC 2002).

7.2 CERVICAL ADENITIS

7.2.1 Clinical aspects

The link between NTM and cervical lymphadenitis was first described by Prissick in 1957 (Prissick *et al.* 1957) when these organisms were isolated from cases of scrofulosis. The first NTM species isolated was named after this lesion: *Mycobacterium scrofulaceum*. Today more than 100 mycobacterial species have been described. Clinical classification separates them into three groups according to their potential to cause diseases in humans: pathogenic; facultative pathogenic; and non- pathogenic species. The most common presentation is cervical adenitis, from which MAC is currently the most frequently isolated agent. Table 7.1 lists the NTM species most frequently encountered in mycobacterial lymphadenitis.

Table 7.1 Selected species of *Mycobacterium* isolated from cervical adenitis in children with normal immunity (in alphabetical order)

Slow growing species	Rapidly growing species
Mycobacterium avium complex	*Mycobacterium abscessus*
Mycobacterium kansasii	*Mycobacterium chelonae*
Mycobacterium malmoense	*Mycobacterium fortuitum*
Mycobacterium scrofulaceum	*Mycobacterium xenopi*

MAC cervical adenitis usually presents as a unilateral, solid, swelling high in the neck that in most cases is not painful to the touch. The overlaying skin might show a purplish discoloration and sometimes a fistula has formed. This swelling is present over a longer period of time (weeks to months) and unresponsive to antibiotic treatment (Wolinsky 1995). Ultrasound examination usually shows no necrolysis and small calcifications might suggest mycobacterial disease. In immune-competent children the disease remains localized, the child appears well and further studies including chest x-ray and blood chemistry are unremarkable.

The most important issue for diagnosis is the differentiation of TB and non-tuberculous disease. In MAC adenitis there is no history of exposure to TB. Skin testing for TB usually shows positive results, even strongly positive reactions in some cases, but might also be variable (Chesney 2002). Thus PPD skin testing does not differentiate between tuberculous disease and MAC cervical adenitis. Also, histopathological changes in immune-competent children do not differ significantly. In addition, mixed infections caused by *M. tuberculosis* and NTM have been described.

Acid-fast staining is not species specific and has a low sensitivity. The gold standard for diagnosis of mycobacterial disease is the isolation of the pathogen by culture. Modern

culture techniques have increased the sensitivity of culture isolation of NTM to about 50%. In addition, the time for a positive culture could be decreased to an average of two to three weeks. Nucleic acid amplification tests to detect MAC and other mycobacterial pathogens directly from the specimen are available. They should only be used as an addition to conventional culture, as they exhibit a lower sensitivity. Susceptibility testing of the isolated mycobacterium is not standardized and – with the possible exception of susceptibility to the newer macrolides (clarithromycin, azithromycin) – does not contribute much to the therapeutic decision (American Thoracic Society Statement 1997).

7.2.2 Epidemiology and risk factors

The epidemiological information on mycobacterial cervical adenitis in immunocompetent children is based on a number of case series performed over different periods of time in different geographical regions. A hallmark review by Wolinsky - and its follow-up publication 16 years later - found this disease mostly among young children below five years of age. Girls were more often affected than boys. The initial manifestation started more often in the winter months, sometimes preceded by an upper respiratory tract infection (Wolinsky 1979, 1995). Other studies also showed an intriguing age distribution of mycobacterial adenitis: while in infants and small children the majority of cases are caused by MAC or other NTM, children older than 10-12 years of age and young adults usually suffer from tuberculous lesions.

There are characteristic differences in the geographical distribution of the species. While MAC is the most common pathogen found in cervical adenitis on a worldwide scale, *M. malmoense* is the second most frequently isolated organism in Europe. In the United States however, *M. scrofulaceum* is second and *M. malmoense* is rarely isolated (Benjamin 1987; Clark *et al.* 1994; Grange *et al.* 1995; Wolinsky 1995; Suskind *et al.* 1997).

In western countries with a low incidence of TB (below 20 per 100 000 population) most studies demonstrate a ratio of non-tuberculous to tuberculous mycobacterial adenitis of 4:1 (Wallace *et al.* 1990; Inderlied 1993; Chesney 2002). However, in other countries scrofula is still caused almost exclusively by TB. It has also been suggested that there might be a negative correlation with BCG vaccination in infancy. This suggestion is supported by the efficacy of BCG in prevention of leprosy.

The maturation of the cellular immune response seems to be responsible for the age distribution in non-tuberculous disease. However, it remains unclear why only a small number of children develop clinical disease even though the organisms are ubiquitous in the environment. The genetic analysis of the syndrome of "mendelian susceptibility to mycobacterial disease", first described by Casanova *et al.* has elucidated some of the underlying specific pathways predisposing to mycobacterial disease (IL-12, INFγ) (Altare *et al.* 1998; Casanova *et al.*, 1999). Other deficiencies in cellular immune defence

such as HIV/AIDS also predispose to disease progression and generalized disease. In addition, iatrogenic immune suppression and – in pulmonary disease – morphological and pathophysiological factors might trigger disease.

7.2.3 Morbidity/mortality

Diseases caused by NTM are not routinely reported in most countries. An exception is Sweden, where incidence data have been published for birth cohorts between 1969 and 1990. During this time an increase in the incidence rate from 1 to 5.7 per 100 000 children younger than 5 years of age was reported (Romanus *et al.* 1995). A similar trend has been reported from clinical observations and laboratory data for other countries, where incidence data are not available (Kuth *et al.* 1995).

7.2.4 Burden of disease

As there is little information about the morbidity caused by mycobacterial adenitis; for most countries, the burden of disease can only be speculated. Even though, mycobacterial adenitis is a rare disease, it seems to be increasing in western countries. This might even happen more rapidly in countries that have stopped BCG vaccination because of the low incidence of TB (Romanus *et al.* 1995). Furthermore, the long-term effect of mycobacterial adenitis and other non-tuberculous disease remains to be studied. One current hypothesis suggests that infection with NTM disease in childhood might play a role in triggering diseases such as CD (Hermon-Taylor *et al.* 1998; see also Chapter 6).

7.2.5 Prevention and treatment

As NTM are ubiquitous, prevention of exposure seems to be difficult, if not impossible, to achieve. For the development of strategies for prevention more surveillance data are needed to estimate the impact of other factors such as BCG vaccination or regional differences on the long-term trend.

As MAC and other NTM species are resistant to antituberculous drugs and most antibiotics, the treatment of choice in localized disease still consists of complete excision of the diseased lymph nodes (Schaad *et al.* 1979; Starke *et al.* 1995). However, depending on the relationship to other anatomical structures, especially the mandibular branches of the facial nerve, surgical intervention carries the risk of transitory or permanent damage to these structures. If the affected nodes cannot be surgically removed or are (inadvertently) drained during the process, local reactivation is likely to occur. In a number of cases, total excision is not feasible and therefore a combined approach including antimycobacterial chemotherapy is used. Usually a triple therapy consisting of a new generation macrolide, a rifamycin derivative, and ethambutol is prescribed for a

period of more than six months. However, there are no large blind randomized trials about the best treatment regimen.

In generalized and reactivated disease the initial treatment approach consists of combination chemotherapy. If surgical revision is indicated — for example because of fistula formation — the procedure requires a very experienced surgeon.

7.3 TENOSYNOVITIS

Soft tissue infections with MAC in immunocompetent patients are rare, but a number of cases of tenosynovitis have been reported, usually following local surgery, trauma or corticosteroid administration (Hellinger *et al.* 1995; see also Chapter 8).

7.4 OSTEOMYELITIS AND SEPTIC ARTHRITIS

Multifocal osteomyelitis caused by MAC has been identified in three patients with a genetic defect of the interferon-gamma receptor and a family history of infections with NTM (Arend *et al.* 2001). Patients had a delayed diagnosis and a protracted illness that responded slowly to multi-drug treatment. In one patient, additional treatment with IFN-gamma was necessary. Macrophages from patients had a reduced responsiveness to IFN-gamma and were heterozygous for a dominant negative mutation in the gene encoding the IFN-gamma binding receptor-1 chain. The infections were limited to skin, bone and lymph nodes. Recurrent MAC osteomyelitis has been associated with a deletion at the 818 residue of the interferon-gamma receptor (Villella *et al.* 2001). Osteomyelitis and septic arthritis can occur in apparently immunocompetent children (Frosch *et al.*, 2000) and adults (Jones *et al.* 1995; Mahan & Jolles 1995; Pombo *et al.* 1998; Weiner *et al.* 1998; Weigl & Haas 2000; Bridges & McGarry 2002). A case of MAC spinal epidural abscess without vertebral osteomyelitis has been reported in a patient with AIDS (Rotstein & Stuckey 1999). Primary septic arthritis and osteomyelitis can occur in AIDS patients (Blumenthal *et al.* 1990; Sheppard & Sullam 1997). Osteomyelitis caused by MAC may be recurrent (Mahan & Jolles 1995; Kourtis *et al.* 1996) and occasionally disseminated (Bender & Yunis 1980; Collert *et al.* 1983; Zammarchi *et al.* 1987; Kwong *et al.* 1991).

Osteomyelitis can follow trauma (Walz & Crosby 1995), surgery, steroid therapy (Pirofsky *et al.* 1993) and sarcoidosis (Sato *et al.* 1992) and is more common in old age (Tanaka *et al.* 1993).

In disseminated MAC infection the organism may be recovered from bone marrow samples (Ohse *et al.* 1997). Although this can lead to osteomyelitis, this does not appear to result from such colonization in most cases.

7.5 MENINGITIS

MAC infections rarely involve the central nervous system, even in patients with widely disseminated disease (Jacob *et al.* 1993; Gyure *et al.* 1995). On the rare occasions when the central nervous system is involved, MAC is the most common NTM cause of meningitis and has a high mortality rate (Jacob *et al.* 1993; Gyure *et al.* 1995; Weiss *et al.* 1995; Flor *et al.* 1996).

7.6 PANCREATIC INFECTION

Pancreatic infection with MAC is rare but can occur in children with HIV infection (Horsburgh *et al.* 1994; Kahn *et al.* 1995).

7.7 SARCOIDOSIS

Mycobacteria, including MTB, MAC and MAP have been implicated in the development of sarcoidosis. Molecular methods to demonstrate mycobacterial nucleic acid in tissues from patients with sarcoidosis have had mixed results (Ikonomopoulos *et al.* 1999; Li *et al.* 1999; Eishi *et al.* 2002), although *M. avium* and MTB sequences have been found in such tissues (Li *et al.* 1999).

7.8 KEY RESEARCH ISSUES

Cases of disseminated *M. avium* in AIDS have become sufficiently unusual that further epidemiologic studies in this population are not likely to be fruitful. Epidemiologic studies have not been conducted to identify possible sources of disseminated *M. avium* infection among HIV–negative patients with defects in cellular immunity. These cases are also rare but epidemiologic studies might explore possible nosocomial transmission from potable water. Nosocomial transmission of *M. avium* has occurred among AIDS patients (von Reyn *et al.* 1994) and nosocomial transmission of other NTM has also been confirmed using molecular methods among various other patient groups (Phillips & von Reyn 2001). Thus molecular epidemiologic studies of disseminated *M. avium* in HIV-negative populations might focus on hospital water supplies and procedures with demonstrated potential for nosocomial transmission of NTM.

Further work on the epidemiology of lymphadenitis is needed to improve our understanding of the routes of transmission and to aid the design of preventative measures.

8

Skin, bone and soft tissue infections

M.A. De Groote and P. Johnson

This chapter describes the multiple environmental mycobacteria species that can cause skin, bone and soft tissue infections. The etiologic organisms, environmental exposures and clinical presentations will be highlighted.

Infection can occur after traumatic inoculation, as part of a disseminated infection, or from a medical or cosmetic procedure. Risk factors include certain hobbies and occupations as well as various forms of immunosuppression. The source of exposure for skin and soft tissue infection by PEM is usually water or soil: person-to-person transmission has not been demonstrated.

An important cause of skin and soft tissue infection is the agent responsible for BU. BU is the third major mycobacterial disease of man after TB and leprosy. The name derives from a region in Uganda where the disease was common in the 1960s and 1970s (Barker 1972). The responsible bacterium, *Mycobacterium ulcerans,* produces a toxin called mycolactone that causes necrosis of skin and subcutaneous fat and has immunosuppressive properties (George *et al.* 1999). Sufferers of BU are generally healthy individuals with no known underlying immune defect.

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Additional details regarding skin infections can be found in the chapter dealing with invasive devices and procedure (Chapter 10). For the interested reader, an excellent review on NTM infections of the skin has recently been published (Weitzul *et al.* 2000).

8.1 CLINICAL ASPECTS

PEM can cause a variety of skin and skin structure infections. The clinical presentation can be quite variable. Most patients are relatively healthy and acquire disease from minor trauma with exposure to the environment (soil or water). Lesions tend to be fairly indolent, but severe disease can occur. The initial exposure can be hard to track as the incubation period can be quite long. Disseminated disease should alert the clinician to an immunocompromising condition such as malignancy, medications used to suppress the immune system, HIV or defects in cytokine pathways (IL-12 and gamma-interferon). Some of the organisms involved require special knowledge of optimal cultivation methods in the laboratory. Examples of a few of the major species and the nature of the disease they cause are described below.

8.1.1 *M. marinum*

M. marinum skin infections have been observed in people who own aquaria, people who work with water, and those who use water for recreational purposes (Huminer *et al.* 1986). It has been associated with both salt and fresh water exposures. Certain occupations such as aquatic farming represent a high-risk group. Also, farmers and nursery garden workers appear to be at greater risk.

Several studies of disease caused by *M. marinum* have been reported. In Australia, 29 patients were reported with *M. marinum* infection following trauma and exposure to an aquatic or timber source (Iredell *et al.* 1992). In a study from Chesapeake Bay, USA, Hoyt *et al.* (1989) reported infection predominantly in males and that cutaneous infection was most common. In contrast, Edelstein (1994) found that in northern California over 50% of the victims with skin infections due to *M. marinum* were female. The major risk factor for infection was contact with fish tanks. A large outbreak of *B. balnei* (later identified as *M. marinum*) infection was identified in an open-air hot springs pool (Collins *et al.* 1985). In this outbreak, and others like it, skin lesions were predominantly located on the elbows and less often the knee, foot, finger and toes. Skin lesions were often superficial and verrucal. Acid-fast stain is often negative in biopsy and drainage samples. Lymphadenopathy is unusual but does occur (Huminer *et al.* 1986). Most lesions healed spontaneously. A

positive skin test to tuberculin has been reported even long after the lesions healed (Judson & Feldman 1974).

There is often a delay between the development of symptoms and proper diagnosis of sporadic cases. The lesions of these infections can be variable in presentation but they often begin as a reddish violaceous nodule that goes on to ulcerate. A common presentation is nodular lymphangitis (or sporotrichoid spread) consisting of subcutaneous nodules with satellite lesions that can ascend up the extremity by lymphangitic spread (Wolinsky *et al.* 1972). While the finding of sporotrichoid disease can be seen in other infectious (including mycobacterial diseases), it has been particularly well described with *M. marinum*. The most frequent location is the right middle finger. Disease is most often limited to the skin but can cause adjacent joint and tendon infections. In addition to nodules, cutaneous lesions include pustules, verrucous lesions, red plaques, abscesses, seromas and ulcers. Synovitis and arthritis are seen rarely. Dissemination can occur in the immunocompromised host (Gombert *et al.* 1981; King *et al.* 1983; Enzenauer *et al.* 1990; Tchornobay *et al.* 1992; Parent *et al.* 1995; Holmes *et al.* 1999; Enzensberger *et al.* 2002). Pathologic specimens reveal granulomas which can be non-caseating; there can be suppurative inflammation, micro-abscesses and necrosis. The immunocompromised patient may have less well-formed granulomas.

Often acid-fast staining of tissue or drainage is negative and the diagnosis is made by culture. The organism has a growth temperature optimum of 30-32 °C, Diagnosis is usually suggested on clinical grounds, but identification of the organism in the laboratory is crucial. Clinicians should inform the laboratory of the suspicion of the organisms so that the laboratory can set up the material for mycobacterial culture at lower temperature in addition to routine cultivation procedures. Susceptibility tests can be performed once the isolate has been grown. Lesions in the immunocompetent host can resolve spontaneously; however, most experts would begin combination antimicrobial therapy when a diagnosis is made. Agents that have been used include rifampin, ethambutol, trimethoprim/sulfamethoxazole, clarithromycin, fluoroquinolones and doxycycline/minocycline. Success has been reported with trimethoprim/ sulfamethoxazole alone. Consultation with an experienced surgeon may be indicated for the removal of infected and devitalized tissues. The optimal duration of therapy is unknown, although three to six months is normally the minimum; many experts recommend a longer course. Tendon and joint infection requires longer periods of therapy with the use of surgical debridement if chemotherapy alone is not successful (Aubry *et al.* 2002). In the hand, surgical treatment is often required to control the infection. Occasionally, heat is applied to the limb in an attempt to inhibit the organism's growth (Sutherland *et al.* 1980).

8.1.2 Rapidly Growing Mycobacteria

Infection of the skin and soft tissues with RGM is not uncommon (Wallace *et al.* 1992). In a recent case control study of *M. fortuitum* furunculosis associated with footbaths in California, the risks for acquiring infection was related to shaving the legs with a razor before pedicure (odds ratio 4.8) (Winthrop *et al.* 2002). Microabrasion with the razor could explain the portal of entry. Cultures of salon tap water yielded other species of RGM (*M. abscessus/chelonae*). Cultures from the footbaths yielded *M. fortuitum*. Some were indistinguishable from the clinical isolates on the basis of PFGE. Some of the infections reported in these individuals were associated with severe scarring furunculosis. This important large community outbreak, and prior reports of rapidly growing mycobacterium skin infections associated with hot tubs and public baths, highlight the important role that waterborne mycobacterial pathogens play in skin infection (Aubuchon *et al.* 1986; Lee *et al.* 2000a). The frequency of isolation of these pathogens and the burgeoning nail salon and cosmetic industry suggests that additional cases may occur.

Injection site abscesses have been reported, most often secondary to *M. abscessus*. Other rapidly growing species such as *M. chelonae, M. smegmatis* and *M. fortuitum* have also been implicated in a variety of similar situations. These have often occurred as the result of iatrogenic exposures, cosmetic procedures and complications related to alternative medicine practices (Wallace *et al.* 1983; Safranek *et al.* 1987; Camargo *et al.* 1996; Murillo *et al.* 2000). *M. abscessus* tenosynovitis has been reported in a health care worker who had a penetrating injury due to a contaminated scalpel (Wolinsky 1992). *M. abscessus* is one of the most common causes of post-surgical mycobacteria infections, especially after plastic surgery. *M. abscessus* and *M. fortuitum* have caused skin infections after iatrogenic procedures such as liposuction, facial blepharoplasty, augmentation mammoplasty and other cosmetic procedures (refer to Chapter 10). A common finding in *M. abscessus* infections of the skin is the formation of violaceous to red tender nodules that can form fistula. Fistula formation can be severe and can track to the surface in multiple sites.

Therapy for RGM infections is generally guided by *in vitro* antimicrobial susceptibility testing. Clarithromycin, cefoxitin and amikacin are among the most effective antimycobacterial agents for *M. abscessus*. Single-drug therapy with clarithromycin has been effective but failures have been reported due to the emergence of drug resistance (Vemulapalli *et al.* 2001). Susceptibility tests for the rapid growing species can also be used to guide therapy to other potentially effective agents such as trimethoprim/sulfamethoxazole and doxycycline, and fluoroquinolones. As with other infections, skilled surgical debridement of necrotic tissues and subsequent wound management is important in the successful outcome of many of these infections.

Other reported cutaneous manifestations of *M. abscessus* include Sweet syndrome and nodular panniculitis (Rotman *et al.* 1993; Choonhakarn *et al.* 1998; Retief & Tharp 1998; Bowenkamp *et al.* 2001). Sweet syndrome presents with fever, elevated white blood cells, erythematous plaque-like skin papules and neutrophils on histopathology. A recent report of disseminated disease due to *M. abscessus* manifest as lymphadenopathy and various organ involvement due to RGM revealed a high incidence of Sweet syndrome (Chetchotisakd *et al.* 2000). These patients were seronegative for HIV. Suspicion of a defect in cell-mediated immunity was raised but not confirmed.

8.1.3 *Mycobacterium avium* complex

Both *M. intracellulare* and *M. avium* have been reported to cause cutaneous, joint, tendon and skeletal disease. Isolated lesions tend to be nodules, which rarely ulcerate. There can also be tenosynovitis, panniculitis, fasciitis, or synovitis. Typically the infections have been localized to soft tissues of the anterior surface of the hand and wrists. Tenosynovitis frequently involves the upper extremity and is a result of inoculation following minor trauma. Incubation times between the event and the presentation to a health care provider can be substantial. The antecedent source is often not clear, and diagnosis can be difficult. Patients are frequently misdiagnosed with arthritis or other inflammatory conditions and have received systemic or local injection of corticosteroids before a proper mycobacteriology diagnosis is made (Hellinger *et al.* 1995; Zenone *et al.* 1999). This can aggravate the disease. The right distal extremity is more often affected. The typical patient is immunocompetent and in the fifth to seventh decades of life. Men and women appear fairly equally affected (Hellinger *et al.* 1995). Symptoms include swelling and violaceous to red discoloration of the overlying skin. The lesions can be tender. Surgical debridement is an important component of diagnosis and therapy. During the operation, the finding of "rice bodies" is felt to be characteristic of mycobacterial infection (Sanger *et al.* 1987). Pathology of tendon infection shows chronic tenosynovitis often with granulomatous inflammation. Other mycobacterial causes of tenosynovitis include *M. kansasii, M. fortuitum, M. terrae, M. chelonae, M. malmoense, M. xenopi* and *M. abscessus* (Zenone *et al.* 1999).

MAC is a rare cause of osteomyelitis and can occur in both immunocompetent and immunocompromised hosts (Chan *et al.* 2001; see also Chapter 7). Localized pain, immobility and, if disseminated disease is present, fever, night sweats and weight loss can occur (Marchevsky *et al.* 1985).

