EPIDEMIOLOGY OF *MYCOBACTERIUM AVIUM* COMPLEX INFECTING AIDS PATIENTS

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ABSTRACT

Organisms of the *Mycobacterium avium* complex cause disseminated infections in 25 to 50% of patients with AIDS. To assess the likelihood of exposure to *M. avium*, we attempted to recover *M. avium* complex from environmental samples in geographical areas (Boston, Massachusetts; Hanover, New Hampshire; Helsinki, Finland; Nairobi, Kenya; and Kinsasha, Zaire) located near *M. avium* infected AIDS patients. Although *M. avium* was recovered from environmental samples at all sites, it was found more frequently in water supply systems in the United States and Finland (8/25, 32%) compared to water supply samples from Africa (0/14, 0%).

To determine if *M. avium* isolates recovered from the same geographical area as AIDS patients shared phenotypic and genetic characteristics with clinical AIDS *M. avium* isolates (recovered by collaborating laboratories), the ability to grow at 43°C, cadmium-

and streptomycin-resistance, and the presence of plasmids were used as epidemiological markers. We found that environmental isolates in this study shared similar characteristics with the clinical AIDS *M. avium* isolates.

Compared to developed countries, the prevalence of *M. avium* infections among AIDS patients in developing countries (i.e., Africa) is very low. To determine if M. avium was absent in the African environment, we attempted to recover the organisms from water and soil in Kampala, Uganda. *M. avium* was recovered from 43% of environmental samples, and these isolates shared similar phenotypic and genetic characteristics with *M. avium* isolates from the United States.

Cigararette smoking was identified as a possible risk factor for HIV infected individuals. *M. avium* isolates were recovered from several brands of cigarettes, suggesting that cigarettes are a possible source of infection.

Dedicated with love to my mother and brother

and

to the memory of my father

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Chapter 1

Introduction

MYCOBACTERIUM AVIUM, M. INTRACELLULARE AND *M. SCROFULACEUM*

Members of the genus *Mycobacterium* are gram-positive acid-fast bacilli (Wayne and Kubica, 1986). Early useful classification of the genus *Mycobacterium* was based on rate of growth, pathogenicity, and pigmentation (Runyon, 1959). Mycobacteria were divided into two divisions on the basis of growth rate; rapid and slow. Rapidly growing mycobacteria are those in which colonies (<1 mm diameter) appear in seven days or less. Slowly growing mycobacteria require greater than seven days for colony formation (Wayne and Kubica, 1986). Members of the *Mycobacterium avium-intracellulare-scrofulaceum* group (i.e., *M. avium, M. intracellulare, M. scrofulaceum*, MAIS) are classified as slow growing, acid-fast bacilli that may produce a yellow pigmentation in the absence of light (Wayne and Kubica, 1986).

Techniques that have been traditionally used for the speciation of putative *M. avium* complex (i.e., *M. avium, M. intracellulare*, MAC) isolates include biochemical and cultural characteristics (Wayne *et al.*, 1991), gas liquid and thin layer chromatography (Brennan *et al.*, 1982), HPLC analysis, DNA relatedness (Baess, 1979), and DNA probes (Saito *et al.*, 1990). *M. avium* and *M. intracellulare* phenotypically cannot be distinguished by biochemical and cultural tests (Good, 1985).

Through the development of non-isotopically labeled DNA probes, a rapid, specific, and safe method for the identification of members of the *M. avium* complex is

available. One such method, the SNAP Culture Identification Diagnostic Kit for *Mycobacterium avium* complex (Digene Diagnostics Inc., Silver Spring, MD), utilizes an alkaline phosphatase labeled single stranded DNA probe specific for *M. avium*, *M. intracellulare*, or *M. X*. *M. X* is a newly discovered, separate species belonging to the *M. avium* complex. Cells are lysed and the DNA is denatured and fixed onto a nylon membrane. Upon hybridization of the labeled probe to the test DNA, the enzyme activity of alkaline phosphatase produces a color change indicating a positive reaction. A second method, developed by Gen-Probe Inc. (San Diego, CA) employs chemiluminescence. When an acridium ester-labeled single stranded DNA probe forms a DNA:RNA hybrid with the target ribosomal RNA, a chemiluminescent reaction occurs upon the addition of an alkaline hydrogen peroxide solution. The excited acridone product decays to a non-excited state emiting light which is measured by a luminometer.

