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

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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 carneau 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 (C₆₀-C₈₀), 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, IS901 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).

IS1245 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 IS1245 fingerprinting has been published (van Soolingen *et al.* 1998). It is not clear whether IS1245 fingerprinting alone will be sufficient to provide unambiguous evidence of identity of patient and environmental *M. avium* isolates. Results of IS1245 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 IS1245 pattern" (Lair *et al.* 1998). These studies, coupled with IS901 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; Khor *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; Mijls *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. IS901, IS1245, 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.