Cutaneous manifestations of MAC infection also include disseminated disease in patients who have defects in immune function including AIDS. Those with severely depressed CD4 cells are most at risk. Since MAC, *M. haemophilum, M. szulgai, M. tuberculosis* and other species have been reported to cause skin manifestations in

patients with AIDS, proper microbiological diagnosis is key for management of these patients. Histopathologically, lesions in immunocompromised patients can be atypical (Bartralot *et al.* 2000). While HAART to improve immune function is the cornerstone in management of MAC infection in AIDS, clinicians should be aware that osteoarticular, cutaneous, and other unusual disease presentations can also develop or exacerbate when cellular immunity is restored (Nalaboff *et al.* 2000).

The diagnosis should be clear for all the above infections if careful attention is paid to proper biopsy, histopathology, microbial identification and susceptibility testing. Therapy is complex and multiple drug-drug interactions occur especially for patients taking other medications (such as HIV therapy). Consultation with experts in treating these infections is recommended. Therapy is typically continued for 12-24 months and consists of multiple antibiotics typically including clarithromycin, rifampin and ethambutol. Additional therapy such as an aminoglycoside (i.e. amikacin) is sometimes included for a limited time. In many cases, especially if there is devitalized tissue, surgery can also contribute to curative therapy. In cases of severe tenosynovitis of the wrist or hand, patients require extensive debridement often along the entire length of the tendon sheath. Splinting immediately after the procedure is often done and aggressive hand physical therapy as soon as wound healing allows is often undertaken.

8.1.4 *M. haemophilum*

This organism is a fairly recently recognized pathogen and causes disseminated cutaneous lesions in immunocompromised patients. The lesions often ulcerate. In addition to the skin it can occur in bones, joints, lymphatics and lungs (Straus *et al.* 1994). The cutaneous lesions are violaceous and most often tender. Abscesses with small amounts of serosanquinous drainage have been reported. The cutaneous lesions can be multiple and tend to cluster on the extremities (Dever *et al.* 1992). They also frequently overlie a joint. These findings are consistent with the preference of the organism for lower growth temperatures. In contrast to *M. marinum*, the distribution is typically not sporotrichoid. People at risk are those with defects in cellular immune function such as those with HIV, transplants, lymphoma or those taking medications to suppress immune function. In one study, infection presented a mean of 16 months after an AIDS diagnosis (Straus *et al.* 1994). Pathology shows minor necrosis, poorly formed granulomas and both extracellular and intracellular acid-fast organisms. In the laboratory, *M. haemophilum* is fastidious, requires iron-supplemented growth media, and has a lower temperature for incubation than most other mycobacteria. These facts have likely resulted in a lower historical detection rate. *M. haemophilum* infections have been sporadic and the reservoirs and mode of transmission are not clear.

8.1.5 *M. ulcerans*

BU due to *M. ulcerans* is a great public threat. In certain geographical regions the incidence of BU is increasing. Children are disproportionately affected. Rates in some villages in Africa are greater than 15% (Marston *et al.* 1995). New data has implicated an aquatic insect in the transmission of the disease and this may be the basis for beginning a control programme (Marsollier *et al.* 2002).

BU is painless and slow to develop. A typical Buruli lesion is an extensive, deeply undermined skin ulcer that heals by scarring. There are other presentations including nodules, plaques, oedematous swelling of a whole limb or the abdominal wall and osteomyelitis (Buntine *et al.* 2002). Death due to BU is rare but permanent deformities are common. The diagnosis of BU is likely if large numbers of acid-fast bacilli are present in smears or histological sections obtained from a suspicious lesion. The presence of *M. ulcerans* can be rapidly confirmed by a specific and sensitive PCR (Ross *et al.* 1997; Russell *et al.* 2002). Culture confirmation may take some weeks.

Patients often delay seeking medical assistance until disease is advanced. The main mode of treatment is surgery, with the aim of totally excising early lesions or, in established disease, removing necrotic tissue and grafting the resulting defect. There is current interest in combination antibiotic therapy as an adjunct to surgery, or alone for very early lesions (Etuaful *et al.* in press).

8.1.6 *M. terrae*

This complex of organisms (including *M. terrae, M. trivale* and *M. nonchromogenicum*) is a rare cause of cutaneous infection (Zenone *et al.* 1999; Bartralot *et al.* 2000). Tenosynovitis is the most commonly reported manifestation (Smith *et al.* 2000). Pathological specimens reveal granulomas with multinucleated giant cells.

In common with several other mycobacterial infections, studies of empiric therapy have not been done. Rifampin and ethambutol containing regimens tended to have a better outcome but did not reach statistical significance (Smith *et al.* 2000). Some of the predisposing exposures for skin/tendon infection presented in the literature include farming, fish tanks and gardening, and other soil exposures.

8.2 OVERALL BURDEN OF DISEASE

BU is an important disease because the incidence is increasing, it is expensive to treat, and it is most common in regions that lack advanced medical facilities. For example, a recent study has estimated the cost per case in Ghana at 780 USD (Asiedu 1998). BU has become a major burden for poor agricultural communities in West Africa. In Australia, one of the very few Organisation for Economic Co-operation and

Development countries where transmission of *M. ulcerans* occurs, the cost per case has been conservatively estimated at 12 000 USD (Drummond 1998).

A recent report from Ghana has estimated a national prevalence of 20.7/100 000 in 1999 (Amofah 2002). In one highly endemic region in Ghana an annual incidence of 280/100 000 was reported - higher than TB in the same region (Ragunathan *et al.* 2001). The disease rates in Uganda have been estimated at 2-5% of the population and in Côte d'Ivoire and Ghana; the rates in some villages have been estimated at 16% and 22% respectively (Amofah *et al.* 1993; Marston *et al.* 1995). Disabilities resulting from BU disease are severe and it has been estimated that 25% of cases are left with some disability in Côte d'Ivoire (Marston *et al.* 1995) and 58% in a recent study in Ghana (Ellen *et al.* 2003).

The global burden of BU has not been established but concern about the emergence of BU prompted WHO to create a specific programme for BU in 1998 (GBUI: Global Buruli Ulcer Initiative).

Disease due to PEM is not reportable in the Unites States. Ascertaining the precise incidence and prevalence data is therefore not possible. While some general conclusions can be gleaned by looking at laboratory-based surveys, such surveys have significant limitations. It is difficult to determine how many patients are involved given that the reporting is culture-based, not patient-based, and a single individual may have more than one specimen submitted. However, since 1993 a Public Health Laboratory Information System method of reporting by State Health Departments has proved useful for tabulating data on the environmental mycobacteria. The population under study are those patients in the United States who have had a specimen submitted for evaluation. Only one isolate for each patient was recorded. Compared to historical rates, it appears that the incidence of *M. marinum* and *M. haemophilum* has increased (Bean *et al.* 1992; Dobos *et al.* 1999).

8.3 DISTRIBUTION

Mycobacteria are found in soil and water and often (but not always) the inoculation event can be traced to a specific exposure. In many of the infections due to these organisms transmission occurs via minor trauma to the skin. For *M. marinum* and *M. ulcerans*, water appears to be the major source. For *M. haemophilum*, water has been suggested as a source but the actual reservoir is not completely understood. Patients infected with other species of PEM may have acquired the infection from soil, water or an otherwise unknown source.

While mycobacterial skin disease is believed to be worldwide, certain infections have limited geographic occurrences. BU has been reported in many tropical and some temperate countries, and it is endemic in parts of sub-Saharan Africa. There are also established foci in the Americas, Asia, Australia and Papua New Guinea (Asiedu *et al.* 2000).

8.4 DESCRIPTIVE EPIDEMIOLOGY

Infection is acquired from the environment and person-to-person spread has not been described. Systematic studies of the epidemiology of infections have not been done. For a few of the infections, some general comments can be made.

BU endemic areas are usually near tropical marshes, rivers or lakes but transmission also occurs in temperate southern Australia (Horsburgh 1997). BU is typically unevenly distributed within an endemic country. New areas of micro-endemicity may appear unpredictably (Johnson *et al.* 1996).

Unlike TB and leprosy, BU is contracted by exposure to a contaminated environment rather than from infected people. Most patients with BU are children below the age of 15. A study from Amansie West in Ghana reported the median age as 12 years, with 49% of cases aged 10-14 years. Only 20% were over 50 years old (Amofah *et al.* 1993). Recent reports from Benin suggest that there is also an increased attack rate of BU in the elderly, resulting in an age-specific incidence similar to that observed for TB (F. Portaels, personal communication). The precise mode of transmission has not been established, but recent work has suggested that aquatic insects and biofilms attached to aquatic plants harbour *M. ulcerans* (Marsollier *et al.* 2002). Transmission of *M. ulcerans* from infected insects to laboratory mice has been demonstrated, but whether this is how humans become infected remains unknown. Transmission by aerosol or through direct contact with contaminated soil has also been proposed. It is possible that more than one mode of transmission exists. It has been suggested that environmental changes such as logging, mining and nutrient enrichment of waterways is contributing to the spread of BU.

Recent serological data from Australia has suggested that the rate of household exposure to *M. ulcerans* within endemic areas may be quite high (Gooding *et al.* 2002). It is not understood why some individuals are affected while the majority remain disease free. There is no convincing evidence of an increased incidence of BU in patients with HIV, diabetes or medically induced immunosuppression.

In the majority of reports the mean age of people with *M. marinum* is the fourth to fifth decade of life and Caucasian males are most commonly affected. Trends in rates of infection are largely unknown; however the recorded incidence of *M. haemophilum* may increase as culture methods improve and as clinicians become more aware of the organism.

8.5 RISK FACTORS

Most infections are thought to occur by local inoculation as a result of accidental or unapparent trauma. Local steroid injections can also worsen undiagnosed skin infections that have occurred as a result of trauma. These infections can occur in immunologically normal individuals.

The main risk factor for BU is contact with an endemic region. The period of exposure can be very short, but most affected people are residents of these areas. There is circumstantial evidence that wearing clothing (trousers) may be protective (Marston *et al.* 1995).

Exposure to contaminated solutions or devices is another risk factor for other PEM and iatrogenic infections are dealt with in the invasive procedures chapter (Chapter 10). In severely immunocompromised patients, it is likely that the skin lesions have occurred as a result of haematogenous dissemination rather than direct inoculation. In summary, immunodeficiency, abnormal defensive barriers as a result of skin injury and exposure to certain sources (i.e. soil, water and contaminated solutions or devices) are risk factors associated with infection.

8.6 CAUSALITY AND ASSOCIATED MICROBES

Table 8.1 contains a list of PEM species that have been reported to cause skin infections:

Table 8.1 Classification of PEM species that have been reported to cause skin infections (Based on the criteria of Runyon*)

Class	Species name
Photochromogens	*M. marinum*
	M. kansasii
Scotochromogens	*M. scrofulaceum*
	M. szulgai
Non-photochromogens	*M. avium*
	M. intracellulare
	M. ulcerans
	M. haemophilum
	M. terrae complex
	M. xenopi
Rapidly growing species	*M. abscessus*
	M. chelonae
	M. fortuitum
	M. smegmatis

*Runyon criteria: Rapid growers grow in seven days or less. Scotochromogens produce pigment when grown in the dark as well as upon exposure to light. Photochromogens produce pigment when exposed to light but not when grown in the dark. Nonchromogens do not produce pigment even after light exposure.

8.7 KEY RESEARCH ISSUES

In common with other mycobacterial infections, it will be important to systematically study the epidemiology and causative microbes. To achieve this, standard case definitions and accurate microbial identification should be emphasized. Use of new molecular tools for species identification and for strain typing of clinical and environmental isolates to understand the epidemiology is important. The prognostic value of *in vitro* antibiotic susceptibility tests for clinical outcome needs to be determined for these and other PEM infections. Given the small numbers of cases in single centres, multicentre studies will be necessary. Since therapy is toxic and expensive, determining the proper agents and duration of therapy will be important. Encouraging pharmaceutical companies to develop better antimycobacterial therapy is important. Studies are required into the role of topical therapy and the use of antibiotic-impregnated polymethacrylate beads and cement placed intraoperatively as a means of attaining a higher local concentration of the antibiotic. If newer potent agents were developed it may lessen the need for such long-term courses of therapy. Elucidation of the reservoirs for *M. haemophilum* and other species will be illustrative. Study of the climatic and other environmental changes that may predispose to favourable conditions allowing these emerging pathogens to exist is an important avenue of future research. Additional studies of unique species-specific virulence factors, host tropism, environmental niches and modes of transmission will be a fruitful area of investigation with far-reaching benefits for patients.

9

Pulmonary infection in non-HIV infected individuals

M.A. De Groote

PEM are an important cause of pulmonary disease and the incidence is increasing in areas around the globe. With certain species the disease may have latitudinal preferences (refer to section 9.3). Although the clinical manifestation of pulmonary infection with PEM can resemble TB, the disease is very different because the host acquires infection by exposure to environment sources of these organisms (e.g. the water and soil). In contrast, disease due to MTB is predominantly spread person to person. Thus TB will not be discussed here except when it is pertinent to understanding certain host predisposing conditions for PEM infection and as it relates to situations of prior exposures and cross-immunity.

The causative microbial agents for PEM disease are numerous. The majority of the description in this chapter will deal with infection due to members of the MAC, given the preponderance of this organism causing disease. Unless

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otherwise specified, MAC is the prototypical infection referred to in the text. However, when appropriate, other species will be highlighted.

Clinically, PEM infection can range from asymptomatic, indolent disease with minimal clinical symptoms to rapidly destructive pneumonic disease with significant morbidity and mortality. Therapy for these infections is difficult and often associated with toxicity and expense. For reasons that are not understood, extrapulmonary dissemination is strikingly rare when the systemic immune system is intact, despite very advanced pulmonary disease. Because PEM are ubiquitous in the environment, actual pulmonary disease needs to be differentiated from colonization or contamination in the laboratory. Finding environmental mycobacteria in respiratory secretions requires supportive clinical (symptoms, signs and radiographic evidence of disease) and microbiological evidence of infection before treatment is commenced. Practically, the decision to treat is sometimes very difficult and requires a mutual understanding between the patient and the responsible physician. Occasionally, when complex situations arise due to diagnostic or therapeutic uncertainties, the patient can be referred to an institution with experience in treating these infections. In this chapter, data will be presented detailing the geographical variation in environmental mycobacterial infection and potential explanations for these observations will be discussed. This topic is also dealt with in the chapters 2 and 3. For the interested reader, there have been a number of excellent reviews on the subject of pulmonary environmental mycobacterial infections (Griffith 1997, 2002; Iseman 2002).

9.1 CLINICAL ASPECTS

Acquisition of pulmonary infection most likely occurs by the aerosol route. Given the prevalence of PEM in the environment, exposure must be fairly universal. The steps that follow and the determinants of in whom disease takes hold are not completely understood. After disease develops, the symptoms can include cough, sputum production, fatigue, weight loss, sweats, haemoptysis, pleuritic and non-pleuritic chest pain. Occasionally symptoms are out of proportion to the amount of disease seen radiographically. For example, patients with significant disease noted on chest radiograph can be surprisingly asymptomatic or those with minimal changes can have debilitating symptoms. Fever is unusual unless bacterial superinfection occurs; however, the presence of another pathogenic organism in the sputum does not always predict the presence of fever. Often it is difficult to sort out symptoms due to the underlying lung diseases (e.g. dyspnea associated with emphysema or sputum production from underlying bronchiectasis) from those due to the underlying mycobacterial infection.

Confirmation of PEM in respiratory secretions is essential before committing the patient to therapy. If sputum specimens are negative, many clinicians will go on to bronchoscopy for diagnosis. Failure to recognize infection and disease and thus withholding therapy may result in unnecessary lung damage; nevertheless, pulmonary disease needs to be differentiated from colonization, which does not require immediate therapy. *M. kansasii* is thought less likely to be a saprophyte when it is found in patients' respiratory secretions than certain other species, although colonization can occur on rare occasions. In 1997, the American Thoracic Society published criteria for diagnosis and treatment of disease caused by the nontuberculous mycobacteria. These criteria are thought to be best suited for MAC, *M. kansasii* and *M. abscessus*. If a patient presents with the appropriate clinical symptoms and radiographs, one of the following criteria must be met for a microscopic diagnosis to be made (American Thoracic Society 1997):

A If 3 sputums/bronchial washings are available in last 12 months:
- a) 3 positive cultures with negative smears or
- b) 2 positive cultures and one positive smear

B If only 1 bronchial wash available:
- a) positive culture with 2+, 3+, or 4+ AFB smear or 2+, 3+, or 4+ growth on solid media

C If sputum/bronchial wash evaluations are nondiagnostic or another disease cannot be excluded:
- a) transbronchial or lung biopsy yielding PEM
- b) biopsy showing mycobacterial histopathologic features (granulomatous inflammation and/or AFB) and one or more sputums or bronchial washings is positive for an NTM even in low numbers.

Expert opinion has varied regarding the usefulness of the above guideline but it is included here given the important role these criteria have played over the last few years by providing a standardized mechanism of diagnosis. In addition, the emphasis on the crucial role of a strong microbiological diagnosis is key to management of these patients. Once a diagnosis is made, the clinician may continue to be challenged by many facets of management of these patients. Respiratory secretions from these patients can harbour a complex suite of organisms such as mixed mycobacterial species, fungi and other co-pathogenic bacteria. This, too, can make appropriate antibiotic selection more difficult as

one tries to decide which organism is contributing the most towards pathogenicity.

The natural history of PEM lung disease is quite variable. There are two prototypical descriptions of disease: an indolent (primary) form that tends to occur in older non-smoking females and a more traditional form, which is usually secondary to underlying structural lung disease. Interestingly, there seems to be a shift in disease presentation from the secondary to the primary form.

9.1.1 Primary and Secondary Pathogenic Pulmonary Environmental Mycobacterial Infection

The epidemiology of PEM appears to be shifting from being predominantly a disease of male smokers (usually with underlying emphysema) to one of non-smoking older females with no obvious underlying lung disease. Given this emerging appreciation of different subsets of patients with PEM disease, it is useful to consider the infection as one of primary vs secondary infection in an attempt to sort out the dynamic nature of PEM pulmonary diseases. PEM pulmonary disease will be discussed in this chapter on the basis of "primary" and "secondary" disease based on terminology first proposed by von Reyn, *et al.* (2001). For the purpose of this monograph, primary disease will refer to the absence of a recognized host predisposition for infection (i.e. no pre-existing lung disease). Recently, a distinct syndrome of primary disease presenting as nodular bronchiectasis has been recognized. In this setting, our current understanding is that the mycobacteria are a cause of the disease manifestations in previously healthy hosts. Secondary PEM disease, on the other hand, occurs in the setting of a well recognized lung abnormality where the PEM takes advantage of the host's pre-existing condition (i.e. it is an illustration of an opportunistic pathogen). Examples of secondary PEM infection include prior structural lung disease such as previous infection, emphysema and bronchiectasis. Secondary disease is relatively common, accounting for up to two thirds of disease in some reports. Regardless of whether PEM cause primary or secondary illness, there is little doubt that PEM can contribute to ongoing clinical illness and pulmonary dysfunction.

9.1.1.1 Primary PEM

In 1989 Prince and co-workers reported a subset of patients with pulmonary PEM disease who had no underlying predisposition (Prince *et al.* 1989). They found that 81% were female, 86% were white and there was a mean age of 66 years. Since the original work by Prince *et al.*, others have described the occurrence of PEM in hosts without obvious predisposing conditions

(Chalermskulrat *et al.* 2002). The patients, similar to the original report, are predominantly female non-smokers, and represent what appears to be a shift from the predominantly male dominated cases of secondary infection presented below (Rosenzweig 1979). These patients do not have obvious abnormalities in lung or immune function. The overwhelming majority are white (or Asian) women who present with nodular-bronchiectatic PEM disease (Reich & Johnson 1992; Kennedy & Weber 1994; Kubo *et al.* 1998; De Groote *et al.* 2001). In fact, the range of female patients in recent studies is between 75-94%. The term Lady Windermere's syndrome has been given to these patients based on the character in Oscar Wilde's play *Lady Windermere's Fan* as her voluntary cough suppression behaviour is considered to be the etiology behind not being able to clear secretions properly (Reich & Johnson 1992). However, this designation has been called into question (Iseman 1996). Whatever the etiology for this process, many experts and reports suggest that there has been a shift to female-predominant primary PEM disease. Most present in the fifth to eighth decade of life. Nodular bronchiectasis is the major clinical presentation in primary PEM disease. Whatever the reasons for this shift, an understanding of the clinical presentation and the gender-specific pathogenesis will be important. Classification of primary disease is based on the exclusion of underlying disorders as we understand them today. As we learn more about these putative predispositions, we may need to re-define these categories.

Patients with the nodular bronchiectatic primary PEM disease typically present with chronic cough and none, some, or all of the following: fever, fatigue, sweats, weight loss, dyspnea, hemoptysis and chest pain. Time from onset of symptoms to diagnosis can be weeks to years. Chest radiographs demonstrate nodular infiltrates and cylindrical bronchiectasis (Aksamit 2002). The characteristic high resolution computed tomography findings are bronchiectasis and ill-defined small nodules that are centrilobular in distribution. The typical description is "tree-in-bud" appearance, which is felt to represent an inflamed bronchiolar wall with fluid (i.e. mucus and inflammatory cells) within the lumen. Radiographic studies indicate that while disease can involve multiple segments of the lung, a very suggestive pattern consists of disease located in the mid-lung (right middle lobe and lingula) zones (Chalermskulrat *et al.* 2002). The computed tomography scan adds considerable information in the diagnosis of pulmonary PEM disease (Figure 9.1). Studies have shown airflow limitation as well as high residual volumes. Described together they represent distal air trapping in the small airways (Kubo *et al.* 1998). Up to 50% of patients can have normal results on pulmonary function testing (Prince *et al.* 1989; Aksamit 2002;). The disease can be fatal. In primary disease described by Prince and co-workers, death occurred in four patients due to progressive uncontrolled pulmonary infection. Relapse is common after

therapy is stopped. Left untreated the nodular bronchiectatic disease can go on to cavitate and cause severe lung destruction. A certain body phenotype has been described consisting of mitral valve prolapse and pectus excavatum. The patients are often thin and have an abnormal narrowing of the anterior-posterior diameter (Iseman *et al.* 1991). Sufficient data supporting a unifying explanation of a "predisposing phenotype" is currently lacking but is an area of interest for future study. Considerations of the role of bacterial virulence, previous exposure to antimicrobials, immunosenescence, host genetic and environmental factors influencing disease are fruitful areas of future research.