Mycobacterium avium and *M. intracellulare* include 28 known serovars (Wolinsky, 1992). DNA relatedness studies have assigned serovars 1 through 6, 8 through 11, and 21 to *M. avium* and serovars 7, 12 through 20, and 25 are *M. intracellulare* (Wolinsky, 1992). Strains isolated from AIDS and non-AIDS patients are most commonly serotypes 1, 4, 8 and 9 (i.e., *M. avium*) (Wolinsky, 1992).

EPIDEMIOLOGY OF *MYCOBACTERIUM AVIUM* COMPLEX

Members of the *M. avium* complex are opportunistic human pathogens whose

source of infection is the environment (Wolinsky, 1979). These pathogens have been recovered in high numbers from water (Falkinham *et al.*, 1980, Kirschner *et al.*, 1992), soil (Brooks *et al.*, 1984, Kirschner *et al.*, 1992) and aerosols (Wendt *et al.*, 1980) in the southeastern United States. These organisms are frequently found in man-made water systems (duMoulin and Stottmeier, 1986), and have been recovered from homes (Songer, 1980) and hospital hot water systems (duMoulin *et al.*, 1988). *M. avium* complex organisms have also been isolated from tuberculous lesions in cattle, swine and chickens in the United States (Thoen *et al.*, 1981). Unlike the "typical" mycobacterial infections caused by *M. tuberculosis* and *M. leprae*, there is no evidence that this non-tuberculous disease is communicable to the general population (Wayne and Sramek, 1992).

Although there is little information on the route of human infection, there is evidence which suggests that environmental and clinical *M. avium* complex isolates are related. *M. avium* complex isolates recovered from the environment have been shown to have similar phenotypic characteristics as non-AIDS isolates (Fry *et al.*, 1986). However, *M. avium* complex clinical isolates recovered from non-AIDS patients are more frequently able to grow at 43° C, without OADC enrichment, and are resistant to streptomycin and cadmium compared to *M. avium* complex environmental isolates. Only isolates from environmental aerosols share those characteristics which are found in clinical non-AIDS *M. avium* complex isolates (Fry *et al.*, 1986). Because pulmonary disease due to *M. avium* is the most common presentation of *M. avium* infection in

patients without AIDS (Wolinsky, 1992), entry of aerosolized *M. avium* isolates into the respiratory tract and penetration into bronchial mucosa is a possible mode of mycobacterial infection in these patients.

Plasmids are frequent among clinical non-AIDS and environmental *M. avium* complex isolates (Meissner and Falkinham, 1986). Plasmids were detected in a higher frequency among non-AIDS clinical (55%) and aerosol (75%) *M. avium* complex isolates as compared to the frequency of plasmids in *M. avium* complex isolates from soil (5%), sediment (<6%), dust (7%), and water (25%) (Meissner and Falkinham, 1986). Although the plasmid profiles of *M. avium* complex isolates are very heterogeneous, there is evidence that plasmids are shared among clinical non-AIDS and environmental isolates plasmids (Meissner and Falkinham, 1986). Because of the frequence of *M. avium* plasmids and their stablity, they are very useful as epidemiologic markers. Four plasmid groups have been identified among small plasmids (i.e., <25 kb) which are commonly found in *M. avium, M. intracellulare,* and *M. scrofulaceum* environmental isolates and isolates recovered from non-AIDS and AIDS patients (Crawford and Bates, 1986; Jucker Ph.D. dissertation, 1992). Jucker (1992) reported that 56% of clinical *M. avium* isolates from AIDS patients had plasmids which hybridized to plasmid group I, 67% had group II-related plasmids.

Although the role of plasmids is not fully understood, *M. avium* plasmids have been shown to encode genes for DNA restriction and modification enzymes (Crawford

et al., 1981), mercuric reductase activity (Meissner and Falkinham, 1983), and copper resistance (Erardi *et al.*, 1987).