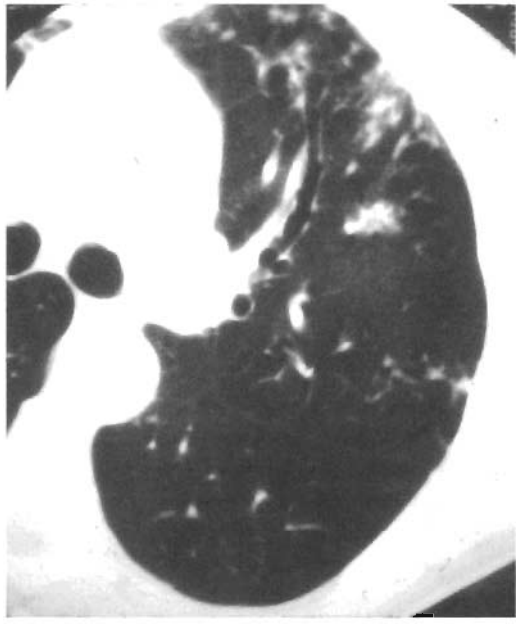


Fig. 9.1 High resolution computed tomography scan of a patient with PEM lung disease demonstrating lingular bronchiectasis, nodules, and infiltrates.

9.1.1.2 Secondary PEM

Unlike primary PEM patients, secondary cases do have some pre-existing lung disease to put them at risk for infection (Aksamit 2002). Secondary PEM disorders can be associated with CF, emphysema, previous TB and histoplasmosis, radiation therapy to the lung, silicosis and other inorganic dust pneumoconiosis, chronic aspiration, malignancies, etc. (refer also to section 9.6). It appears that regional factors in areas of lung damage predispose to

infection. It is postulated that abnormal blood flow, altered anatomy and local impairment of host defences may contribute. Many of these principal disorders result in fibrosis and scarring that is felt to become colonized/infected with PEM. In these cases, disease occurs at the site of previous damage. In smoking-related emphysema, disease is usually in the upper lung zones in areas of pre-existing bullae and can present with fibrocavitary disease. The symptoms can include cough, sputum production, fatigue, weight loss, sweats, hemoptysis, pleuritic and non-pleuritic chest pain. Fever is unusual unless secondary bacterial infection occurs. Often it is difficult to differentiate between symptoms due to underlying lung diseases (i.e. dyspnea associated with emphysema) and those of the superimposed mycobacterial infection. The radiographic manifestations can also include cavities, pleural thickening, nodular infiltrates, consolidation and various forms of bronchiectasis. Since nodules can also signify malignancy (to which smokers are predisposed) it is important to discriminate infection from malignancy. In emphysema related PEM disease, there is a predominance of male smokers. It remains to be seen if the rise in female smoking incidence will be reflected in increased secondary PEM in this group in the future. The patients with secondary PEM pulmonary infection are also in the older age range (sixth to eighth decade). The patients often have grossly abnormal measures of lung function (Aksamit 2002). The patients with secondary PEM tend to have cavities that are thinner walled when compared to those of TB (Prince *et al.* 1989).

Of particular interest is the association of PEM and CF. It is felt that the very viscous respiratory secretions of this disease contribute to recurrent pulmonary infections. Prior to the 1990s, the recovery of PEM from sputum of patients with CF was rare. Longevity into the third and fourth decades due to better antibiotics, inhaled medications, nutrition and improved sputum clearance techniques has occurred. With time there has been a rise in the incidence of other important pathogens including drug resistant *Staphylococcus aureus, Burkholderia cepacia, Achromobacter xylosoxidans, Stenotrophomonas maltophilia* and PEM. Olivier *et al.* (2002) found a prevalence of PEM of 13% in the sputum of CF patients. Of these, MAC accounted for 75% of PEM isolates. However, the contribution to respiratory decline in CF patients is disputed. With a follow up of 15 months they found no effect on lung function, but high resolution computed tomography scan of the chest abnormalities suggestive of infection with the PEM was predictive of disease progression. In a large study of multiple CF centres, patients who were infected with the PEM showed no significant differences in gender or frequency of pancreatic enzyme use. Only 37% of the patients met the American Thoracic Society microbiological criteria for disease. Those harbouring an *M. abscessus* were more likely to meet the criteria. Those with PEM were more likely to have

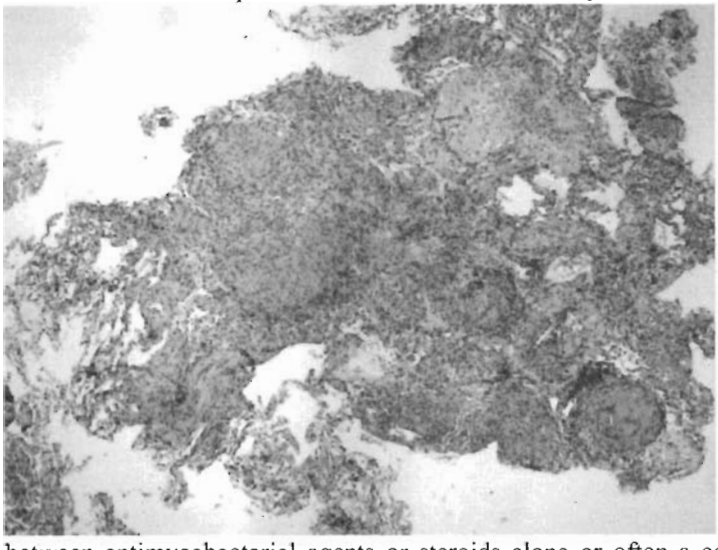
S. aureus infection and have a higher prevalence of *Aspergillus* co-infection. The clinical significance of infection is incompletely defined. There are reports substantiating the important pathogenic role of PEM when found in CF secretions. Autopsy studies have demonstrated granulomas and caseating necrosis felt to contribute to a patient's demise (Boxerbaum 1980). The clinical and radiographic manifestations of PEM in CF patients are similar to those in other cases of pulmonary disease. Chest computed tomography scans demonstrate bronchiectasis as well as cysts (or cavities) consolidated areas and peripheral nodules (including in a tree-in-bud pattern). However, these patients often have another infection (e.g. *Pseudomona aeruginosa*) that can cause the same symptoms. Experts in the field recommend that multiple positive sputum cultures for the same organism and consistent symptoms and worsening spirometry as well as traditional radiographic findings be present before consideration for treatment (Olivier *et al.* 1996, 2001; Olivier 1998; Ebert & Olivier 2002). In rare circumstances where the patient is stable, a period of close clinical observation can be tried with a low threshold to begin therapy if any parameters change. Given the severity of the disease due to *M. abscessus* and the therapeutic difficulties, these patients are likely to require therapy without delay. The most common isolate in nearly all studies of CF patients is MAC but *M. kansasii* and members of the rapidly growing species (particularly *M. abscessus*) have also been reported. Since infection with the PEM is an indolent process, it is likely that with longer follow up significant clinical impact of infection will be demonstrated.

9.1.1.3 Hypersensitivity pneumonitis

Another form of PEM disease which has been recently recognized is the phenomenon of hypersensitivity pneumonitis seen in immunocompetent people with aerosol exposure to mycobacteria. This form does not have any known gender bias. Typically it is linked to hot tubs and indoor swimming pools and particularly strikes healthy subjects such as lifeguards and those who use these aquatic sources for recreation or work (Embil *et al.* 1997; Mangione *et al.* 2001; Mery & Horan 2002). Aerosols are felt to be the major route of infection in hypersensitivity pneumonitis. Likely due to their hydrophobic nature, these organisms are found in greater quantities in the air above the pool than in the pool water. High hydrophobicity can lead to adherence to surfaces. *M. avium* can also adsorb to air bubbles in water resulting in high concentrations of the organism at the air water interface (discussed in Chapters 2 and 3).

The precise pathogenesis is not completely understood, but investigators have attributed the pathology to both a component of bacterial infection and host immune response to mycobacterial antigens. A relatively distinct clinical

syndrome occurs after inhalation. Symptoms include cough, dyspnea, fatigue, impaired exercise tolerance and sputum production. The chest radiograph and computed tomography scans demonstrate hazy or ground glass opacities and peripheral small nodules often with a tree-in-bud pattern (Rickman *et al.* 2002). The AFB smear from sputum is insensitive but the culture is more sensitive (Khoor *et al.* 2001). MAC is the most common offender but other species have been implicated. Bronchiolitis obliterans and other features of hypersensitivity, including non-necrotizing granuloma, are seen on pathology (Figure 9.2). Discontinuation of exposure to the source is mandatory. Treatments have varied



between antimycobacterial agents or steroids alone or often a combination of both (Khoor *et al.* 2001). Discontinuation of hot tub use has led to prompt improvement in symptoms, pulmonary function and radiographic abnormalities without the use of antimycobacterial agents (Rickman *et al.* 2002). Hypersensitivity pneumonitis secondary to PEM as an occupational lung disorder has been well described and causes a similar syndrome. It has been seen most often in individuals using metal working fluids that are contaminated with mycobacteria (Hodgson *et al.* 2001; Wallace *et al.* 2002a).

Fig. 9.2 Histopathology of a patient with hypersensitivity pneumonitis. Intense inflammation, bronchiolitis obliterans and non-caseating granulomatous infiltrates are seen.

9.1.2 Selected treatment issues

As mentioned above, the criteria for diagnosis of disease usually include clinical signs and symptoms, sputum mycobacteriology and radiographic studies. Failure to recognize infection and disease and thus withholding therapy may result in unnecessary lung damage. Likewise, giving therapy when no evidence of disease is present only puts the patient at risk from unnecessary toxicity. The 1997 American Thoracic Society guidelines on diagnosis and therapy are a useful starting point. Our understanding of the optimal considerations for treatment continues to evolve.

In the era of the newer macrolides such as clarithromycin and azithromycin, greater treatment success is now achievable. Macrolides have the most predictable *in vitro* activity against MAC and have added a great deal to the treatment of infection (Griffith *et al.* 1996; Wallace *et al.* 1996). A typical course of antibiotics includes three or four drugs for many months. Preferred agents include clarithromycin (or azithromycin), rifampin (or rifabutin), ethambutol and possibly an aminoglycoside for a short period of time at the beginning of therapy. Thrice weekly regimens have been studied and are effective (Griffith *et al.* 1998, 2000). For previously untreated PEM disease due to MAC, a regimen of clarithromycin or azithromycin, rifampin, ethambutol and amikacin is often recommended. The amikacin is given for a period of two to three months with careful attention to renal, audio and vestibular toxicity. Toxicities with rifampin include orange discoloration of the secretions and urine, staining of contact lenses, nausea, vomiting, hypersensitivity syndrome (fever, rash), hepatitis, leukopenia, flu-like illness, thrombocytopenia, drug-induced lupus and renal failure. In addition, multiple drug interactions can occur manifested by increased hepatic metabolism of numerous agents by induction of the cytochrome P450 system. Rifampin combined with clarithromycin results in less bioavailability of clarithromycin. Rifabutin has similar toxicities to rifampin and can also cause polymyalgia and polyarthritis. Rifabutin has somewhat less effect on the P450 system. Clarithromycin can cause toxic accumulations of rifabutin due to its ability to inhibit the elimination of rifabutin (which can be associated with uveitis). Ethambutol may cause optic neuritis (loss of red/green colour discrimination and loss of visual acuity) and rash. This side effect is often dose related. Given the complexity of therapy, consultation with experts in the field should be considered.

With the exception of clarithromycin, the role and the predictability of *in vitro* susceptibility testing of MAC is an area of controversy (Iseman 2002). In contrast to TB, where susceptibility testing has an undisputed role in management of disease, in PEM disease it is somewhat debatable. The American Thoracic Society statement recommends against testing of agents other than clarithromycin. A study done at the National Jewish Medical and Research Center found that there

was a correlation between treatment response (as measured by consecutive sputum culture negativity for three months) and the number of antimycobacterial drugs that the patient was treated with that had demonstrable *in vitro* susceptibility (Iseman 2002). If a patient has had prior therapy with a macrolide or is failing therapy, *in vitro* susceptibility testing for clarithromycin (at a minimum) should be performed. Testing of rifampin susceptibility for *M. kansasii* and a panel of antimicrobial agents for other mycobacteria, such as the RGM, is recommended (American Thoracic Society 1997). The recommendations for duration of therapy are based on a high rate of relapse if treatment is terminated early (Wallace *et al.* 1996). Anywhere from 12-24 months is the usual suggested duration of treatment, although shorter courses may yet be possible using macrolide containing regimens. Additional details and other variations of these recommendations based on unique underlying patient characteristics can be found in the review by Iseman (Iseman 2002).

Special considerations exist in the diagnosis and management of *M. kansasii* disease. This subject has been extensively reviewed (Griffith 2002). *M. abscessus* may be the most difficult pathogen to treat in patients with and without CF. It is difficult to render patients with this infection culture negative despite the apparent *in vivo* susceptibility of the isolates in the laboratory. It is possible that this has to do with an enhanced resistant state (i.e. a biofilm mode of growth) in the host compared to the planktonic culture used for *in vitro* susceptibility testing (Bardouniotis *et al.* 2003). Treatment recommendations for *M. abscessus* includes amikacin, cefoxitin and usually a third agent such as clarithromycin is added. The duration of intravenous therapy is typically six to eight weeks with a period of longer-term suppressive therapy. Patients with CF have altered drug absorption and pharmacokinetics. Consideration should be given to measuring serum antimicrobial concentrations for patients with and without CF, which may dictate altering drug dosages to avoid toxicity and achieve therapeutic success. Serum concentrations for most of the antimycobacterial agents can be obtained from the Pharmacokinetic Laboratory at the National Jewish Medical and Research Center (+1-303-398-2603). A multicentre treatment trial involving inhaled interferon-gamma is nearing completion and results should soon be available.

Surgical therapy continues to play a role in the management of these patients; however, it is largely reserved for patients who have failed medical therapy, or for those with severe symptomatic disease. When present, localized disease lends itself best to surgical intervention. Predictably, those that have poor preoperative lung function do less well. Complications arising from treatment, such as bronchopleural fistulas, etc., do occur (Iseman *et al.* 1985; Pomerantz *et al.* 1991, 1996; Nelson *et al.* 1998; Shiraishi *et al.* 2002): those that undergo pneumonectomy (especially for right-sided disease) seem to have a higher risk of severe postoperative complications (Pomerantz *et al.* 1991).

9.1.3 Selected microbiological issues

In the clinical context, microbiological data is the foundation of a proper diagnosis. Specimen processing in a laboratory capable of performing up-to-date and timely identification and susceptibility testing is important (refer to 9.4). Isolation of PEM from the sputum of patients with CF is often difficult due to the common finding of overgrowth of more rapidly growing bacteria and fungal species. In order to enhance recovery of PEM, oxalic acid is sometimes added to the decontamination step in specimen processing and can increase the yield of PEM (Bange & Bottger 2002). If a high index of suspicion exists, multiple specimens should be submitted to the laboratory for analysis.

Advances in laboratory methodologies have enabled more rapid and reliable differentiation of mycobacterial species. Also, new and emerging species have recently been described that cause pulmonary disease. Additional insights into PEM infections are gleaned from applying molecular typing tools. In a study of pulmonary nodular bronchiectasis infection involving *M. intracellulare* isolates that had the same colony morphology on the plate, PFGE demonstrated multiple different fingerprints (i.e. a polyclonal infection) (Wallace *et al.* 1998a). In patients with secondary PEM infection, especially disease due to underlying emphysema with fibrocavitary disease, there was often only a single isolate over time. If therapy with a macrolide was continued long enough in those with nodular bronchiectatic disease and relapse, the relapse strain was macrolide susceptible and represented acquisition of a new strain (Wallace *et al.* 2002). Considering the relatively high numbers and wide diversity of *M. avium* and *M. intracellulare* in the environment (i.e. drinking-water and soil), it is not surprising that some patients would be infected by more than a single strain.

9.2 OVERALL BURDEN OF DISEASE

The precise incidence of PEM is difficult to ascertain. MAC is estimated to have a range of infection of 1-2.5/100 000 population and is rising (Marras & Daley 2002). The upward trend is not due exclusively to AIDS, because PEM occurred before and independently of the appearance of HIV. There are multiple potential reasons for an increase in PEM disease. These include, but are not limited to: 1) more sputum cultures are being ordered; 2) more computed tomography lung scans are being ordered and when the classic radiographic pattern is seen (i.e. bronchiectasis, tree and bud), cultures are more likely to be requested; 3) the use of more liquid media resulting in higher recovery rates compared to the use of Lowenstein-Jensen agar media. The organisms most commonly implicated in human infections are MAC, *M. kansasii*, and the RGM especially *M. abscessus*. In some regions, *M. kansasii* is one of the most common PEM isolated. It has

annual infection rates of 0.5-1/100 000 population. In certain areas it has higher rates (i.e. 2.4-17.6 per 100 000). It occurs in geographic clusters and affects primarily white men but it can occur in any race or at any age.

9.3 DISTRIBUTION

PEM lung disease has been reported from all over the globe. MAC has been most commonly studied and is found in nearly all regions of the world where it has been looked for. However, it has been found in the primary disease form particularly in patients from the south-eastern United States coastal states of the gulf (M. Iseman, G. Huitt, personal communication). In a study done by the CDC, the rates of MAC isolation tended to be highest in states bordering the Atlantic ocean and the Gulf of Mexico (> 4.8/100 000) (Good & Snider 1982). *M. kansasii* tends to occur in an inverted "T" in the United States with a broad area of disease in Texas, Oklahoma and up into the Midwest. *M. xenopi* is an endemic problem in Britain and France; however, sporadic cases have been described elsewhere, including in the United States. *M. malmoense* is also more prevalent in Europe. Other slowly growing mycobacteria, including *M. simiae,* appear to be on the rise as well. The distribution of disease due to this species and to some of the rarer species is unknown.

9.4 DESCRIPTIVE EPIDEMIOLOGY

The epidemiological study of pulmonary infections is challenging. Growth of a PEM in culture can result from contamination in the laboratory or from any point in the process of specimen collection. In order to reliably discern the incidence and prevalence of disease associated with each isolate, clinical data is required to understand more comprehensively the true incidence of disease. In contrast to infection caused by *M. tuberculosis*, disease due to the environmental mycobacteria is not reportable in the United States. Thus data is not available to ascertain the precise incidence and prevalence.

In the early 1980s O'Brien *et al* (1987) carried out a survey of the state and city health departments. Disease due to the PEM occurred in 1.8/100 000. Limitations of the study include incomplete sampling from across the population of the United States and possible under-recognition of the nodular bronchiectatic form of disease at the time the survey was done. However, other national surveys, such as one performed in Switzerland, showed fairly consistent profiles of disease prevalence of around 1/100 000 population (Debrunner *et al.* 1992). A superb analysis of the world's literature has recently been published (Marras & Daley 2002). The authors have analysed data where laboratory based surveys also contained clinical data allowing some

ascertainment of presence or absence of disease. Nearly all studies performed in Europe, North America, Asia, Africa and Australia found a rising proportion of potentially pathogenic mycobacteria in laboratory-based surveys. For instance, in one province of British Columbia the annual incidence of PEM pulmonary disease rose at an exponential rate (from 0.08/100 000 in 1960 to 0.6/100 000 in 1980). In Massachusetts in 1972 non-*M. tuberculosis* species accounted for 12% of mycobacterial isolations; by 1983 they accounted for 70%. The incidence rates in recent decades range from 1.7-4.5 per 100 000 people. In Europe, reports of disease rates range from 0.3-18/100 000 people. African, Asian and Australian studies have shown a rate between 0.3-78/100 000 in certain areas. In all regions, most reports would suggest that concomitant with the decline in TB, the incidence of PEM has increased.

9.5 CAUSALITY AND ASSOCIATED MICROBES

It is important to accurately identify the organism to ensure proper diagnosis and to make proper treatment selections. Advances in laboratory methodologies have enabled more rapid and reliable differentiation of mycobacterial species and new species with the potential to cause pulmonary disease are being described all the time. In the United States MAC was most commonly seen followed by *M. kansasii* (20%) and rapid growers (10%) (Marras & Daley 2002). MAC includes *M. avium* and *M. intracellulare.* These two species are indistinguishable using routine laboratory criteria such as biochemical testing and morphology. Newer methods based on sequencing and chromatographic patterns of mycolic acids are employed in reference labs. Molecular tools have allowed distinction on the basis of 16SrRNA sequences using commercially prepared kits (GenProbe, Accuprobe). Each species should be described separately as they are likely to have different biologies in the environment, unique antibiotic susceptibilities and particular virulence characteristics. Recently, by sequencing the *hsp65* gene of representative isolates, it was shown that there are unique pathogenic capabilities of human isolates compared to the environmental strains (Smole *et al.* 2002).

9.6 RISK FACTORS

Risk factor analysis needs to consider host factors, microbial factors and environmental factors.

While the precise details are not well worked out, it is generally believed that patients acquire mycobacteria from their environment, including waters and soils. The number of mycobacteria isolated from drinking-water can be very high: up to 100 000 cfu/cm^2 can be found in water distribution systems (refer to Chapter 3). Since the combined length of water pipes in an average distribution system can be hundreds

of miles, the potential human exposure is immense. In addition, the mycobacteria are relatively resistant to chlorination, which may explain their success and persistence in drinking-water.

Pulmonary infections disproportionately affect the elderly. Some of the areas with the highest incidence of PEM disease are also the areas with the greatest proportion of older adults (data from the United States Census Bureau). The proportion of people over 65 years of age was 1 in 20 in 1930, it increased in the mid eighties to 1 in 10, and projections for 2020 are that 1 in 5 individuals will be 65 years and older. With an ageing population, we may see more pulmonary PEM disease. It may also be that the current trend in some countries for elderly people to retire to warm, moist environments may put these people at risk. Additional risk factors include those with underlying host conditions such as patients with CF and smoking-related emphysema. These and additional risk factors for pulmonary disease are listed in Table 9.1.

Table 9.1 Risk factors for PEM disease (adapted from De Groote & Iseman 2003)

Traditional lung disorders	Heritable Conditions	Body Habitus	Prior lung infections	Aspiration
emphysema	CF	pectus excavatum	TB	gastro-oesophageal reflux
dust pneumoconiosis	disordered ciliary motility	scoliosis	histoplasmosis	achalasia
fibrosis, ankylosing spondylitis, rheumatoid arthritis	tracheo-broncheomegaly	slender body type	coccidiodo-mycosis	swallowing disorders
radiation lung injury, alveolar proteinosis, pulmonary embolism	alpha-1 anti-trypsin disorders	mitral valve prolapse	aspergillosis	mineral oil ingestion

Host defences against mycobacterial infections include intact epithelial surfaces, the mucociliary lining of the respiratory tract, neutrophils, macrophages and lymphocytes. Factors that negatively affect any of these defences predispose patients to mycobacterial infections.

9.7 PREVALENCE OF ASYMPTOMATIC DISEASE

Most of the data regarding asymptomatic disease comes from skin test surveys. This test depends on the development of a delayed hypersensitivity to skin test

antigens derived from PPD of mycobacteria. In two separate and large studies, a consistent and striking epidemiological finding was the geographical differences in exposure, with particular high rates in the south-east United States (Marras & Daley 2002). The first used the PPD of *M. tuberculosis* at low and high doses (5 and 250 TU). A positive response to the 250 TU skin test was felt to be related to exposure to antigenically similar mycobacterial species. Those subjects whose residence was in zones encompassing Texas, North and South Carolina, Georgia, Florida, Tennessee, Alabama, Mississippi, Louisiana, Arkansas, Georgia and Oklahoma had the highest degree of reactivity. Similarly, skin test surveys done in the 1960s using a battery of skin test antigens including the Battey bacillus (MAC) revealed a higher percentage of reactivity in Navy recruits from the south-eastern United States. Von Reyn *et al.* (2001) have recently shown a high incidence of skin test positivity in the United States with higher rates among adults in the southern states. Limitations in interpreting this data related to asymptomatic disease include absence of supportive clinical data to rule out active disease. Both large studies were done on presumably healthy young people, so this is likely just a theoretical concern. Secondly, there is non-specificity in the antigenic response to antigens in these preparations and cross reactivity can occur. Cross immune protection to MTB and other environmental mycobacteria probably occurs. Newer tests using gamma interferon production by whole blood lymphocytes may yield additional discriminative information.

9.8 KEY RESEARCH ISSUES

There are a number of research priorities to be considered in dealing with the issue of PEM pulmonary infections. Some of these are listed below:

- design prospective, safe, multicentre controlled studies of natural history, risk factors, treatments and predictors of outcome;
- develop and standardize methods for unique identification to the species level and study microbial correlates to pathogenesis and outcomes;
- define the nature of exposure in the environment to advise high-risk individuals how to avoid exposure;
- define the role of pharmacokinetic and *in vitro* susceptibility studies in the routine management of PEM.

10

Disease resulting from contaminated equipment and invasive procedures

M.A. De Groote

As ubiquitous inhabitants of the environment and frequent colonizers/contaminants in nosocomial settings, PEM are an important and emerging cause of human disease. The persistence of these organisms in municipal and hospital water supplies coupled with their intrinsic resistance to disinfection is the likely explanation for these cases (Falkinham 2002). Procedures that place people at risk of infection are commonly performed every day in large numbers in health care and cosmetic settings throughout the world. Fortunately, rates of acquiring infection are relatively low, but laboratories and clinicians will occasionally be faced with such patients. Due to the ubiquity of the organisms in the environment, microbiological specimens can become contaminated. It is sometimes difficult to determine accurately if the isolation of mycobacteria is associated with disease, or if it is an accidental

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contaminant. In addition to these unique individual patient considerations, such cases could also herald a "real" or "pseudo-outbreak" resulting from a shared contaminated device or solution. Laboratory workers, infection control practitioners, clinicians, and occasionally even public health personnel play important roles in the evaluation of each case.

This chapter outlines exposures resulting from contaminated devices and invasive procedures and gives representative examples of each. Selected aspects of the clinical presentation, treatments and outcomes will be highlighted. While infections due to various members of the PEM are addressed, special attention is given to the rapidly growing species due to the central role these organisms play in the etiology of device-related infection. In some cases there is overlap with the data presented in the accompanying skin infection chapter in this book and the reader is referred to this chapter for complementary information (Chapter 8). Finally, there are several excellent recent reviews on nosocomial outbreaks/pseudo-outbreaks that have been published recently (Fraser 1981; Wallace *et al.* 1998; Phillips & von Reyn 2001).

10.1 CLINICAL ASPECTS

10.1.1 General Comments

Though the problem has been recognized for decades, the number of reports of pathogenic mycobacterial disease caused by the use of contaminated devices or from an invasive procedure has been increasing. Some of the highest rates of potable water contamination are found in hospitals, dental offices and hemodialysis units (Carson *et al.* 1988; Schulze-Röbbecke *et al.* 1995; Fujita *et al.* 2002).

Difficulty can arise in making a diagnosis due to the indolent nature of the clinical presentation and a long incubation period. Because the original exposure may have occurred some time before clinical presentation, heightened clinical suspicion is often needed to make a proper diagnosis. Once considered, the organisms are usually not difficult to cultivate in the laboratory.

When a microbiological specimen yields PEM, it is important to discriminate between colonization, true infection and pseudo-infection (Phillips & von Reyn 2001). Colonization indicates the presence of the organism within or on the patient but, by definition, occurs in the absence of any identifiable disease. Pseudo-infection results when mycobacteria are isolated in culture from a clinical specimen as a result of contamination. In pseudo-infection, there is no evidence of infection or disease. Pseudo-infections can represent an isolated event or a pseudo-outbreak involving many people. These scenarios are summarized in Table 10.1.

Table 10.1 Potential situations associated with PEM isolation from a clinical specimen (Adapted from Phillips & von Reyn 2001)

Situation	Definition
Colonization	Isolation of potentially pathogenic EM without signs or symptoms of disease attributed to the organism.
Infection/disease	Clinical evidence of infection attributed to the organism.
Pseudo-infection	No evidence of infection or colonization and isolation in the laboratory has resulted from contamination of the environment (can occur at any point: from a contaminated device, in obtaining the specimen, or up through the final cultivation in the laboratory).

These scenarios are important to identify because the patient(s) could be spared unnecessary antimicrobial agents if a "pseudo-infection" is present. Examples of pseudo-outbreaks include contaminated endoscopes and bronchoscopes as well as other types of instrumentation (CDC 1991; Canadian Disease Weekly 1991; Maloney *et al.* 1994; Griffiths *et al.* 1997). Bronchoscopic contamination is a common source and can occur due to inadequate cleaning and disinfection of the scope or by contaminated solutions or water used in the process (Sniadack *et al.* 1993). The organisms most commonly involved in bronchoscopic pseudo-outbreaks include *M. abscessus, M. fortuitum,* MAC (both *M. avium* and *M. intracellulare*), *M. gordonae* and *M. xenopi*. Automated bronchoscope disinfecting machines can become heavily contaminated with mycobacteria that are resistant to normal disinfection procedures (Wallace *et al.* 1998). Bronchoscopes contain components that are small and difficult to sterilize. For example, contamination of the suction valve (an area of the scope that is not easily accessible) has been documented (Wheeler *et al.* 1989). There exists potential for biofilm growth in many areas of the scope including the small lumens and multiple ports. In addition to pseudo-infection, there is a theoretical risk of true infection upon exposure to a contaminated instrument especially if a patient is immunocompromised or has a significant underlying lung disease (Prakash 1993). In this situation the patient should be monitored carefully for signs of disease. Other cases of pseudo-outbreaks have been reported as a result of laboratory cross-contamination, contaminated microbiological equipment, media, supplements and tap water (Wallace *et al.* 1998). Laboratories and practitioners need to be aware of these possibilities and in the absence of a compatible clinical syndrome a pseudo-infection should be considered.

True infection/disease has occurred in many different types of clinical settings. While not meant to be comprehensive, some examples of disease due to PEM are shown in Table 10.2.

Table 10.2 A representative listing of mycobacterial species associated with infections in the nosocomial setting.

Type of infection	*Mycobacterial species*	Source
Venous catheter infection	*M. fortuitum* *M. abscessus* *M. chelonae* *M. avium*	Catheter or solutions
Pacemaker or automatic implantable cardiac defibrillator infection	*M. avium* *M. abscessus*	Device
Sternal wound infection	*M. fortuitum, M. abscessus*	Water/ice cardioplegic solution
Cardiac porcine valve infection	*M. chelonae*	Contamination during sterilization of valve
Abscess after adrenal cortex extract (ACE)	*M. abscessus*	Contaminated ACE (non-FDA approved)
Plastic surgery (facial blepharoplasty and augmentation mammoplasty) wound infection	*M. abscessus*	Contaminated instruments, or solutions (i.e. gentian violet)
Peritonitis in peritoneal dialysis patients	*M. abscessus*	Identical to water isolate by strain typing
Haemodialysis related infection	*M. abscessus and M. mucogenicum*	Water used in rinsing dialysis equipment
Otitis media	*M. abscessus*	Possible contaminated instruments or tap water
Discitis	*M. xenopi*	Hospital hot water

Some species are rarely associated with disease, such as the occurrence of *M. kansasii* and *M. gordonae* with peritoneal dialysis infections. Other less commonly isolated species include *M. smegmatis, M. neoaurm, M. gastri, M. genavense, M. szulgai* and *M. immunogenicum.* Excellent reviews of this subject are available (Wallace *et al.* 1998; Phillips & von Reyn 2001).

10.1.2 Specific infections

10.1.2.1 Infection of intravascular catheters, pacemakers

Vascular devices such as indwelling venous access lines and vascular shunts can become infected with both RGM and other species (Katz *et al.* 2000; Schinsky *et al.* 2000; Rodriguez-Gancedo *et al.* 2001; Bouza *et al.* 2002). Infections can result in skin and soft tissue involvement (including tunnel site infections) with or without bacteraemia. Symptoms include local manifestations of line infections such as erythema and occasionally drainage at the site. There may be fever and other signs and symptoms of bacteraemia. The usual treatment is to remove the device and administer appropriate antibiotics (typically for a period of 2-12 weeks or more). Pocket infections involving pacemakers have also been seen with PEM, with MAC and especially *M. abscessus* predominating (Amin *et al.* 1991; Katona *et al.* 1992; Cutay *et al.* 1998; Verghese *et al.* 1998).

10.1.2.2 Dialysis related infection

Dialysis related PEM infection has been reported in both intravascular and peritoneal mechanisms of renal replacement therapy. RGM species are the most commonly implicated. Contaminated aqueous solutions used to sterilize the re-usable dialysis filters have been involved in many cases. PEM representatives have been isolated from water supplies of haemodialysis centres (Carson *et al.* 1988). In 1982, 27/140 patients receiving haemodialysis developed infection after being exposed to mycobacteria in water used to prepare dialysis fluids (Bolan *et al.* 1985).

Peritonitis can occur in patients undergoing chronic ambulatory peritoneal dialysis (Band *et al.* 1982). In this setting it can involve the catheter insertion site, tunnelling tract and/or the peritoneum itself. MAC and the rapid growers are the most commonly isolated species (Band *et al.* 1982; Soriano *et al.* 1989; Lowry *et al.* 1990; Vera & Lew 1999). Patients with end stage renal disease from any cause are likely to be more prone to infection due to impaired lymphocyte and neutrophil activity in the face of uraemia.

Overall, NTM accounts for a relatively small number of peritonitis infections, but this may be an underestimate given the frequency of culture negative cases. In fact if routine cultures are negative one should consider the diagnosis of PEM peritonitis (Hakim *et al.* 1993). Symptoms are insidious and often involve fever, abdominal pain and cloudy dialysate fluids. There can be catheter dysfunction, nausea, vomiting, diarrhoea and weight loss. The white blood cell can be elevated and is often of neutrophilic predominance. Catheter removal improves the rate of cure and antibiotics are necessary to prevent clinical failure. Sequelae include adhesions and sometimes difficulty replacing the catheter (Hakim *et al.* 1993).

10.1.2.3 Injection abscesses

Injection site abscesses have been caused by a variety of contaminated materials (Vandepitte *et al.* 1969; Wenger *et al.* 1990; Villanueva *et al.* 1997; Galil *et al.* 1999). Complementary and alternative medicine settings, such as injection of non-FDA approved substances that are contaminated by environmental mycobacteria, have been implicated as the source of infection (Wallace *et al.* 1998). For example, post-injection infection occurred in a large number of individuals who received adrenal cortex injection as part of a programme to lose weight (Galil *et al.* 1999). These contaminated substances have led to localized skin and soft tissue abscesses. The solutions themselves may be contaminated, and needles that are re-used or rinsed in tap water have also been implicated (Nolan *et al.* 1991). Contaminated multiple-use vials are especially suspect if there is more than one similar case. Self-administered cutaneous injections (i.e. patients with diabetes) can also present with PEM skin abscess. One diabetic patient developed *M. smegmatis* from insulin injections self-administered immediately after sitting in a hot tub that probably was contaminated with mycobacteria (De Groote, unpublished observation). A report of an outbreak of *M. abscessus* in an alternative medicine practice involving young Hispanic females undergoing cosmetic procedures with non-traditional agents including avocado products (D. Ashford, CDC, unpublished) is an example of this emerging problem. A case of *M. chelonae* infection as a result of acupuncture needles has been reported (Woo *et al.* 2001). This category of infections is likely to increase with enhanced utilization of alternative and complementary medical practitioners.

Often the diagnosis is not immediately obvious and obtaining proper cultures is delayed. It is not infrequent that a patient presents with an indolent inflammatory lesion that is unresponsive to antimicrobials. Administration of inappropriate antibiotics or corticosteroids can delay the diagnosis and may be misleading because it can temporarily provide some clinical relief and improvement. Because they are anti-inflammatory, corticosteroids can also worsen disease (Kermosh *et al.* 1979; Hellinger *et al.* 1995; Zenone *et al.* 1999). A heightened awareness, especially if there is a history of an injection, will help to ensure an accurate and timely diagnosis. Symptoms after injection can be very indolent with incubation period of 7-120 days. The lesions are reddened and vary in consistency. There is often some mild discomfort from the lesions, but in rare cases, the pain can be more severe. Antimicrobial agents and occasionally surgical incision and drainage are indicated.

10.1.2.4 Cosmetic industry

While strictly not an invasive device, an outbreak of RGM involving a whirlpool footbath used in a pedicure salon is presented as an example of contaminated equipment that can result in a large community outbreak (Winthrop *et al.* 2002). The outbreak involved over 100 pedicure salon

customers who developed furunculosis in the lower extremity. Patrons routinely underwent a period of soaking the lower extremity in the bath prior to the pedicure. A major risk factor for acquisition of infection was shaving the legs prior to the footbath treatment. Shaving probably caused micro-abrasions that provided the organism with a means of access into the skin. Multiple species of RGM were isolated from the footbath and the skin lesions of affected patients. Strain typing of *M. fortuitum* obtained from selected samples of the footbaths and patients revealed identical patterns. In this outbreak it was concluded that the contamination resulted from the municipal water which supplied the footbath. It was speculated that further growth on sloughed skin and other organic debris present in the bath resulted in high numbers of organisms. Infections ranged from mild to severe. Most patients were treated with antimicrobials and all resolved the infection, although some were left with scars.

Cases of PEM infections after body piercing have been documented (Jacobs *et al.* 2002). For example, one healthy young female presented with a breast mass that resulted from a nipple piercing carried out 10 months previously. On tissue pathology, no acid-fast organisms were seen in the granulomatous inflammation, but *M. abscessus/ chelonae* was isolated in culture (Trupiano *et al.* 2001).

10.1.2.5 Miscellaneous medical and surgical procedures

Post-surgical infections with PEM have been reported in many settings (Robicsek *et al.* 1977, 1978, 1988; Hoffman *et al.* 1981; Safranek *et al.* 1987; Wallace *et al.* 1989; Jarvis 1991; Grange 1992; Syed *et al.* 1997). They typically arise from solutions or instruments that have been inadequately sterilized (Phillips & von Reyn 2001). Mediastinitis and sternal wound infections due to *M. fortuitum* or *M. chelonae* have been reported after cardiothoracic surgery (Hoffman *et al.* 1981; Kuritsky *et al.* 1983). One outbreak was traced to contaminated iced cardioplegic solution used during the procedures. Prosthetic or implantable material such as porcine heart valves have been contaminated with RGM and are associated with a particularly bad outcome (Robicsek *et al.* 1988; Grange 1992).

An outbreak of otitis media in children has been reported after placement of tympanic membrane tubes. Infection due to *M. chelonae* was felt to have resulted by transmission between patients from contaminated instruments (Lowry *et al.* 1988).

Spinal infections have been reported after epidural injections and also after spinal surgery. A large outbreak after discovertebral surgery in France involving 58 patients in a single hospital in Paris occurred over a period of more than 10 years. This was a result of chronic contamination of the hospital water supply with *M. xenopi* (Astagneau *et al.* 2001).

An emerging scenario involves the development of mycobacterial infection after laser vision correction surgery. One example is keratitis after *in situ* keratomileusis surgery (Frueh *et al.* 2000; Chandra *et al.* 2001; Garg *et al.* 2001; Solomon *et al.* 2001; Alvarenga *et al.* 2002; Fulcher *et al.* 2002; Giaconi *et al.* 2002; Holmes *et al.* 2002; Maloney, 2002; Pushker *et al.* 2002; Seo *et al.* 2002). Even though mycobacteria are presumably of low virulence, a sight-threatening infection can occur (Bullington *et al.* 1992). A typical case is one of indolent, slowly progressive corneal disease. Reports of crystalline opacities seen in the corneal stroma are thought to be highly suggestive of mycobacterial infection (Alvarenga *et al.* 2002). Inflammation is rare. Whether the absence of inflammatory changes is due to a particular feature of the mycobacterial organisms causing the corneal infections or the use of topical corticosteroids is uncertain.

Other surgical procedures reported to result in PEM infection include augmentation mammoplasty, liposuction, and other plastic surgical procedures (Murillo *et al.* 2000). *M. abscessus, M. chelonae* and *M. fortuitum* infection after such procedures have all been described. In liposuction, for example, the cannulae used for tissue suctioning can become contaminated, with infected tracts appearing in areas where the cannulae were introduced (Murillo *et al.* 2000). Clinically, the patients present with localized abscesses including microabscess formation, purulent drainage and fistulae formation. Cellulitis has also been reported. Fever can be present or absent.

Many different types of aqueous solutions have been reported as the source of infection (epidural injections, steroids, tap water). Wound infections have been reported post surgery after using contaminated gentian violet skin marking solution. This outbreak of wound infections was caused by *M. abscessus,* and the organism was recovered from the solution (Safranek *et al.* 1987). In addition to solutions, instrumentation and implants that have been implicated include lacrimal duct probes, tympanostomy tubes, epidural catheters and graft materials. The potential for contamination exists in a multitude of surgical procedures (Wallace *et al.* 1998; Phillips & von Reyn 2001).

These infections can be very difficult to eradicate. They require cooperative management between those with infectious disease expertise and surgical colleagues to ensure adequate debridement and appropriate antimicrobial therapy. All surgical instruments should be sterilized according to manufacturer regulations, conform to standard practice, and should be overseen by the hospital infection control committee. If an outbreak is confirmed in the US, related to a device or product, the incident is reported to the MedWatch programme at the Food and Drug Administration (http://www.fda.gov).

10.1.3 Selected microbial factors

For reasons that are not entirely clear, RGM species, including *M. abscessus, M. chelonae* and *M. fortuitum* have a propensity for iatrogenic infections. As mentioned above, they account for most infections reported with post injection infection and wound infection post-procedures. Potential explanations include the ability of these organisms to adapt to a low nutrient tap water environment, to form biofilms as a successful mode of growth, and their relative resistance to standard disinfection.

10.1.3.1 The role of disinfectant resistance

Contamination of hospital water has been recognized for many years, especially hot water systems (du Moulin *et al.* 1988; El Sahly *et al.* 2002; Fujita *et al.* 2002; Labombardi *et al.* 2002; Zhang *et al.* 2002). These waters can become a source of infection for patients. Rapidly growing and other mycobacteria are difficult to eradicate with common decontamination practices and can continue to exist in water pipes in hospitals and operating rooms (Lowry *et al.* 1990; Wenger *et al.* 1990). Most PEM are relatively resistant to standard disinfectants such as benzalkonium chloride, CPC and quaternary ammonium compounds, chlorine, glutaraldehyde, formaldehyde, heavy metals, alcohols, peroxides and iodophores (Russell 1996; Griffiths *et al.* 1997, 1999; Manzoor *et al.* 1999; Falkinham 2002). A thorough review of this topic is presented in recent papers (Wallace *et al.* 1998; Phillips & von Reyn 2001), and in chapter 11. At their recommended concentrations for use, however, many of these agents can be mycobactericidal. Even when adequate disinfection has occurred, regrowth of the organism could again result in human infection. An interesting idea has been put forward by Falkinham that, given the relative resistance of certain PEM to disinfection, it survives relative to its competitors and is able to acquire nutrients that it would not otherwise have access to in the absence of disinfectants. It is important to not reuse disinfectants.