EPIDEMIOLOGICAL MARKERS FOR *M. AVIUM*

While there have been several epidemiologic techniques developed to prove that the source of *M. avium* in AIDS patients is the environment, these techniques have not been successful due to several limitations.

Serotyping has been the most commonly used epidemiological marker. However, there are several factors which limit this method in epidemiologic studies. The seroagglutination test can only be used with smooth colony-forming strains. Rough colony-forming mycobacteria, which lack certain cell specific antigens, spontaneously agglutinate alone (Tsang *et al.*, 1983). Further, there are some smooth colony-forming isolates which react with more than one anti-serum (Grange *et al.*, 1989). Because some *M. avium* strains are not typable, there may be other serovars which have not yet been characterized. Although most types have been classified, there is still debate on the classification of types 22-24 and 26-28.

Phage-typing has proven to have limited utility in discriminating between strains of *M. avium-M. intracellulare* complex strains (Crawford *et al.*, 1981) although it has been used in the epidemiology of *M. tuberculosis* (Snider *et al.*, 1984). Crawford *et al.* (1981) attempted to phage-type several hundred *M. avium*, *M. intracellulare*, and *M.*

scrofulaceum strains and found that only one-third of the isolates were susceptible to the mycobacteriophages (Crawford *et al.*, 1981). Thus the lack of susceptibility of many strains of mycobacteria to phage infection is an important limitation in this technique.

There are several physiologic characteristics (e.g., cadmium and streptomycin resistance and the ability to grow at 43°C) which have been found to be useful epidemiologic markers. This is because of the high frequency of clinical *M. avium-M. intracellulare* isolates that are able to express these characters compared to environmental *M. avium* isolates (Fry *et al.*, 1986). Although phenotypic characters of *M. avium* may be useful in epidemiological studies, they are not rapid methods due to the time it takes to grow *M. avium* isolates.

Plasmids can also serve as epidemiologic markers of *M. avium* (Meissner and Falkinham, 1986; Crawford and Bates, 1986; Jucker and Falkinham, 1990). Plasmids are common among clinical AIDS and non-AIDS *M. avium* isolates (Crawford and Bates, 1986; Meissner and Falkinham, 1986), and are less frequent in environmental *M. avium* isolates (Meissner and Falkinham, 1986). Plasmids are not only important in determining a possible source of human *M. avium* infection, but they can also be used to rapidly identify patients with polyclonal *M. avium* infection. The stability of *M. avium* plasmids (Meissner and Falkinham, 1986), the uneven distribution of plasmids among clinical and environmental *M. avium* isolates, and the ability to detect rapidly *M. avium* plasmids through the use of DNA probes, provides an ideal tool for epidemiologic studies.

However, plasmids may be limited for use in epidemiologic studies because not all *M. avium* strains carry plasmids.

The separation of large restriction fragments of mycobacterial genomic DNA by pulsed-field gel electrophoresis has been shown to be a useful technique in distinguishing between strains of *M. avium*. Because a specific banding pattern is generated for each isolate, it has been demonstrated that strains of *M. avium* cause both monoclonal (Mazurek *et al.*, 1993) and polyclonal (Arbeit *et al.*, 1993) infections in patients with AIDS. In addition, isolates recovered from a single patient have been shown to be unique to that patient (Mazurek *et al.*, 1993). However, the disadvantage to this technique is that the analysis of the banding patterns is laborious.

An insertion sequence, IS901, has been useful in distinguishing between pathogenic strains of *M. avium* complex. Analysis of strains of M. *avium* isolated from clinical, veterinary, and environmental sources for the presence of the mycobacterial insertion sequence demonstrated that IS901 is specifically associated with animal pathogenic *M. avium* complex strains (Kunze *et al.*, 1992). In contrast, most clinical non-AIDS *M. avium* strains, all AIDS-derived strains and all environmental *M. avium* isolates lacked IS901 (Kunze *et al.*, 1992). Analysis of *M. avium* complex strains for the presence of absence of IS901 may be useful in the determination of possible host-ranges and virulence properties.