10.1.3.2 The role of biofilms

A biofilm is a consortium of microbes that adhere to either abiotic or biotic surfaces. Rapidly growing species are especially adept at forming biofilms (Hall-Stoodley & Lappin-Scott 1998). They produce an extracellular matrix that makes them more difficult to eradicate compared to their planktonic counterparts. Different species vary in their susceptibility to biocides (e.g. *M. marinum* biofilm cells are more sensitive, and *M. fortuitum* is more resistant to biocides than their single cell forms) dictating that each needs to be studied and considered separately (Bardouniotis *et al.* 2003). Some of the PEM are thermotolerant and likely also to form biofilms, especially *M. avium* and *M. xenopi*, which can grow at temperatures around 45 °C (Falkinham 2002). In addition, RGM can readily form biofilms on silastic rubber, which is a common

material used in medial instruments. NTM has been recovered from dental instruments at 400 times the concentration in drinking-water (Schulze-Röbbecke *et al.* 1995).

10.1.3.3 Role of water temperature

Multiple mycobacterial species have been described in piped water systems. *M. avium* and *M. xenopi* prefer hot water systems and can grow at temperatures around 45 °C (Falkinham 2002). In hot water systems several of these thermophilic mycobacteria can survive and have been reported to cause outbreaks or pseudo-outbreaks (du Moulin *et al.* 1988). In some cases temperatures of up to 70 °C are required to inhibit the organism. However, this high water temperature creates a risk of scalding injury. In cold water systems, *M. kansasii, M. gordonae, M. fortuitum, M. chelonae, M. abscessus* and *M. mucogenicum* have been found. Since cold and hot water temperatures exist in hospital settings, it is not surprising to see an array of mycobacterial species responsible for nosocomial infection. As an example of the remarkable spectrum of persistence, mycobacterial organisms have been isolated from taps, numerous contaminated instruments, solutions, showerheads and a hospital ice machine (du Moulin *et al.* 1988; Labombardi *et al.* 2002).

10.2 OVERALL BURDEN OF DISEASE

In many countries precise incidence and prevalence data is not available. Several laboratory surveys have been conducted in the United States and Europe. However, the limitation of these surveys is that most are culture-based, not patient-based, so it is difficult to know if each isolate represents a single individual occurrence. Since 1993, there has been a reporting system for State Health Department Laboratories, which has yielded important insights (Bean *et al.* 1992). Short of a standardized, mandatory reporting of outbreaks and individual case infections, we are often left with anecdotal reports. Despite such limitations, infection due to contaminated fluids and devices continue to be reported from all across the globe. Nevertheless, it is likely that the incidence of disease is underreported. In areas of the world where there is inadequate mycobacteriology laboratory support the true incidence may be even higher than is currently recognized.

Care providers make frequent changes to their practices and it is likely that alternative medicines and traditional medical practices will become more popular among their patients. Patients are making more frequent visits to providers of alternative medicine and placing more reliance on outpatient procedures, including those that are done outside a large hospital facility. Some of these centres are not routinely monitored by the infection control committees or equivalent oversight bodies. If these centres use less rigorous disinfection protocols, the incidence could become even higher. As advances in medical technology occur and as the population of immunosuppressed patient continues to increase there will undoubtedly be more cases reported.

10.3 DISTRIBUTION

Information regarding the distribution of infection is difficult to obtain. An analysis of data from the United States Emerging Infectious Diseases Network relating to RGM infections showed that invasive infections have been reported from all regions of the country (De Groote *et al.* 2002). RGM infection associated with medical procedures was the most common predisposing condition for infection. Given the ubiquity of these environmental pathogens and their relative resistance to disinfectants it is not surprising to find such outbreaks scattered around the globe. Disease has also been reported from Europe, Africa, Central America, Australia and Asia.

10.4 DESCRIPTIVE EPIDEMIOLOGY

Males and females appear to be equally susceptible to these infections. However, in adrenal cortex injection infections and pedicure salon infections the majority of patients are females, reflecting the group who are most likely to seek out such treatments. In other outbreaks such as post-surgical infections, there is no race or gender bias reported. There has been no person-to-person transmission demonstrated for these infections.

10.5 RISK FACTORS

In the majority of cases no high-risk group has been identified and most people are healthy. Presumably all people exposed to contaminated solutions or equipment are at risk, but the attack rate is not always the majority and the reasons for this are not completely known (Astagneau *et al.* 2001). However, underlying disease or immunosuppression would predispose the patient to more severe disease. Any exposure to contaminated solutions, medical or surgical devices, or other equipment is a risk, especially if there is disruption of defensive barriers caused by invasive procedures or microtrauma (Winthrop *et al.* 2002). Certainly those of increasing age, underlying immunosuppression and those who simply have more contact with the health care setting are more likely to become infected. However, many of the examples presented in this chapter include perfectly healthy young individuals. Unifying problems with specific disinfectants, instruments, etc., has not been uniformly observed.

10.6 CAUSALITY AND ASSOCIATED MICROBES

The microorganisms that are associated with infection are numerous and our understanding of disease mechanisms is likely to improve with better tools for species identification and molecular epidemiology. The RGM have been implicated most often. *M. abscessus, M. chelonae* and *M. fortuitum* appear to have the greatest

propensity for iatrogenic infections related to contaminated devices or solutions. *M. avium, M. mucogenicum* and *M. xenopi* are also common isolates in nosocomial disease. New species are being described all the time. A recent paper by Wilson *et al.* (2001) demonstrates that a newly described rapidly growing species, *M. immunogenicum* is responsible for a significant percentage of pseudo-outbreaks. *M. simiae, M. terrae, M. haemophilum, M. genavense* and *M. malmoense* are also capable of survival in water and have the potential to cause nosocomial outbreaks. It is important to point out that sometimes laboratories do not differentiate between the very closely related *M. chelonae* and *M. abscessus* making species-specific conclusions somewhat difficult (Wallace *et al.* 1997).

10.7 KEY RESEARCH ISSUES

In the future, timely recognition and reporting of real infections or pseudo-outbreaks will continue to be important. Updating and adding new surveillance methods within institutions will be important. Good communication between laboratory workers, clinicians and infection control practitioners will be a significant contribution to the care of our patients. With more PEM species described all the time, the laboratory and clinicians will need to be ever diligent, and incorporate accurate species identification into the routine workload in the laboratory. If such capabilities are not present in the laboratory where the infections occur, sending the isolate to a reference facility for identification and susceptibility testing may be indicated. Also, an enhanced role for strain typing in outbreak investigation will be important to track epidemics, common source exposures, and "pseudo-infections". If feasible, setting up a national and possibly an international cooperative system for reporting and tracking will be important for outbreak investigation. Investigations into the relative virulence of the organisms responsible for outbreaks should be carried out. Toward this end, strain collections should be maintained, so that in the future they may help to advance our understanding of such infections.

Studies into the dose response will be important when setting guidelines for standard disinfection (i.e. what is an acceptable contamination level to prevent infections). With this in mind, proper protocols for disinfection of instrumentation are very important. Easier and more effective sterilization solutions should be investigated, as well as more attention to proper training of personnel involved in instrument decontamination and reprocessing. Protocols for proper disinfection of equipment outside of the hospital setting are important (e.g. nail salon whirlpool baths). A greater understanding of the role of biofilms in and on devices and how to perform efficient eradication of biofilm cells will be paramount.

11

Control, treatment and disinfection of *Mycobacterium avium* complex in drinking water

M.W. LeChevallier

11.1 INTRODUCTION

Three basic mechanisms govern the occurrence of pathogenic microorganisms in treated drinking-water: 1) the microbes break through the treatment process from the source water supply, 2) the microbes regrow from very low levels, typically in biofilms, and 3) the organisms result from a recontamination of the treated water within the distribution pipeline system. These mechanisms are incorporated in the concept of multiple barriers for water treatment, the cornerstone of sanitary engineering. These barriers are selected to duplicate removal capabilities by succeeding process steps so that sufficient backup systems are available to permit continuous operation in the face of normal mechanical failures. Traditionally, the barriers have included:

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- source water protection
- coagulation, flocculation, sedimentation
- filtration
- disinfection
- protection of the distribution system.

Because mycobacteria can originate from a variety of environmental sources (refer to Chapters 2 and 3), little information is available to develop source water protection plans for this organism, although the basic premise applies: that water used for drinking-water should originate from the highest quality source possible. Available information for MAC and other mycobacteria are limited for some of the multiple barrier protection mechanisms, but information available for other microbes can help fill in gaps to develop an overall control strategy.

There are a myriad of microbes (in addition to mycobacteria) that may be of concern in source waters or within the distribution system; developing a monitoring scheme for each would be an impossible task. The food and beverage industry has used the HACCP approach to determine the key processes within the manufacturing chain where contamination can be measured and prevented (Table 11.1). Researchers have proposed a similar concept to prioritize the key contamination points within the treatment and distribution system to focus the utility's resources on monitoring and correcting these locations (Bryan 1993; Sobsey *et al.* 1993). The WHO has prepared a document outlining the development of a Water Safety Plan that is based on HACCP principles (WHO 2003). The following information will be useful in identifying critical control points for mycobacteria. The specific application of HACCP procedures to the control of mycobacteria in water systems is discussed in Chapter 12.

Table 11.1 Application of HACCP to water supplies

Step	Activity
1	Define the scope of the hazard analysis
2	Set up a multidisciplinary team
3	Perform detailed analysis of the process
4	Obtain information on raw materials and distribution conditions
5	Produce detailed flow diagram
6	Perform hazard analysis, prioritize hazard in order of importance and probability of occurrence
7	Identify critical control points for each hazard
8	Specify criteria for each critical control point
9	Identify means of monitoring critical control points to ensure control
10	Identify actions to be taken if tolerances are exceeded
11	Document all control and monitoring procedures
12	Train personnel involved in the process

11.2 REMOVAL OF MYCOBACTERIA BY WATER TREATMENT PROCESSES

As detailed in the WHO report entitled *Water Quality and Drinking-water Treatment: the impact of treatment processes on microbial water quality* (LeChevallier & Au 2003), microbes are controlled during water treatment processes by two principle mechanisms: 1) physical removal by coagulation, sedimentation and filtration and 2) inactivation by disinfectants. The unique characteristics of the mycobacteria affect these processes in ways that both improve and impair treatment.

11.2.1 Physical removal by coagulation and filtration

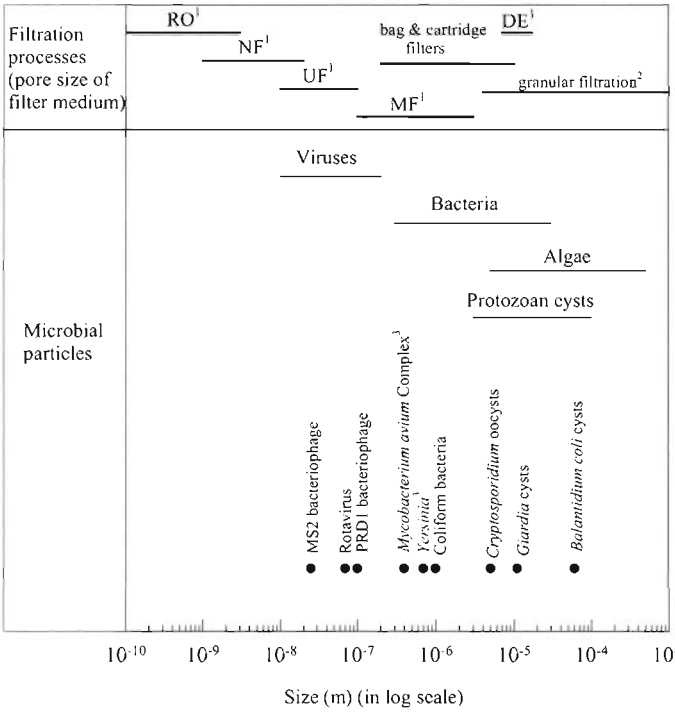
The hydrophobic nature of the mycobacteria cell wall promotes attachment of the organism to surfaces. In this regard, most mycobacteria in untreated raw water will be attached to suspended particulate material and treatment processes that reduce particle counts and turbidity will be effective for removal of mycobacteria. Falkinham *et al.* (2001) reported that the concentration of *M. avium* in raw surface water samples was significantly associated with turbidity levels greater than 2 NTU ($r^2 = 0.93$, $p < 0.0001$); similarly, the detection rate of *M. avium* in raw water was associated with turbidity levels greater than 2 NTU ($r^2 = 0.63$, $p < 0.02$).

The addition of coagulants destabilizes microbial particles (e.g. neutralize or reduce the surface electrical charge on the microbe, enmesh the microbes in a floc particle or bridge between separate particles) and allows particles to contact. Flocculation of these particles results in aggregates with sufficient settling velocities to be removed in the sedimentation basin. Coagulation conditions (dose, pH, temperature, alkalinity, turbidity and the level and type of natural organic matter) impact the efficiency of microbial removal, with slightly better overall reductions under pH conditions (5 to 6.5) optimum for removal of total organic carbon (Harrington *et al.* 2001; Bell *et. al.* 2002).

With proper design and operation, filtration can act as a consistent and effective barrier for microbial pathogens. The pore size of the most commonly used filtration processes in potable water treatment are listed in Figure 11.1. The relative size of MAC is compared to other bacterial, viral and protozoan pathogens. These size spectra provide insights for developing strategies to remove microbes by different filtration processes.

Removal of mycobacteria by six conventional treatment plants is shown in Table 11.2. The treatment plants used a variety of coagulants, filter media and disinfection regimes. Mycobacteria, *M. avium* and *M. intracellulare* were detected in all raw water sources (based on 18 monthly samples), but MAC

organisms were not detected in any plant effluent samples. Slow growing mycobacteria were detected in drinking-waters treated with monochloramine after filtration, with the highest frequency of occurrence in waters treated with chloramines and GAC. It is likely that the mycobacteria detected in the plant effluent samples originated from the filter media. Monochloramine is a slow reacting disinfectant (LeChevallier & Au 2003) that would likely result in little inactivation of the organism.



[1]RO: reverse osmosis; NF: nanofiltration; UF: ultrafiltration; MF: microfiltration; DE: diatomaceous earth filtration.

[2]Including slow sand filtration. Slow sand filter has a lower pore size than rapid-rate filter.

[3]These bacteria are rod-shape. The sizes shown represent the smallest dimension.

Fig. 11.1 Pore size of filter medium and size of microbial particles

Table 11.2 Occurrence of mycobacteria and *M. avium* complex in raw and filtered water samples (Adapted from LeChevallier *et al.* 2001)

Site	Coagulant	Filter Media	Disinfectant Type*	Sample	Total Samples	% Samples Mycobacteria		% Samples *M. avium*	% Samples *M. intracellulare*
						Slow	Rapid		
1	Ferric chloride	Anthracite	Ozone/Free Chlorine	Raw	17	35	6	6	6
				Plant Effluent	17	0	0	0	0
2	Ferric chloride	Anthracite/ sand	Free Chlorine	Raw	18	17	0	0	0
				Plant Effluent	18	0	0	0	0
3	Alum	Anthracite/ sand	Free Chlorine	Raw	18	11	6	6	0
				Plant Effluent	18	0	0	0	0
4	Polymer	Sand	Free/Mono-chloramine	Raw	17	53	18	29	6
				Plant Effluent	17	6	0	0	0
5	Ferric sulfate	Anthracite/ sand	Free/Mono-chloramine	Raw	18	39	0	11	0
				Plant Effluent	18	11	0	0	0
6	Alum	GAC/sand	Free/Mono-chloramine	Raw	18	56	22	22	0
				Plant Effluent	18	22	0	0	0

*Disinfectants are listed as the primary disinfectant followed by the residual disinfectant, if different.

Species of rapid and slow growing mycobacteria detected in the six conventional raw and plant effluent samples are listed in Table 11.3. A wide variety of slow and rapidly growing *Mycobacterium* subspecies were recovered, with the most predominant isolates identified as representatives of *M. terrae* and *M. coronae* (Table 11.3). The greatest variety of species was recovered from those water samples that yielded the highest numbers of *Mycobacterium* subspecies. There did not appear to be a species that was restricted to any particular site or type of sample.

Table 11.3 Identification of rapid or slowly growing *Mycobacterium* spp. in raw or plant effluent water samples (Adapted from LeChevallier *et al.* 2001)

Site	Sample	*Mycobacterium* spp.	Percent Occurrence
1	Raw	*M. gordonae*	18
		M. terrae	18
		M. chelonae	6
		slow unknown	6
2	Raw	*M. gordonae*	11
		M. terrae	11
3	Raw	*M. terrae*	11
		M. aurum	6
4	Raw	*M. terrae*	29
		M. gordonae	18
		slow unknown	12
		M. flavescens	6
		M. fortuitum	6
		M. marinum	6
		M. smegmatis	6
	Plant Effluent	unknown	6
5	Raw	slow unknown	22
		M. gordonae	17
		M. terrae	17
		M. marinum	6
	Plant Effluent	*M. terrae*	11
6	Raw	slow unknown	28
		M. gordonae	22
		M. fortuitum	11
		M. abscessus	6
		M. aurum	6
		M. flavescens	6
		M. shimoidei	6
		M. szulgai	6
		M. terrae	6
		rapid unknown	6
	Plant Effluent	*M. terrae*	17
		slow unknown	6

The ability of mycobacteria to grow within the treatment process was also reported by Le Dantec *et al.* (2002a) from a study of two treatment plants located in the Paris suburbs. The concentration of mycobacteria was between 20 and 20 000 cfu/l

following the clarification step (pre-ozone, coagulation-flocculation and rapid sand filtration) of the Orly plant, but was reduced to 10 to 20 cfu/l at the end of the water treatment process (ozone, GAC filtration, chlorine disinfection). All samples after rapid sand filtration were positive for mycobacteria, including *M. gordonae, M. peregrinum* and unidentified strains. After prefiltration (pre-ozone, coagulation-flocculation and rapid sand filtration) at the Ivry plant the concentration of mycobacteria was between 20 and 2000 cfu/l, but by the end of the treatment process (slow sand, ozone, GAC filtration, chlorine disinfection), the maximum value observed was only 60 cfu/l. Mycobacteria detected after slow sand filtration included *M. peregrinum, M. flavescens* and *M. chelonae*. Only one sample after ozonation was positive, at 10 cfu/l of *M. nonchromogenicium*; however, when ozone-treated water was filtered through GAC filters, all samples were positive for mycobacteria. Slow sand filter effluent samples typically contained lower levels of mycobacteria than did rapid sand filters, although it is difficult to attribute this difference to the physical removal of the organisms by the filter medium or the lower nutrient levels following the biological activity of the slow sand filters. No treatment plant samples were positive for either *M. avium* or *M. intracellulare* and finished plant effluent samples were typically negative for mycobacteria (Le Dantec *et al.* 2002a).

GAC used in point-of-use treatment devices can accumulate bacterial nutrients and neutralized disinfectant residuals, thus providing an ideal environment for microbial growth (Tobin *et al.* 1981; Geldreich *et al.* 1985; Reasoner *et al.* 1987; LeChevallier & McFeters 1988). The presence of a silver bacteriostatic agent did not prevent the colonization and growth of HPC bacteria in GAC filters (Tobin *et al.* 1981; Reasoner *et al.* 1987). Rodgers *et al.* (1999) observed the growth of *M. avium* in point-of-use filters in the presence of 1000 μg/ml of silver. Ridgway *et al.* (1985) reported that hydrophobic surface interactions between bacterial cell surfaces and cellulose acetate membrane filters resulted in a 25-fold more effective adhesion of *Mycobacterium* subspecies than did the hydrophilic *E. coli* strain tested. The strong hydrophobic surface properties of mycobacteria result in these organisms being the predominant membrane-fouling microorganism, particularly in the initial operation of the filter (Ridgway *et al.* 1985).

11.2.2 Disinfection

The high concentration of mycolic acid and the hydrophobic surface characteristics of mycobacteria are also primarily responsible for the high resistance of the group to chemical disinfection. Often, mycobacteria and other mycolic acid-producing genera (*Nocardia, Rhodococcus)* are the only bacterial organisms surviving chlorine disinfection of drinking-water supplies (Norton & LeChevallier 2000). Most species of mycobacteria survive challenge with 1 mg/l free chlorine (Carson *et al.* 1978, 1988a). du Moulin *et al.* (1988) found *M. marinum* to be resistant to 10 mg/l free chlorine. Haas *et al.* (1983)

found that there was no reduction in acid-fast bacterial numbers following chlorination in the water treatment plant examined.

Taylor *et al.* (2000) examined the disinfection resistance of five strains of *M. avium* to free chlorine, monochloramine, chlorine dioxide and ozone (Table 11.4) and determined the disinfectant concentration multiplied by the time for a 3-log or 99.9% inactivation ($CT_{99.9\%}$). The authors applied centrifugation techniques to minimize mycobacteria clumping that would interfere with disinfection. Free chlorine $CT_{99.9\%}$ values for the *M. avium* strains were 700- to 3000-times greater than that for *E. coli*. Similarly, the $CT_{99.9\%}$ values of the *M. avium* strains for chlorine dioxide and ozone were at least 100- and 50-fold greater (respectively) than the *E. coli* strain. The *M. avium* strains could be divided into two groups based upon their susceptibility to chloramines. Three of the strains were at least six times more resistant than the *E. coli* C strain, whereas two of the *M. avium* strains were as susceptible to monochloramine as was *E. coli* C.

Table 11.4 Calculated disinfection $CT_{99.9\%}$ (mg·min/l) for *E. coli* C and *M. avium* strains* (Adapted from Taylor *et al.* 2000)

Disinfectant	***Mycobacterium avium* strain**					
(Culture condition)	**Control *E. coli* C**	**A5**	**1060**	**1508**	**5002**	**5502**
Chlorine (M7H9)	0.088 ± 0.003	106 ± 9	204 ± 36	164 ± 28	126 ± 27	51 ± 10
Chlorine (water)	Not Done	1552 ± 403	1445 ± 238	596 ± 292	962 ± 431	551 ± 290
Monochloramine	73 ± 28	97 ± 9	458 ± 152	548 ± 62	1710 ± 814	91 ± 34
Chlorine Dioxide	0.015 ± 0.003	Not Done	8 ± 3	Not Done	11 ± 2	2 ± 0.1
Ozone	0.002 ± 0.002	Not Done	0.17 ± 0.14	Not Done	0.12 ± 0.01	0.10 ± 0.01

* Cells were exposed to the disinfectants in demand-free phosphate buffer (pH 7.0) at 23 °C

Data published by Taylor *et al.* (2000) also demonstrated large variations in sensitivity to disinfection among *M. avium* isolates. Two epidemiology-linked strains (5502 water isolate and 5002 AIDS patient isolate) with the same PFGE pattern showed markedly different sensitivity patterns to free chlorine, chloramines and chlorine dioxide. Some of the variability between strains (and various research studies) can be attributed to the degree of clumping, growth conditions and physiological properties. The authors reported that strain 5502 (water isolate) grew faster than strain 5502 (clinical isolate), and that the growth rate could be related to the increased resistance to disinfection. The authors demonstrated that isolates grown in low-nutrient tap water were between 4 and 15 times more resistant to free chlorine disinfection than isolates propagated in Middlebrook 7H9 broth. Le Dantec *et al.* (2002) reported that the inactivation rate of

M. gordonae increased 9-fold when the concentration of the growth medium was diluted 10-fold. Increased disinfection resistance has been observed for other low-nutrient grown waterborne microbes (LeChevallier *et al.* 1988).

The USEPA requires surface water treatment plants to apply filtration and disinfection, primarily as a means of controlling *Giardia* (USEPA 1989, 1998). Facilities that use conventional coagulation, sedimentation, and filtration are required to apply sufficient disinfection to achieve at least 0.5 log inactivation of *Giardia* cysts. Data in Table 11.5 shows *M. avium* (average of the five strains in Table 11.4) to be more resistant than *Giardia* to free chlorine disinfection, but equal or more sensitive than *Giardia* to monochloramine, chlorine dioxide and ozone. However, both organisms are more sensitive to disinfection than are *Cryptosporidium* oocysts (LeChevallier & Au 2003). Similar comparisons were made by Jacangelo *et al.* (2002) for 2-logs (99%) inactivation of *M. fortuitum* and *Giardia* (Table 11.6). The observed level of inactivation for *M. fortuitum* is higher than other reports, in part because the researchers did not attempt to reduce or eliminate bacterial clumps. However, similar to data in Table 11.5, mycobacteria were 3 to 10 times more resistant than *Giardia* to free chlorine disinfection.

Table 11.5 Comparison of disinfection conditions ($CT_{99.9\%}$ in mg·min/l) for *Giardia* cysts and *M. avium*

Disinfectant	*Giardia* cysts	*M. avium*
Chlorine	46	130
Monochloramine	700*	580
Chlorine Dioxide	11	7
Ozone	0.48	0.13

Data are for pH 7.0, 23-25 °C. Average *M. avium* data based on Taylor *et al.* 2000.

* Extrapolated.

Table 11.6 Comparison of free chlorine disinfection ($CT_{99\%}$ in mg·min/l) for *Giardia* cysts and *M. fortuitum*

Temperature (°C)	pH	*Giardia* cysts	*M. fortuitum*
5	6.0	70	> 320-1000
5	7.0	99	320-630
5	8.0	144	> 320- > 1000
15	6.0	35	90-320
15	7.0	50	500- > 1000
15	8.0	72	> 320-1000
25	6.0	17	50- > 320
25	7.0	25	130- > 320
25	8.0	36	130- > 320

Data for free chlorine at 1.0 mg/l, based on Jacangelo *et al.* 2002

Data for disinfection of other NTM by free chlorine are shown in Table 11.7. Cells were initially cultured on Middlebrook 7H10 agar but resuspended in sterile reverse osmosis water for four to five weeks to adapt the strains to low nutrient conditions. Similar data by Le Dantec *et al.* (2002) are shown in Figure 11.2. Although the CT values differ between different investigators and replicates of the same species (e.g. *M. chelonae*) showed large variations in disinfection sensitivity, the relative rank of chlorine sensitivity was similar (*M. fortuitum* and *M. chelonae* were more resistant than *M. gordonae)* and, in general, the isolates were more sensitive to free chlorine disinfection than the *M. avium* strains shown in Table 11.4.

Table 11.7 Free chlorine disinfection of mycobacteria

Organism	CT value (mg·min/l)	Log Inactivation
M. fortuitum	2	0.04
	42	2.9
M. chelonae	2	0.03
	10.5	2.5
	21	4.75
	42	2.8
M. gordonae	2	0.07
M. scrofulaceum	2	0.08

Data based on Carson *et al.* 1978; 1988a. Data are for pH 7.0, 22-25 °C.

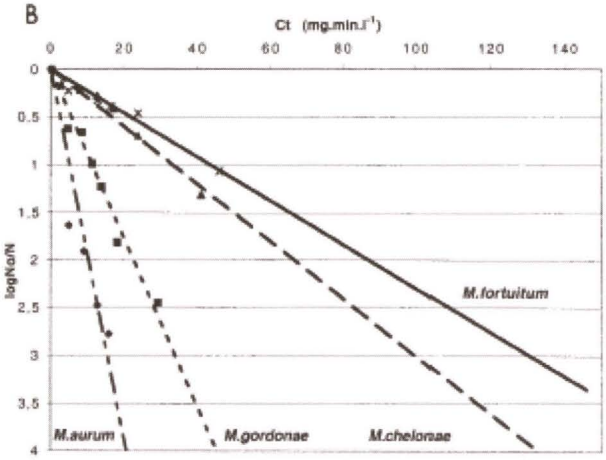


Fig. 11.2 Disinfection of mycobacteria by free chlorine. Experimental conditions: pH 7.0, 25 °C, initial free chlorine concentration 0.5 mg/l. Reprinted from Le Dantec *et al.* 2002a.

Disinfection of a variety of mycobacteria by chloramines was reported by Pelletier *et al.* (1991) (Table 11.8). The decreasing CT for 0.5-log inactivation with higher chloramine doses suggests that clumping or disinfectant demand may be influencing these results. However, *M. avium* were more resistant than the other mycobacteria examined.

Application of ultraviolet light for microbial inactivation is gaining increased attention, especially for control of *Cryptosporidium*. Inactivation data for mycobacteria by UV light is shown in Table 11.9. Although sensitivities vary among *Mycobacterium* species, the values are generally in the range for other vegetative bacteria (http://www.iuva.org). David *et al.* (1971) reported that *M. tuberculosis* and *M. marinum* were capable of photoreactivation, with an increase of 40-56% following irradiation with visible light for one hour. McCarthy and Schaeffer (1974) reported a 1.7-log increase in *M. avium* counts following exposure to 9 mJ/cm^2 and a 3-hour photoreactivation. They also noted that the 14-day post-exposure incubation period for *M. avium* provided ample opportunity for significant dark repair of UV lesions.

Table 11.8 Chloramine disinfection of mycobacteria for 0.5 log inactivation

Organism	Chloramine (mg/l)	CT (min)	CT value (mg·min/l)
M. avium 743	1.0	600	600
	3.0	240	720
	6.5	30	195
M. avium 723	1.0	600	600
	3.0	240	720
	6.5	30	195
M. intracellulare	1.2	258	310
	3.0	120	360
	6.5	30	195
M. chelonae	1.6	48	76
	3.0	36	108
	6.5	6	39
M. gordonae	1.6	60	96
	3.0	40	120
	6.5	10	78
M. kansasii	1.2	60	72
	3.0	10	30
	6.5	9	58
M. fortuitum	1.5	36	54
	3.0	24	72
	6.5	2	19.5

Data based on Pelletier *et al.* 1991. Data are for pH 7.0, 17 °C

Table 11.9 Inactivation of mycobacteria by ultraviolet light

Strain	Log inactivation	UV dose (mJ/cm^2)	Reference
M. tuberculosis H37Rv	1	5.7	David *et al.* 1971
M. tuberculosis Erdman	1	2.4	Collins 1971
M. tuberculosis	1	2.8	David 1973
*M. avium DM9**	2	7	McCarthy and
M. avium-intracellulare	2	14	Schaeffer 1974
*T-931-72**	1	8.4	David 1973
M. avium-intracellulare			David 1973
*T-931-72**			
M. bovis BCG	1	2.4	Collins 1971
M. fortuitum strain 1	1	3.2	David *et al.* 1971
M. fortuitum strain 2	1	8.9	David *et al.* 1971
M. fortuitum strain 56	1	6.8	David 1973
M. phlei strain 44	1	7.6	David 1973
M. kansasii (avg. of 6 strains)*	1	13.3	David 1973
M. marinum strain 1	1	17.8	David *et al.* 1971
M. marinum strain 1	1	17.0	David *et al.* 1971
M. flavescens	1	12.0	David 1973
M. smegmatis	1	24.3	David *et al.* 1971
M. smegmatis	1	10.8	David 1973

* Data following photoreactivation

A comparison performed by Jacangelo *et al.* (2002) of mycobacteria inactivation to other emerging pathogens is shown in Figures 11.3-11.7. The data show that in general, *Cryptosporidium* is the controlling organism for surface water disinfection. For UV, adenoviruses are the most resistant microbe. However, for nearly all disinfectants, mycobacteria are among the most resistant microbes. The ubiquitous occurrence of mycobacteria in the aquatic environment and their ability to grow within water treatment filters means that effective disinfection is an important part of the multiple barrier approach to reducing the risk of these organisms in drinking-water supplies.

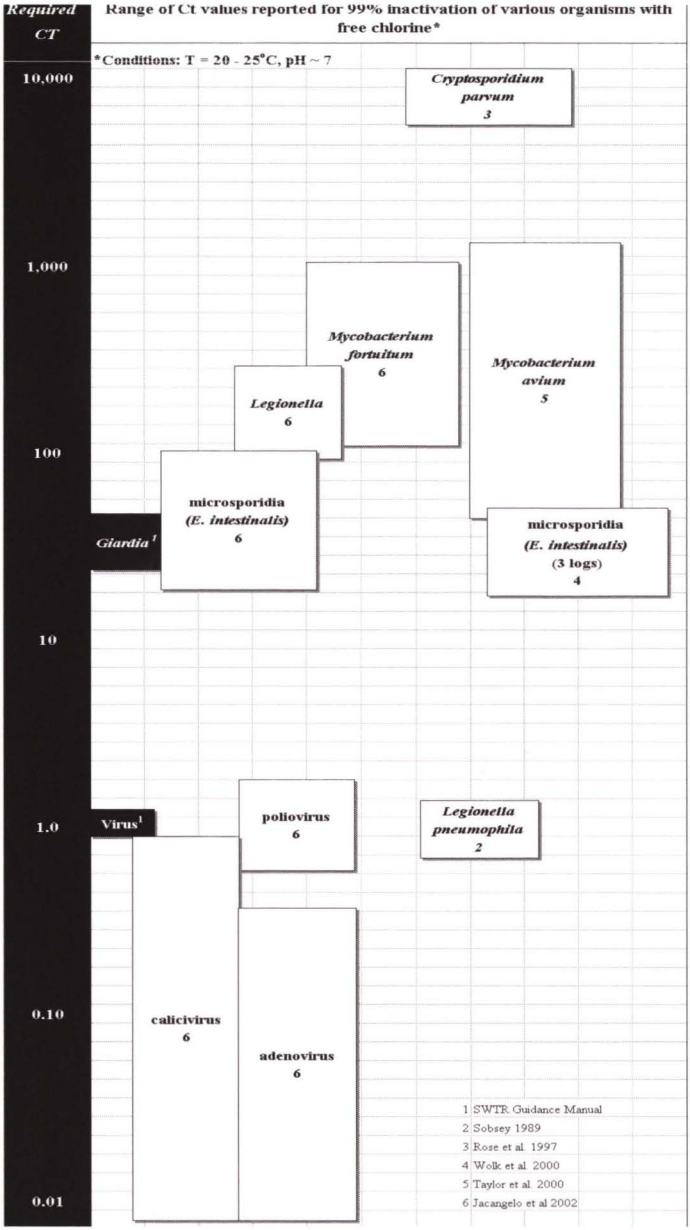


Fig. 11.3 Comparison of CT values for microbial inactivation by free chlorine. From Jacangelo *et al* 2002

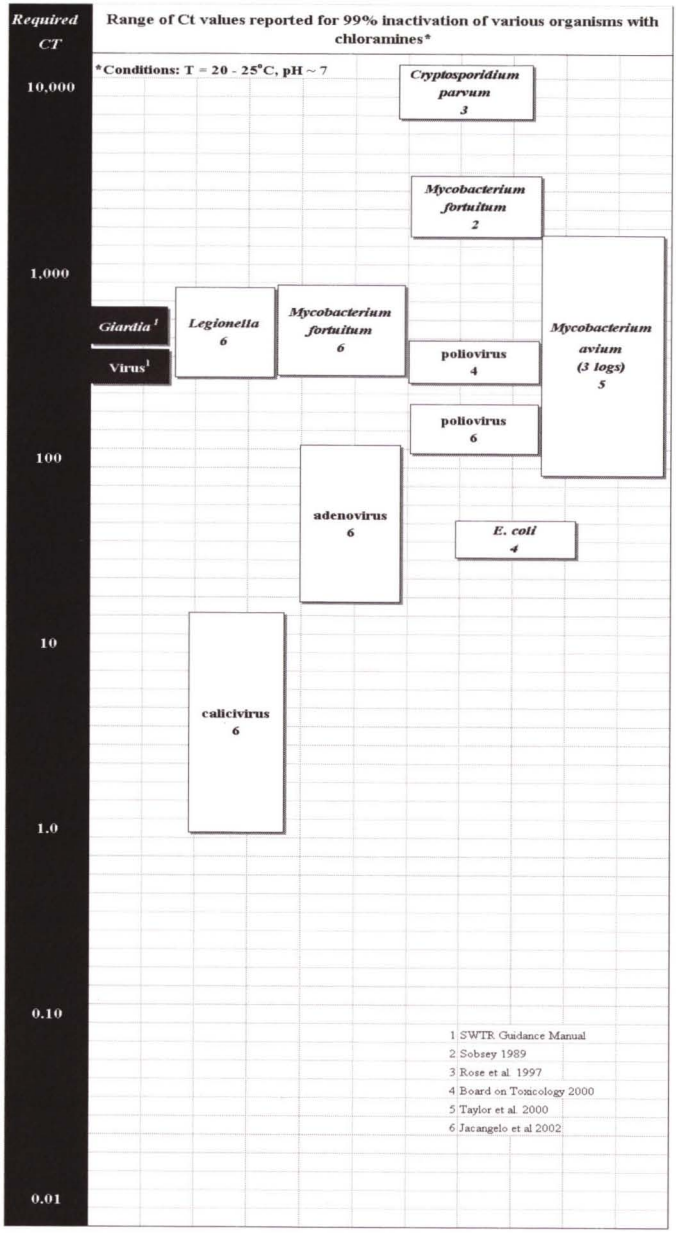


Fig. 11.4 Comparison of CT values for microbial inactivation by chloramines. From Jacangelo *et al* 2002

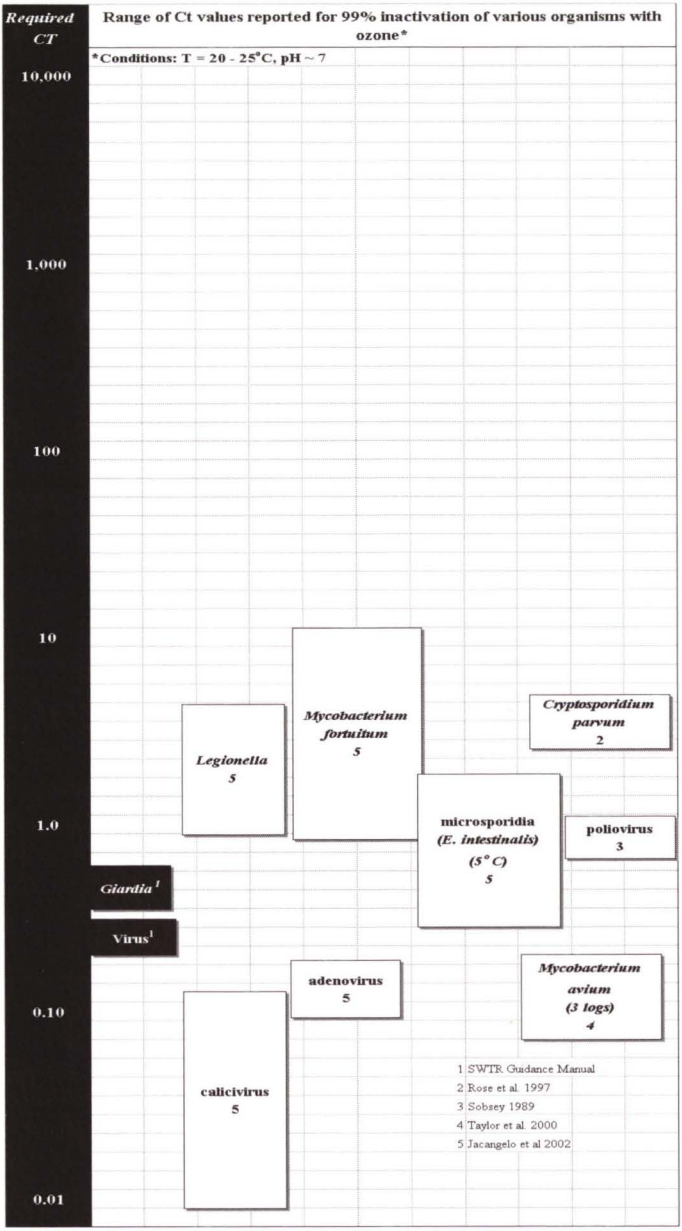


Fig. 11.5 Comparison of CT values for microbial inactivation by chlorine dioxide. From Jacangelo *et al* 2002

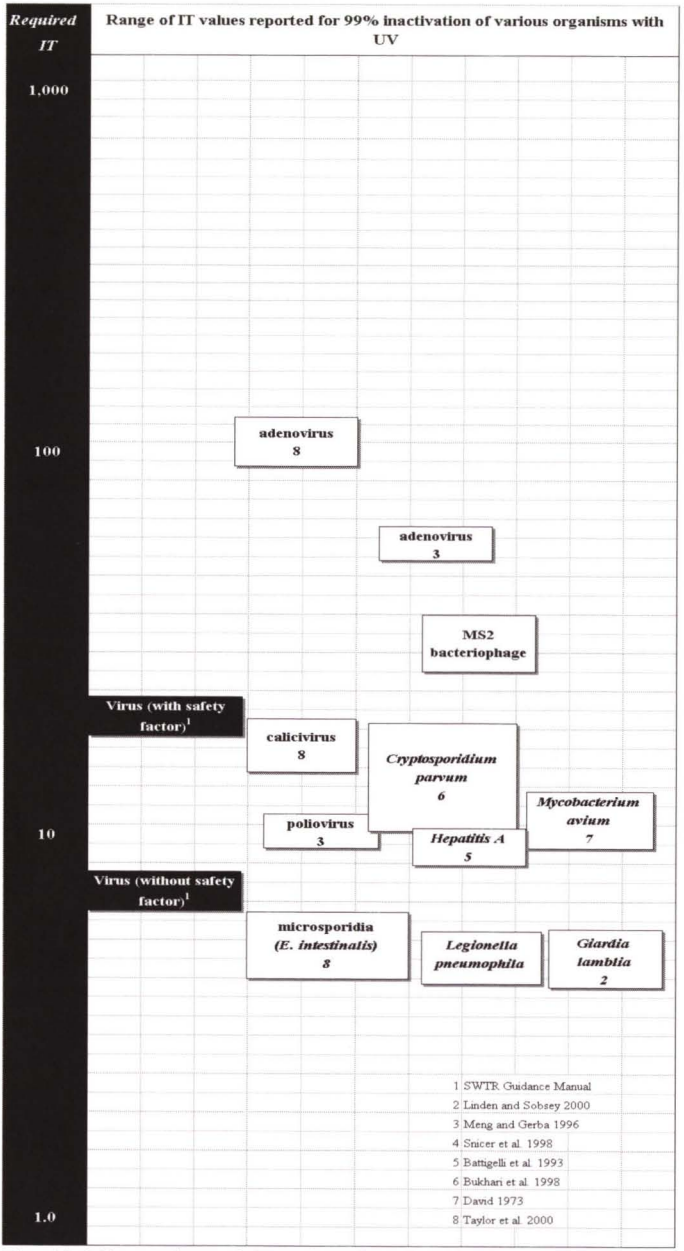


Fig. 11.6 Comparison of CT values for microbial inactivation by ozone. From Jacangelo *et al* 2002

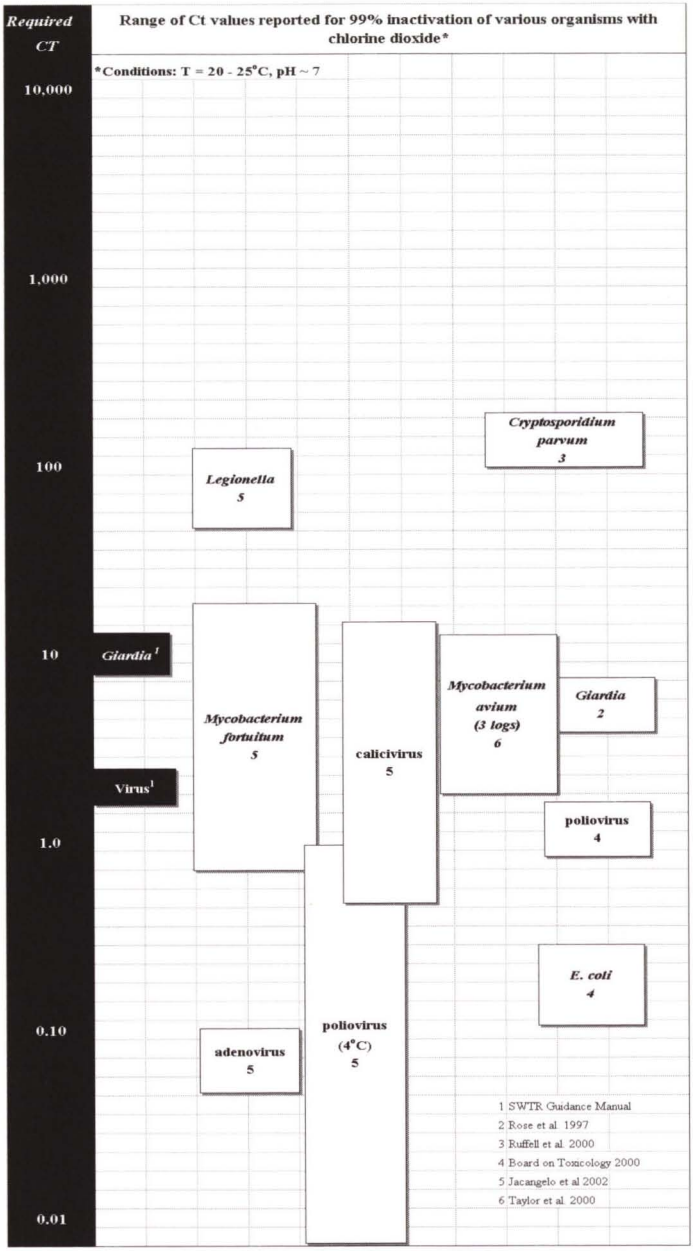


Fig. 11.7 Comparison of CT values for microbial inactivation by ultraviolet light. From Jacangelo *et al* 2002

11.3 REGROWTH OF MYCOBACTERIA IN DRINKING WATER

Bacteria can grow in high-quality drinking-water supplies utilizing trace levels of minerals and organic carbon to produce cellular material (biomass). Studies have demonstrated that bacterial survival and growth in drinking-water distribution systems is dependant upon a complex interaction of parameters (LeChevallier 2003; van der Kooij 2003). These factors are summarized in Table 11.10. For example, temperature is widely recognized as an important controlling factor in influencing bacterial growth. In climates where water temperatures are warm, bacterial growth may be very rapid.