M. AVIUM DISEASE IN NON-AIDS PATIENTS

Prior to the AIDS epidemic, *M. avium* was more frequently associated with human disease than any of the other non-tuberculous mycobacteria. Although infections with nontuberculous mycobacteria were not reportable in the United States (Inderleid *et al.*, 1993), approximately 33.2% to 73.3% of all pathogenic *Mycobacterium* isolates recovered belonged to the *M. avium* complex (Good and Snider, 1982). In patients without AIDS, the *M. avium* complex causes pulmonary disease, lymphadenitis and, rarely, disseminated disease (Wolinsky, 1992). *M. avium* infection was found in patients with predisposing conditions such as pheumoconioses, previous tuberculosis, chronic bronchitis and emphysema, bronchiectasis, and malignancies (Wolinsky, 1979). Because of the growing population of individuals who are immunocompromised due to cancer, organ transplant, or steroid administration, there has been an increase in the incidence of disseminated disease due to *M. avium* complex since 1986 (Wolinsky, 1992).

M. AVIUM DISEASE IN AIDS

In the United States and Europe, 25-50% of persons with late stage HIV infections are infected with organisms of the *M. avium* complex (Ellner *et al.*, 1991). Disseminated *M. avium* infections most commonly occur in HIV positive patients whose CD4 counts are < 10 per mm^3 (39%) (Horsburg *et al.*, 1992). Further, 47-50% of patients at autopsy were found to have been infected with *M. avium* complex (Armstrong *et al.*, 1985).

Common symptoms of disseminated *M. avium* infection include persistent fever, night sweats, fatigue, anorexia or chronic wasting syndrome, malaise, anemia, and diarrhea, (Wolinsky, 1992; Hawkins *et al.*, 1986). Although all organs of the body may be infected with *M. avium*, the gastrointestinal tract, bone marrow, lymph nodes, spleen, and liver are more commonly infected (Wolinsky, 1992). Studies have indicated that in AIDS patients the primary route of entry may be the gastrointestinal tract (Damsker, 1985). The adsorption of *M. avium* onto intestinal epithelial cells has been demonstrated *in vitro* and may be due to an immune defect that is unique to HIV-infected persons (Mapother and Sanger, 1984). Colony counts may range from 10^1 to as high as 10^6 colony-forming units/ml (CFU/ml) of whole blood. Possibly, infected tissue may contain 10^2 to 10^5 times the number of *M. avium* than circulating blood (Hawkins *et al.*, 1986).

Treatment of *M. avium* complex infection in patients with AIDS is often difficult because of the the drug-resistance of the organism (Horsburgh *et al.*, 1985). *M. avium* is often resistant to standard anti-TB drugs. However, susceptibility to anti-mycobacterial drugs such as rifabutin, clofazimine, ciprofloxacin, ofloxacin, imipenem/cilastatin, rifapentine, cycloserine, amikacin ethambutol, roxithromycin, clarithromycin and azithromycin has been observed *in vitro* (Cynamon and Klemens, 1989).

M. AVIUM DISEASE IN DEVELOPING COUNTRIES

The frequency of *M. avium* infection among AIDS patients in developing countries

is considerably lower than that among AIDS patients in developed countries (Okello *et al.*, 1990). Indeed, in two studies of 50 late stage AIDS patients (Okello *et al.*, 1990) and 45 AIDS patients (Morrissey *et al.*, 1992) in Uganda, *M. avium* was absent. This suggests that the incidence of disseminated *M. avium* infections among African patients with AIDS does not exist. There are several possible explanations for the absence of *M. avium* in African AIDS patients: (1) *M. avium* complex is absent from African soil and water, (2) *M. avium* isolates in the African environment lack a characteristic which makes them virulent in late stage HIV patients, (3) African AIDS patients are different in some way from AIDS patients in developing countries which makes them resistant to *M. avium* infection, (4) African AIDS patients are dying of other infections before infection by *M. avium* occurs. Confirmation of the hypothesis that *M. avium* complex is absent from the African environment could be accomplished by attempting to recover these organisms from environmental samples collected from Africa. Although there is little known about characteristics of *M. avium* complex isolates associated with virulence, there is evidence that certain physiologic (i.e., cadmium-, and streptomycin-resistance, and growth at 43°C) and genetic (i.e., plasmids, serotype, and IS901) characters are more frequently associated with clinical *M. avium* isolates than environmental isolates (Fry *et al.*, 1986; Crawford and Bates., 1986; Meissner and Falkinham., 1986; Kunze *et al.*, 1992). Therefore, analysis of *M. avium* complex isolates for these characters would be useful in determining if African *M. avium* complex isolates express similar characters as *M. avium*