Table 11.10 Factors associated with bacterial growth/occurrence in drinking-water

	Factor
a	Treatment process, filtration
b	Temperature
c	Disinfectant type, concentration
d	Biodegradable organic matter
e	Pipe material, corrosion
f	Water chemistry
g	System maintenance, flushing
h	Flow velocity, reversal, system shear

11.3.1 Biodegradable organic matter

The presence of biodegradable organic matter in water has been associated with the growth of heterotrophic bacteria. Biodegradable organic matter is commonly measured as AOC or BDOC. AOC is determined using a bioassay (van der Kooij 1990, 1992) and measures the microbial response to biodegradable materials in water. AOC levels (geometric means) in 90 North American drinking-water systems ranged from 20 to 214 µg/l with a median of 100 µg/l (LeChevallier *et al.* 1996; Volk & LeChevallier 2000). BDOC is the difference in the concentration of dissolved organic carbon before and after bacterial growth in a sample and measures the amount of nutrient readily available for bacterial growth (Joret & Levi 1986). Levels of BDOC in 30 North American water systems ranged from 0 to 1.7 mg/l, with a median level of 0.38 mg/l (Volk & LeChevallier 2000). AOC and BDOC levels tend to be higher in surface water supplies and lower in groundwaters, where microbial activity removes biodegradable organic matter as the water percolates through the soil.

To examine the effect of source water type (surface or ground), post-treatment disinfectant (free chlorine or chloramines), and biodegradable organic matter, researchers (Falkinham *et al.* 2001; LeChevallier *et al.* 2001) examined eight well-characterized drinking-water systems (Table 11.11). Samples were collected monthly for 18

consecutive months from the raw water, plant/well effluent, a distribution system mid-point and a dead-end site. *M. avium* and *M. intracellulare* were frequently isolated from drinking-water biofilm samples (Figure 11.8). The data showed that *M. avium* levels were reduced by conventional water treatment, but increased due to regrowth in the distribution system. Increases in *M. avium* levels in drinking-water correlated with levels of AOC ($r^2 = 0.65, p = 0.029$) and BDOC ($r^2 = 0.64, p = 0.031$) (Falkinham *et al.* 2001).

Table 11.11 Summary of nutrient levels for full-scale systems

Site	Source water	Disinfectant type (pre/post)	n	Expected level	AOC levels (μg/litre)[1] Observed	Range	BDOC levels (mg/litre)[2] Observed	Range
1	Surface	Ozone/ Free chlorine	16	High	234 (59)[3]	161–383	0.48 (0.19)	0.15–0.71
2	Surface	Free chlorine	17	Medium	113 (41)	66–220	0.28 (0.17)	0.06–0.58
3	Surface	Free chlorine	17	Low	61 (86)	21–391	0.04 (0.09)	0.0–0.34
4	Ground	Free chlorine	17	Low	28 (43)	5–168	0.07 (0.18)	0.0–0.70
5	Surface	Free chlorine/ Monochloramine	16	High	215 (110)	127–484	0.70 (0.22)	0.31–1.0
6	Surface	Free chlorine/ Monochloramine	18	Medium	109 (80)	37–301	0.34 (0.20)	0.0–0.81
7	Surface	Free chlorine/ Monochloramine	17	Low[4]	98 (53)	49–270	0.40 (0.16)	0.01–0.76
8	Ground	Free chlorine/ Monochloramine	17	Low	17 (9)	6–33	0.06 (0.08)	0.0–0.26

[1]Geometric mean; [2]Average values; [3]Numbers in parentheses represent standard deviations; [4]Actual levels of AOC and BDOC were higher than expected for this site.

In controlled pilot plant experiments, the effect of biodegradable organic matter on the growth of *M. avium* and natural HPC bacteria was investigated (Norton *et al.* 2004). Biofilms of *M. avium* and HPC bacteria formed within one week and appeared to stabilize over a two to three month period (Table 11.12). The results showed that the density of biofilm bacteria increased with increasing AOC levels, but that biofilms of *M. avium* readily grew in the absence of a disinfectant residual at AOC levels of 40 μg/l. Therefore, minimizing the level of biodegradable organic matter in drinking-water can help limit the density of mycobacteria, but probably cannot prevent their occurrence. Carson *et al.* (1978) reported growth of *M. chelonae* in commercial distilled water to levels of 10^5 to 10^6 cells/ml and levels were maintained over a one-year period. Collins *et al.* (1984) reported detection of mycobacteria in water softening resins and in water from deionizers. Groundwater typically has low levels of biodegradable organic matter due to the natural removal of nutrients as the water percolates through the soil. However, Covert *et al.* (1999) reported detecting *M. gordonae* in 31% of 16 groundwater systems.

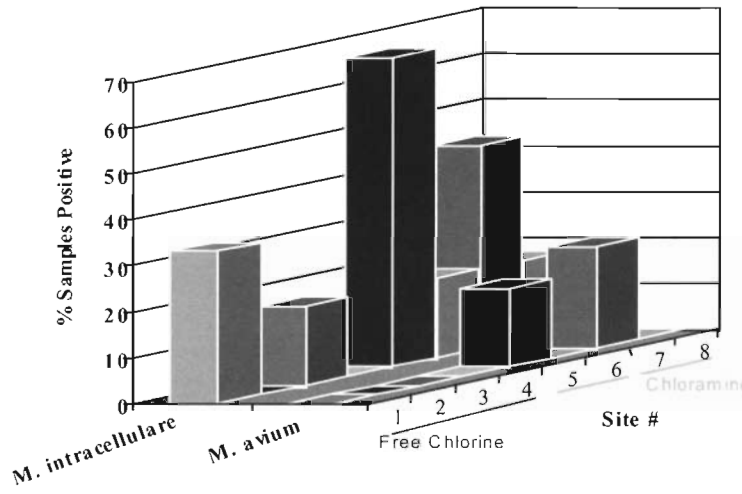


Fig. 11.8 Occurrence of *M. avium* and *M. intracellulare* in distribution system biofilm samples ($N = 55$)

Table 11.12 *M. avium* and HPC biofilm levels in a pilot distribution system supplied with various levels of AOC (Adapted from Norton *et al.* 2004)

Weeks Sampled	Baseline, 42 µg/l		85 µg/l AOC		103 µg/l AOC		213 µg/l AOC	
	HPC	*M. avium*	HPC	*M. avium*	HPC	*M. avium*	HPC	*M. avium*
1	5.27	4.24	5.04	3.86	5.62	4.41	5.08	3.68
4	5.95	4.10	6.27	3.89	6.40	4.03	6.82	4.89
9	5.99	3.17	6.08	4.44	6.64	3.85	7.16	5.36
10	5.51	4.90	6.31	4.44	6.74	4.96	6.88	5.96
11	6.51	5.07	6.33	4.27	6.50	3.91	6.98	4.04
Avg	5.85	4.30	6.01	4.18	6.38	4.23	6.58	4.79

Values are log cfu/cm^2; AOC values are geometric means; no disinfectant was applied; pH 7.2, 24 °C

11.3.2 Impact of water contact materials

Various water contact materials may leach substances that support bacterial growth. For example, pipe gaskets and elastic sealants (containing polyamide and silicone) can be a source of nutrients for bacterial proliferation. Coating compounds for storage reservoirs and standpipes, bitumen, chlorinated rubber, epoxy resin or tar-epoxy resins

can contribute organic polymers and solvents that may support regrowth of heterotrophic bacteria (Schoenen 1986; Thofern *et al.* 1987). In general, the larger surface to volume ratio in smaller diameter pipes (compared with larger pipes) results in a greater impact of biofilm bacteria on bulk water quality. The greater surface area of small pipes also increases reaction rates that deplete chlorine residuals.

The pipe surface itself can influence the composition and activity of microbial biofilm populations. Studies have shown that biofilms developed more quickly on iron pipe surfaces than on plastic PVC pipes (LeChevallier *et al.* 1993; Camper 1996) and that iron pipes support a more diverse microbial population than PVC pipes (Norton & LeChevallier 2000). The impact of pipe composition on the biofilm growth of *M. avium* is shown in Figure 11.9. *M. avium* levels were higher on iron and galvanized pipe than on PVC or copper surfaces. The resistance of *M. avium* to zinc may enhance its survival on galvanized pipe surfaces (Kirschner *et al.* 1992).

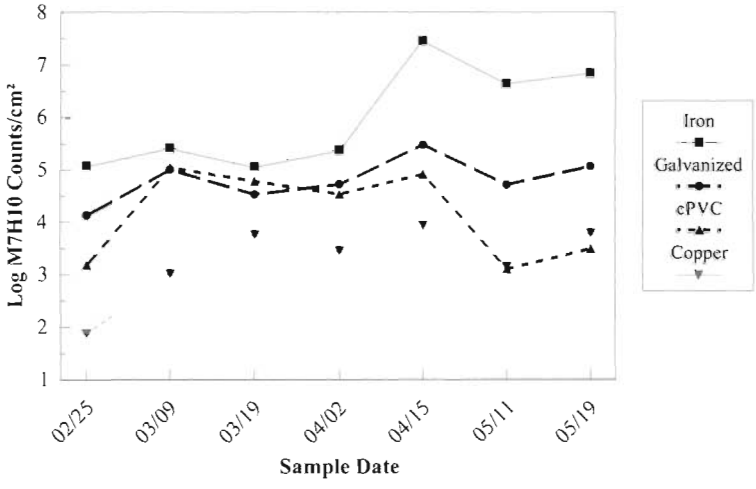


Fig. 11.9 Impact of pipe composition on biofilm growth of *M. avium*. Adapted from LeChevallier *et al.* 2001

The effect of the combination of biodegradable organic matter, disinfectant type and pipe composition on the survival of *M. avium* and HPC bacteria is shown in Table 11.13. The lowest level of biofilms occurred on copper under low nutrient and free chlorine conditions. Chloramines controlled biofilms on iron pipes better than free chlorine, particularly under low-nutrient conditions. Pelletier *et al.* (1991) suggested that use of chloramines as a disinfectant may select for the presence of *M. avium* in drinking-water supplies, although many systems that convert to chloramines do so because of high levels of organic carbon in the water supply that form chlorinated disinfection by-products.

Table 11.13 Impact of nutrient level, disinfectant and pipe material on *M. avium* and HPC levels

AOC Level	Disinfectant Type	Disinfectant Residual (mg/l)	Copper Pipe [1]		Iron Pipe [1]	
			HPC	*M. avium*	HPC	*M. avium*
85 µg/l AOC	Free chlorine	0.6	1.76	0.18	6.02	5.85
	Chloramine	2.2	2.44	2.38	5.21	4.92
213 µg/l AOC	Free chlorine	0.3	2.17	0.37	5.93	5.50*
	Chloramine	1.4	2.43	2.10	5.89	5.20*

[1]Values are log cfu/cm^2 ; *Corrosion products interfered with these analyses. Adapted from Norton *et al.* 2004

11.4 OTHER ENVIRONMENTAL AND CONTROL FACTORS

11.4.1 Temperature

The resistance of mycobacteria to heat and freezing may influence its survival in water systems and in treatment residuals. Certain thermophilic species (*M. chelonae*, *M. avium* or *M. xenopi*) survive at temperatures above 55 °C; whereas, under the same conditions, *M. kansasii* or *M. marinum* are quickly destroyed (Merkal & Crawford 1979; Schulze-Röbbecke & Buchholtz 1992). It has been speculated that differences in sensitivity to heat could account for some of the seasonal variability between different isolates of mycobacteria (Dailloux *et al.* 1999). Du Moulin *et al.* (1988) recovered *M. avium* in a hospital hot water system with water temperatures between 52 and 57 °C. Von Reyn *et al.* (1994) reported that the same strain of *M. avium* was recovered from two hospital recirculating hot water systems, with average or minimum temperatures of 49 °C, over a 24- to 41-month period. Isolates with the same PFGE pattern were isolated from both the hot water systems and AIDS patients. Schulze-Röbbecke & Buchholtz (1992) reported that treatment of *M. avium* suspensions at 60 °C for 4 minutes reduced viable counts by 1 log (Table 11.14). Norton *et al.* (2004) showed that the effectiveness of the heat treatment was dependant upon the pipe material, the level of nutrients and the effluent temperature (Figure 11.10). Treatment was most effective for low-nutrient, hot water (50 °C), in copper pipes. For iron pipe, biofilms of *M. avium* was inhibited when effluent temperatures reached 52 °C (inlet temperatures were 63 °C). For bacteria suspended in the water column, *M. avium* was detected at effluent temperatures up to 52 °C in the low nutrient system, but persisted at temperatures up to 53 °C in the high nutrient system.

Table 11.14 Decimal reduction times *(D)* of mycobacteria at 50, 55, 60 and 70 °C (Adapted from Schulze-Röbbecke & Buchholtz 1992)

		D (s) at temp of			
Species	**Strain**	**50 °C**	**55 °C**	**60 °C**	**70 °C**
M. avium	DSM 43216	60 750	3210	240	2.3
M. chelonae	DSM 43283	10 130	1360	260	5
M. fortuitum	DSM 43271	6330	1520	220	2
M. intracellulare	DSM 43224	32 950	1470	91	4.5
M. kansasii	Water isolate	3970	560	59	< 10
M. kansasii	Water isolate	4700	350	27	< 10
M. marinum	ATCC 927	4510	750	60	< 10
M. phlei	DSM 750	NR	4210	420	2.8
M. scrofulaceum	NCTC 10803	56 030	3650	320	5.6
M. xenopi	NCTC 10042	NR	20 730	1980	22.5

DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC: American Type Culture Collection; NCTC: National Collection of Type Cultures; NR: no reduction in cfu over a period of 48 hours

Freezing is not normally thought of as a water treatment process although water treatment sludges can be dewatered through a freeze/thaw process. In addition, biofilms from distribution systems and institutional pipelines can become detached and contaminate ice machines. Mycobacteria can survive freezing conditions for prolonged periods with counts actually increasing after freezing (-75 °C in nutrient broth), presumably due to disaggregation of bacterial clumps (Iivanainen *et al.* 1995).

11.4.2 Control of free living amoebae

M. avium is capable of survival and growth in phagocytic protozoa (*Tetrahymena pyriformis*) and amoebae (*Acanthamoeba polyphaga* and *Acanthamoeba castellanii*), and such growth can increase its virulence and provide protection from antimicrobial agents (Cirillo *et al.* 1997; Miltner & Bermudez 2000). In fact, protozoa intracellular growth rates of *M. avium, M. intracellulare* and *M. scrofulaceum* were 4- to 40-fold faster than compared to water-grown isolates (Strahl *et al.* 2001). Therefore, it is possible that efforts to control the growth of free-living amoeba and protozoa will have beneficial effects for reducing the risk of mycobacteria in drinking-water. Few studies have specifically focused on the control of free-living protozoa or the effect of control measures on *Mycobacterium* risk, although it is likely that efforts to reduce biofilms levels in drinking-water pipelines would also result in lower amoeba and protozoa levels because these organisms feed on biofilm bacteria. Additional studies are needed in this area.

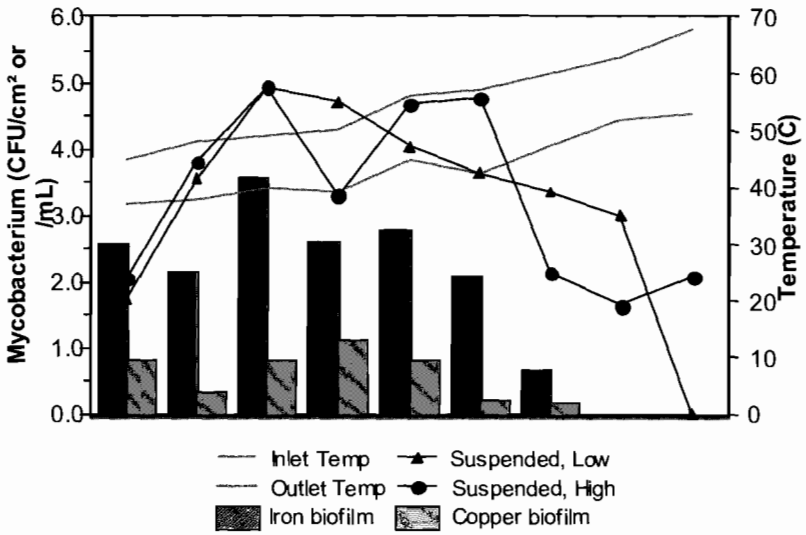


Fig. 11.10 Impact of heat treatment on biofilm and suspended levels of *M. avium* in water. Low and high refer to AOC levels of 85 and 213 µg/l. Adapted from Norton *et al.* 2004.

11.5 ROLE OF DISTRIBUTION SYSTEM RECONTAMINATION

Because mycobacteria exist with a wide variety of aquatic and soil environments (refer to Chapter 3), there is a potential for these organisms to recontaminate the distribution system from cross-connections, backflows, and intrusion events. From 1981 to 1998, the CDC documented 57 waterborne disease outbreaks related to cross-connections, resulting in 9734 illnesses (USEPA 2002). These include 20 outbreaks (6333 cases of illness) caused by microbiological contamination, 15 outbreaks (679 cases of illness) caused by chemical contamination, and 22 outbreaks (2722 cases of illness) where the contaminant was not reported. Craun & Calderon (2001) report that 30.3% of waterborne disease outbreaks in community water systems during 1971-1998 were caused by contamination of water in the distribution systems and 50.6% were due to cross-connection or backflow events. Because documented health impacts most often involve acute gastrointestinal disorders, slow developing infections like those caused by *M. avium* would not be recognized.

Increasing attention is being focused on the occurrence of transient negative pressure events in distribution system pipes caused by sudden changes in water velocity, typically caused by pump shutdowns, or main breaks or fire flows

(LeChevallier *et al.* 2003). During a negative pressure event, contaminated water and soil exterior to the pipe can enter into the distribution system through cracks, seals or pipeline leaks. Karim *et al.* (*J. Am. Water Works Assoc.* In Press, 2003) reported on a study that examined 66 soil and water samples collected from eight utilities in six states. The samples were collected immediately adjacent to the drinking-water pipelines to determine the presence of microbial contaminants in the soil. Total coliform and faecal coliform bacteria were detected in water and soil in about half of the samples, indicating the presence of faecal contamination. Viruses were detected using either culturable or molecular methods in 56% of the samples. Although samples in this study were not analysed for mycobacteria, it is likely that leaking sewer lines could provide the high organic environment favourable for growth of mycobacteria (Brooks *et al.* 1984a; Kirschner *et al.* 1992; Iivanainen *et al.* 1997).

During the repair of main breaks it is likely that soil and water can enter the pipe network. Traditional decontamination efforts using a chlorine solution to swab pipe and fitting surfaces probably do little to inactivate mycobacteria due to their extreme resistance to chlorine (Tables 11.5 to 11.7). Although flushing the affected pipe section may be sufficient to remove sediment from the system, the high velocities may shear biofilms of mycobacteria from pipe surfaces resulting in a net increase of mycobacteria in the water column. For new construction, it is recommended that a foam swab be installed during construction so that debris can be removed from the pipeline prior to placing it into service (Geldreich & LeChevallier 1999).

Given the level of external contamination by mycobacteria and the dilution that would occur in drinking-water networks, the impact distribution system recontamination is probably greatest as an inoculum to drinking-water biofilms, rather than as a direct public health concern. Therefore, efforts to minimizing growth of mycobacteria in drinking-water systems may reduce the concern with low-level recontamination from external sources.

11.6 KEY RESEARCH ISSUES

Much of the research on mycobacteria in drinking water is still at the "observational" stage, where investigators monitor a variety of water supplies and report their findings of various mycobacteria species. Research on control and treatment must move to the mechanistic and predictive stages where treatment systems can be designed based on the ecology, survival, and risk of mycobacteria in water. Improvements in the methodology for detection and enumeration of mycobacteria in water and biofilm samples are necessary so that the efficacy of treatment is based reliable data. A list of key research issues is provided below:

- Because proper chemical pretreatment is crucial for effective microbial removal by sedimentation and filtration, studies need to examine optimal

coagulant doses for mycobacteria. Studies should understand how changes in microbial surface properties due to antecedent growth conditions, and the degree of aggregation, might influence removal efficiencies.