complex isolates recovered from areas in which the prevalence of disseminated *M. avium* infections is higher.

Specific Objectives

1. Isolate, enumerate, and identify *M. avium* complex organisms from water, soil, and other environmental samples collected by collaborating laboratories from the local environment of AIDS patients in Boston, Massachusetts; Hanover, New Hampshire; Helsinki, Finland; Nairobi, Kenya; and Kinshasa, Zaire.

2. Determine the frequency of *M. avium* complex isolates recovered from AIDS patients (sent from collaborating laboratories) and from environmetal samples able to grow at 43° C and which are cadmium- and streptomycin- resistant.

3. Determine the presence or absence of plasmids of homology groups I and II in *Mycobacterium avium* complex isolates recovered from AIDS patients and their environment.

4. Isolate, enumerate, and identify *M. avium* complex organisms recovered from water and soil from Kampala, Uganda. Determine the frequency of Ugandan *M. avium* complex isolates that are able to grow at 43°C, and which are cadmium- and streptomycin-resistant. Determine the presence or absence of plasmids of homology groups I and II and IS901.

5. Determine if cigarettes are a possible risk factor to patients with late stage HIV infections by attempting to isolate *Mycobacterium avium* complex from cigarette fractions.

Chapter 2

Isolation of *Mycobacterium avium* complex from water samples collected in the United States, Finland, Zaire and Kenya.

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INTRODUCTION

Disseminated mycobacterial disease is probably the most common bacterial infection associated with the current epidemic of the acquired immunodeficiency syndrome (AIDS) (Ellner *et al.*, 1991). Despite the high prevalence of *M. avium* infection among AIDS patients in developed countries, disseminated *M. avium* has not been found in clinical and autopsy studies of AIDS patients in Africa (Okello *et al.*, 1990; Morrisey *et al.*, 1992). *M. avium* organisms are widely distributed in nature and have been isolated from environmental waters (Falkinham *et al.*, 1980), as well as municipal (duMoulin and Stottmeier, 1986) and hospital hot water systems (duMoulin *et al.*, 1988). Although no specific source of *M. avium* infection in humans is known, studies indicate that water and aerosols are the most probable sources of infection (Horsburg, 1991). Because of daily activities which involve the use of water (i.e., showering, drinking, recreational uses of natural sources, etc.), exposure to *M. avium* by patients with AIDS is inevitable, thus explaining the 25 to 50% of AIDS patients that are infected with the organism. However, the absence of disseminated *M. avium* infection in African AIDS patients may be due to (1) low numbers or the absence of *M. avium* in the African environment, (2) differences in *M. avium* isolates from Africa which make it less virulent in immunocompromised individuals, (3) differences in African AIDS patients that makes

them resistant to *M. avium* infection or the (4) death of African AIDS patients of other infections before being infected with *M. avium*.

In this study, *M. avium* was recovered from environmental samples from developed countries and developing countries. The locations sampled represent areas where the prevalence of disseminated *M. avium* infection in AIDS patients is high (United States and Finland) and low (Kenya and Zaire). By evaluating the locations of water samples containing *M. avium* we were able to assess the likelihood of exposure by humans to *M. avium*.

MATERIALS AND METHODS

Collection of water samples - Water samples were collected from municipal water supplies as well as environmental sites such as puddles, streams, lakes, springs, and rivers. Samples were collected in sterile 250 ml wide-mouth autoclavable, plastic bottles at ambient temperature and shipped to Virginia Tech for processing.