- Studies need to examine the effectiveness of filter operation and backwashing procedures to optimize the removal of mycobacteria, but at the same time minimize the growth of the organisms within the filter beds. The studies should focus on the effectiveness of backwashing procedures for limiting the occurrence of mycobacteria within the filter beds.
- Although there is extensive literature on the inactivation of mycobacteria by various disinfectants, little field information is available to demonstrate disinfection of mycobacteria in natural waters. Because disinfection is affected by antecedent growth conditions and the degree of aggregation, field validation of bench-scale experiments is imperative.
- Additional research is needed to demonstrate that reduced levels of biodegradable organic carbon can minimize the levels of mycobacteria in drinking water biofilms. The opportunity for greatest impact may be in warm water institutional plumbing systems. The role of pipe materials should also be considered.
- Research should examine the impact of control procedures for free-living protozoa and amoeba on mycobacteria occurrence and virulence.
- The efficacy of standard cleaning and disinfection protocols used during main repairs should be assessed with respect to control of mycobacteria.
- Infectious dose data and risk assessment models for mycobacteria, and in particular *M. avium*, are needed to establish public health goals for potable water supplies. These goals are necessary to determine acceptable limits for exposure to mycobacteria in drinking water, and in turn establish objectives for control procedures. Finally, these health-related goals would enable researchers to evaluate various pathways of exposure and concentrate on those with the greatest risk.

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12

Approaches to risk management in priority setting

T. Ford, J. Hermon-Taylor, G. Nichols, G. Cangelosi and J. Bartram

12.1 INTRODUCTION

Traditionally, the role of the public health community is in prevention rather than treatment of disease. Increasingly, it has been recognized that the HACCP approach used extensively in the food industry (Doores 1999), may also be appropriate for prevention and control of waterborne disease (Deere *et al.* 2001). Water source protection, water treatment, disinfection and distribution all provide critical control points for applying the HACCP approach. Critical levels can be established through dose-response modelling and monitoring approaches developed for specific pathogens or surrogate markers of microbial contamination. The field of molecular epidemiology

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has begun to provide the tools to monitor for the presence of pathogens that have yet to be cultured (Rose & Grimes 2001).

However, in order to effectively use the HACCP approach for protection of public health from a specific waterborne pathogen, fundamental criteria need to be met. These include the following: 1) the pathogen needs to be established as a hazard to human health by the specific exposure path of interest; 2) dose-response studies need to be conducted to establish critical control levels; 3) effective monitoring techniques need to be available to evaluate those control levels; and 4) effective treatment must be available at each critical control point. To date, it is unclear whether this approach could be effective for control of diseases caused by environmental mycobacteria.

12.2 PUBLIC HEALTH RESPONSE

To date, the public health response to the environmental mycobacteria has been directed at disease management and not at prevention. This fact is not surprising, as current epidemiological data has failed to provide a convincing link between exposure to waterborne mycobacteria and disease. As a result, prevention of mycobacterial growth within water distribution systems and domestic hot water systems has received little attention. Most outbreaks that have been epidemiologically-linked to water have been small in scale, and none are directly linked to ingestion of tap water.

Although it is certainly true that data is as yet insufficient to provide a link between ingestion of tap water and mycobacterial disease, there is now considerable evidence that environmental mycobacteria present a health hazard through exposure of abraded skin to swimming pools, spas, hot tubs, footbaths, and aquaria (refer to Chapter 8). These exposures generally lead to skin and soft tissue infections (Collins *et al.* 1984). Inhalation of contaminated aerosols has also been linked to illnesses that range from hypersensitivity pneumonitis to pneumonia (Embril *et al.* 1997; Shelton *et al.* 1999; see also Chapter 9). The hospital environment also poses specific routes of exposure to environmental mycobacteria. Contaminated tap and deionized water used in bronchoscopy procedures has been linked to mycobacterial infections (see Chapter 10) that have been readily controlled by installation of microbiological filters in the water source (Stine *et al.* 1987; Graham *et al.* 1988). In the case of recreational and hospital exposures to mycobacteria-contaminated water, the management response has been relatively straightforward. For control of recreational exposures, rigorous adherence to appropriate disinfection treatment, including superheating of water and education to minimize abrasions, is recommended (WHO 2000). For hospital water, microbiological filtration at tap water sites has been relatively effective.

It is instructive to examine the public health response to *Legionella pneumophila*, an opportunistic pathogen with similar etiology. *L. pneumophila* is similar in many aspects to mycobacterial species in that it is truly an environmental pathogen, it is relatively resistant to water treatment and disinfection, it survives intracellularly within

amoebae, it survives within biofilms, it is prevalent within hot water systems and can be transmitted by the aerosol route, through direct ingestion or through skin abrasion (Kramer & Ford 1994). Table 12.1 lists the similarities and differences between these environmental pathogens.

Table 12.1 Similarities and differences between *Legionella* and *Mycobacterium* species.

	***Legionella* spp.**	***Mycobacterium* spp.**
Present in source waters	Yes	Yes
Resistant to treatment	Somewhat	Very
Survives intracellularly	Yes	Yes
Intracellular survival affects virulence	Yes	Yes
High mortality risk	Legionellosis, Yes (15%) Pontiac Fever, No	No (accept for HIV/AIDS)
Biofilm survival	Yes	Yes
Aerosol survival	Yes	Yes
Growth range	20-45 °C	Similar, depending on species
Maximum temperature	< 60 °C	Probably similar, depending on species[1]

[1] *M. tuberculosis* can survive at 60°C for 10 minutes, environmental mycobacteria may be even hardier

The public health response to *L. pneumophila* has been mixed, and its effectiveness is debatable. This is in part due to the relatively low rate at which legionellosis is diagnosed. It is thought today that a large percentage of hospital-acquired pneumonias are due to *L. pneumophila.* The CDC estimates that between 8000 and 18 000 people get Legionnaires disease each year in the United States, with mortality ranging from 5 to 30%. Legionella is one of the very few named organisms on EPA's National Primary Drinking Water Regulations List, with a Maximum Contaminant Level Goal of zero, and a Maximum Contaminant Level currently defined as a treatment technique that can effectively remove/inactivate *Giardia* and viruses. The rationale for including legionella on this list is relatively clear. Legionellosis has a high mortality risk and the routes of exposure are relatively clearly defined. It can be isolated from source and drinking-water, and outbreaks have been directly linked to cooling towers, whirlpools and showers. As a consequence, there are many resources available to public health practitioners seeking guidelines for minimization of health risks from *Legionella.* For example, Australia has published "Health (Legionella) Regulations 2001" that are specifically designed to:

- prescribe procedures for the maintenance and testing of cooling tower systems and warm water systems;
- require owners and people who have the management or control of cooling tower systems and warm water systems to keep records on the maintenance

and testing of those systems and to make those records available for inspection by an authorized officer on request;

- enable the Secretary to the Department of Human Services to—
  a) substitute different procedures in certain circumstances;
  b) require additional procedures to be undertaken when a system is suspected or implicated in the spread of the prescribed infectious disease, legionellosis.

Other countries have published their own guidelines and codes of practice; for example, the Health and Safety Commission of the United Kingdom has published an approved code of practice and guidance for Legionnaires disease (HSC 2001).

A number of resources are available for hospitals. In the United States, there are numerous web-based resources to allow hospitals to develop management plans for *Legionella*. In fact, the Joint Commission on Accreditation of Healthcare Organizations specifically includes minimization of risks from "organizational-acquired illness" in regulation JCAHO EC 1.7. This standard also requires that health care facilities are responsible for "management of pathogenic biological agents in cooling towers, domestic hot water, and other aerosolizing water systems." In the industrial setting, the OSHA provide links to information on Recognition, Evaluation, Control and Compliance. This includes information on cooling towers, heating, ventilation and air-conditioning systems, hospital control and specific industrial issues that include exposure of workers to *Legionella* in the plastic injection moulding industry and workers in contact with metal-working fluids (http://www.osha.gov/SLTC/legionnairesdisease/index.html).

The argument has apparently not yet been made for mycobacterial species, although there is clear evidence for outbreaks of disease related to many of the same exposure paths. Indeed, control for the mycobacteria may in part be achieved through control for *Legionella*, and it is certainly likely that utility management plans in hospitals that are designed to reduce risk from *Legionella* will reduce risks from the pathogenic mycobacteria in drinking and shower water, hydrotherapy, spa pools, etc. However, it should also be recognized that the mycobacteria are probably more resistant to disinfectants than *Legionella*.

12.3 MANAGEMENT OPTIONS

12.3.1 Drinking/bathing water

Management options for municipal water used for household uses such as drinking and bathing should be aimed at a level that minimizes risk to the most susceptible populations. In some countries, for example, pregnant women may bathe for extended periods (hours), particularly where hot water supply is centrally controlled and essentially free (Egorov, personal communication). The major problem is the inability

to quantify risk. A number of studies have shown colonization of water systems with environmental mycobacteria, including MAC (Glover *et al.* 1994; Montecalvo *et al.* 1994; von Reyn *et al.* 1994) and *M. gordonae* (Le Dantec *et al.* 2002). However, the epidemiological link between presence of MAC in potable water and disease has not been effectively made. In fact, major studies designed to address sources of disseminated MAC infection in people with HIV/AIDS have failed to find an association with home water, including home showering (Horsburgh *et al.* 1994; von Reyn *et al.* 2002). The more recent publication concludes that "MAC infection results from diverse and likely undetectable environmental and nosocomial exposures."

The undeniable presence of environmental mycobacteria in municipal water presents a number of exposure routes within the home, including drinking, bathing, food preparation and even toilet flushing (Arbeit, personal communication). However, without the epidemiological link with disease, the role of public health and water management is unclear. Water treatment itself is likely to be ineffective due to the high level of resistance of mycobacteria to disinfection (Le Dantec *et al.* 2002) and other environmental stressors such as starvation, desiccation and temperature extremes (Archuleta *et al.* 2002). The ability of mycobacteria to survive intracellularly within amoebae (Cirillo *et al.* 1997), and within biofilms (Hall-Stoodley *et al.* 1999), would additionally suggest that current treatment technologies are unlikely to remove mycobacteria from drinking-water, or prevent persistent colonization within the distribution system (see Chapter 11).

What then are the alternatives for water management? In the United States, MAC is listed on the CCL developed in 1998 to direct future regulatory efforts of the USEPA. It was not, however, included on the unregulated contaminant monitoring rule of 1999, presumably because the epidemiological link with disease had not been made. It remains to be seen as to whether MAC will remain on the CCL for 2003, or be considered in the second round of unregulated contaminant monitoring rule, scheduled for 2004. However, without a stronger public health connection, it will be difficult to justify its further regulation. The similarity between the etiology of environmental mycobacterial disease and the etiology of legionellosis is remarkable. *L. pneumophila* is a regulated contaminant in drinking-water, yet most outbreaks of legionellosis are linked to recreational, industrial and institutional exposures. The major difference between mycobacterial infections and legionellosis is the high mortality rates associated with the latter disease, the well-documented survival of *Legionella* in warm/hot water systems, and the now extensive number of epidemiological studies that link specific outbreaks to cooling tower aerosols.

As with *L. pneumophila*, the source of many environmental mycobacteria is likely to be potable water and exposure may predominantly occur through inhalation of contaminated aerosols or aspiration of drinking-water. Until a stronger epidemiological link is made, no definitive regulations are likely to be acceptable, or

even possible to implement. For the most susceptible populations, recommending point-of-use microbiological filtration provides the only management option to date.

12.3.2 Recreational water

Management options for recreational waters are relatively straightforward. In general, most outbreaks of mycobacterial infections (as with *Legionella*) can be related to overcrowding in the facility, insufficient disinfection, poor filter and piping maintenance. Swimming pools and hot tubs can be periodically shock-treated with much higher levels of disinfectants than can be used for a potable water system. A number of guides for disease prevention are available on-line and essentially provide comprehensive management instructions for both private and public swimming pools, hot tubs and spas (e.g. Freije 2000). Careful maintenance and cleaning schedules are critical, as piping and filter material can provide an ideal environment for microbial colonization. It is also suggested that bathers should be encouraged to avoid any behaviour that could result in scrapes and scratches (WHO 2000). It is probably also appropriate to caution immunocompromised individuals about their elevated health risks from recreational water exposure to mycobacteria (and other pathogens) (WHO 2000). Superheating water to 70 °C on a daily basis (Embil *et al.* 1997) can also be an effective mechanism for reducing environmental mycobacteria (as well as *Legionella*).

12.3.3 Industrial exposure

The industrial environment in the United States is regulated by OSHA. OSHA provides a wide range of resources for minimizing risks of worker exposure to *Legionella* (e.g. OSHA 1999). Environmental mycobacteria present similar problems, but may be harder to control in the work environment due to their greater resistance to disinfection, and the potentially wider range of environments that support mycobacterial growth.

The National Institute of Occupational Safety and Health have produced a criteria document 98-116 (NIOSH 1998) to recommend standards for occupational exposures to metal-working fluids. Although microorganisms, including *Legionella*, are mentioned as metal-working fluid contaminants, no exposure limits are currently established. This is likely to change in the near future given the increasing number of publications that link environmental mycobacteria (and most specifically *M. immunogenum*) to diseases such as hypersensitivity pneumonitis.

The specific issue of cooling towers raises interesting questions. Potentially pathogenic mycobacteria have been isolated from cooling tower water, but an epidemiological link with disease has not yet been established. As research on exposure routes to environmental mycobacteria continues, it is likely that cooling tower aerosols will be increasingly suspect as sources of infectious agents. If this is the

case, it will be instructive to see whether the extensive guidelines and regulations currently in place to reduce the legionellosis risks are equally effective in eliminating the more disinfection resistant mycobacteria.

12.3.4 Institutional exposure

Essentially, management of exposure to environmental mycobacteria in hospital and health care settings is similar to management for *Legionella.* Minimize the opportunities for exposure through hydrotherapy, showering and other hot water use. In addition, point-of-use filtration on tap water used for laboratory reagents, deionized water and other hospital uses has been shown to be very effective at minimizing pseudo-outbreaks linked to *M. avium, M. gordonae* and *M. scrofulaceum* (Stine *et al.* 1987; Graham *et al.* 1988). Infections linked to contaminated ice-water have also been effectively controlled by cleaning and disinfection of the ice machines (Labombardi *et al.* 2002; Gebo *et al.* 2002).

Control of *Legionella* in hot water systems has received considerable attention in recent years. Temperatures above 45 °C are considered to increase risks of scalding, yet they are also at the upper temperature range for growth for both *Legionella* and the mycobacteria. Control of these pathogens can be achieved in part by storage of hot water at temperatures that kill the organisms; generally above 60 °C for *Legionella* (although the organism has been isolated from thermal effluent at 63 °C) (Fliermans *et al.* 1981). To prevent risks of scalding, hot water has to be mixed with cold water to achieve a safe temperature prior to point of use. To reduce risks from both the thermotolerant environmental mycobacteria and *Legionella,* the United States CDC and the American Society of Heating Refrigeration and Air-conditioning Engineers recommends that hot water is stored above 60 °C and "circulated with a minimum return temperature of 51 °C" (CDC 2001; ASHRAE 2000). They also recommend that cold water is stored and distributed below 20 °C to minimize growth of these organisms. These guidelines are primarily based on studies with *Legionella,* and do not appear to have been rigorously tested for their effectiveness at reducing environmental mycobacterial infections.

12.3.5 The high risk groups

Both morbidity and mortality from environmental mycobacterial infections are dramatically amplified in high risk groups. As environmental mycobacteria are primarily opportunistic pathogens, this group requires the highest level of vigilance. However, it should be remembered that environmental mycobacterial diseases are increasing in populations with no predisposing factors; they have, therefore, become a public health concern for all groups. It is certainly reasonable to suggest that high risk groups (very young, elderly, immunocompromised, pregnant) should consider

minimizing exposure at public baths and spas. There are also reasonable arguments to suggest that the most susceptible should avoid consumption of tap water without point-of-use filtration (carefully regulated) or boiling. However, these are general recommendations to prevent any infection in these high risk groups and not specific for the mycobacteria.

There are specific groups of people in tropical countries who primarily through geographic distribution are susceptible to the devastating effects of Buruli ulcer (refer to Chapter 8). At present the public health response is limited to early detection and treatment as the exact routes of exposure are yet to be defined.

12.4 THE HACCP APPROACH TO MANAGEMENT

Can we identify a HACCP approach to managing environmental mycobacteria? The seven principles of HACCP begin with identification of hazards and preventative measures (Table 12.2).

The second step in the HACCP approach is to identify critical control points. As for any pathogen found in municipal water systems, there is a flow chart of potential critical control points from source to point of use/point of exposure: Source protection → optimization of water treatment → ensure residual disinfection → maintain distribution system integrity and water pressure → point-of-use protection (Ford 1999).

Table 12.2 Hazards linked to waterborne exposure to environmental mycobacteria and associated preventative measures

	Hazards	**Preventative measures**
Identify hazards and preventative measures	Exposure to contaminated aerosols	Disinfection and microbiological filtration. Placement of cooling towers, etc.
	Aspiration of contaminated water	Disinfection and microbiological filtration. Education in bathing habits and use of, for example, whirlpool tubs
	Ingestion of contaminated water	Disinfection, microbiological filtration and monitoring programs. Education in risks from tap-water consumption, particularly for the immunocompromised
	Infection of abraded skin	Education around swimming pools and spas. Appropriate disinfection, filtration or heating
	Transmission through aquatic insects (postulated for Buruli ulcer)	Insect control programs

Source minimization of environmental pathogens is difficult due to the multiple environmental pathways of source water contamination. In other words, point source control is unlikely to be effective, with the exception of MAP (see below). Coagulation/flocculation/filtration may be effective at reducing numbers of environmental mycobacteria in drinking-water distribution. However, chlorination is unlikely to be an effective method of control due to the chlorine resistance of the organisms, and their ability to survive and possibly proliferate in protozoa and biofilms (Cirillo *et al.* 1997; Field *et al.* 1997; Hall-Stoodley 1999). Given the inability of water treatment to provide 100% removal of microorganisms, the water treatment plant is unlikely to be the most effective means of environmental mycobacterial control.

The distribution system itself can become rapidly recontaminated through leakage, back-siphonage or cross-connections (Ford 1999). With ageing water distribution systems worldwide, the only effective pathogen control within the distribution system is maintenance of sufficient water pressure (to minimize back-siphonage or cross-connection through leaks) and high levels of residual disinfection. Again, with the environmental mycobacteria, residual chlorine is considered ineffective at inactivation. In contrast to many other waterborne pathogens, where regrowth within the distribution system is minimal or does not occur, point-of-use protection would appear to be the most important critical control point for environmental mycobacteria.

For organisms whose transmission path is thought to be primarily through the inhalation route, point of use/point of exposure includes public use of municipal pools and spas, showering, hot tub use, whirlpool baths and toilet flushing. In addition, although the epidemiological link does not appear to have been made for the mycobacteria, potentially PEM have been found in cooling tower water and, therefore, cooling tower aerosols are also a potential point of exposure. Clearly, in these instances, point-of-use filtration for the drinking-water faucet is insufficient and the management options discussed in the previous sections become the critical control points.

The next steps in the HACCP approach include establishing critical limits, effective monitoring, remediation responses, evaluation and documentation; however, we are still not at a point where even critical limits and effective monitoring can be established. For each environmental mycobacterial species, these critical limits will differ. They will also be dramatically affected by the susceptibility of the exposed population. Until research has allowed us to determine the appropriate ranges of critical limits, and quantitative monitoring of the different mycobacterial species is possible, critical control can only be based on rigorous cleaning, maintenance and disinfection programs – and the absence of disease.

MAP is a special case. The organism is known to be shed in the hundreds of millions in Johne-infected cattle faeces (see Chapter 6). There is clearly a role for water management in preventing run-off from cattle feedlots entering water sources. There are also extensive HACCP related guidelines to minimize the risks of JD in

cattle. Although designed to minimize disease and its economic implications in animal husbandry, these same guidelines should also be partially protective of water sources and the hypothesized connection with human disease.

12.5 KEY RESEARCH ISSUES

- The public health response to MAC has been directed at disease management and not at prevention.
- Current epidemiological data fails to provide a convincing link between ingestion of water and mycobacterial disease.
- The public health response to MAP has been primarily in support of "herd management", and a water route of exposure is not currently considered, due in part to the inability to conclusively describe the causative agent of CD.
- If comprehensive epidemiological studies can provide firm evidence that pathogenic mycobacteria are transmitted by the water route and contribute significantly to both morbidity and mortality, then the public health response is clear: 1) for drinking-water: regulations, treatment and monitoring are necessary; 2) for recreational exposures: regulations, treatment where possible, monitoring and education; 3) for institutional exposures: guidelines/strict regulations, prevention, treatment, monitoring and education; 4) for industrial exposure: guidelines/strict regulations, prevention, treatment, monitoring and education.
- For each of the above, critical control points should be identified in order to apply the HACCP approach to water management.
- Each of the above becomes particularly important where susceptible populations are involved.
- If the link between MAP and CD is firmly established, this has enormous implications for agricultural practice, and in particular discharge of wastewaters. The trend in recent years towards confined animal feeding operations has made the issue of surface and groundwater pollution particularly acute.

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Environmental mycobacteria can be found in diverse environments around the world and most appear to exhibit a saprophytic lifestyle. However, some have the ability to infect animals, birds and humans, and have evolved mechanisms by which they can invade and grow within host cells: the pathogenic environmental mycobacteria (PEM). Although some of the diseases caused by these organisms have been known for many years, it is only recently that the potential significance of PEM as waterborne pathogens has been appreciated.

Pathogenic Mycobacteria in Water describes the current knowledge of the distribution of PEM in water and other parts of the environment. The routes of transmission that lead to human infection are discussed and there is a detailed analysis of the most significant disease symptoms that can follow infection. Many species of PEM are difficult to isolate in culture and so detection and identification rely upon the use of modern techniques such as those based on selective nucleic acid amplification, such as PCR. The classical and modern methods of analysis are described. The book concludes with a discussion of the issues surrounding the control of PEM in drinking-water and the assessment and management of risks.

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