Processing of water samples - Twenty-five ml samples were centrifuged at 5,000 x *g* for 30 min at 22°C in sterile 50 ml screw cap centrifuge tubes. The supernatant was decanted and its pH measured. The pellet was suspended in 1 ml of sterile distilled water and 0.1 ml of the liquid was spread to dryness onto Tsukamura-based Tween 80 Complex (TTC) (George and Falkinham, 1986) agar medium plates containing 0.073 M KH_2PO_4, 0.002 M $MgSO_4 \cdot 7H_2O$, 0.02 M $(NH_4)_2SO_4$, 1% (v/v) Tween 80 (Sigma Chemical Co., St. Louis, MO) and 1.5% agar (BBL Microbiology Systems, Cockeysville, MD). The media was adjusted to a pH of 5.5 with KOH, and sterilized by autoclaving. To prevent fungal growth, cycloheximide (Sigma Chemical Co., St. Louis, MO) at 500 mg/liter was added to cooled, sterile medium before plates were poured. After the liquid was spread the plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37°C. Colony formation was observed daily up to 30 days.

Isolation

Identification of Mycobacteria - Acid-fast colonies were identified by Ziehl-Neelson staining. Those acid-fast colonies of the same morphology were counted and representatives streaked on Lowenstein-Jenson (LJ; BBL Microbiology Systems, Cockeysville, MD) or Middlebrook and Cohn 7H10 (M7H10; BBL Microbiology Systems, Cockeysville, MD) agar slants and incubated at 37°C. Growth, as judged by the appearance of colonies, was followed daily for up to 60 days. Rapidly growing mycobacteria (i.e., growth occurring by 7 days) were discarded. Slowly growing mycobacteria (i.e., growth in more than 7 days) were kept for identification. Pigmentation was noted and isolates were then grown in 10 ml Middlebrook and Cohn 7H9 broth (M7H9; BBL Microbiology Systems, Cockeysville, MD) containing 10% oleic acid albumin (OAA) for identification by DNA probes. Isolates were also streaked on Middlebrook and Cohn 7H10 agar medium (M7H10; BBL, Microbiology Systems, Cockeysville, MD) plates to insure a pure culture.

Putative *M. avium* complex isolates were identified using the SNAP DNA Probe following the manufacturers instructions (Digene Diagnostics INC., Silver Spring, MD). Organisms which hybridized with the MAC probe were then tested with probes to distinguish between *M. avium*, *M. intracellulare*, and *M. X*.

RESULTS

Recovery of *M. avium* complex isolates. *M. avium* complex isolates were recovered from the five study sites as listed in Table 1. The numbers of *M. avium* complex positive samples varied between the developed countries and the developing countries: the United States and Finland had higher numbers of *M. avium* complex positive samples (18/52, 35%) compared to samples from Zaire and Kenya (4/39, 10%). Concentrations of mycobacteria were similar between all geographic areas tested and ranged from 0.2-1000 cfu/ml with median concentrations of 0.4 to 40 cfu/ml. *M. avium* complex were recovered at concentrations of 1,000 cfu/ml in two environmental samples: the Charles River (Boston) and lake water from a bucket in a Finnish sauna.

***M. avium* complex in hospital and clinic water systems.** Among water supply systems, *M. avium* complex was recovered in higher numbers in the United States and Finland (8/25, 32%) compared to the fourteen clinic and hospital water samples tested from Africa (0/14, 0%). Both hot (4/11, 36%) and cold (3/12, 25%) water systems from the United States and Finland yielded *M. avium* complex.

Table 1. Recovery of *Mycobacterium avium* by geographic site and sample type.

SITE	No. of MAC positive samples/No. of samples (%)		
	ENVIRONMENTAL[b]	WATER SUPPLY[c]	TOTAL
New Hampshire	2/13 (15%)	1/9 (11%)	3/22 (14%)
Boston	5/13 (38%)	2/8 (25%)	7/21 (33%)
Finland	3/8 (37%)	5/11 (45%)	8/19 (42%)
Zaire	1/3 (33%)	1/5 (20%)	2/8 (25%)
Kenya[a]	3/31 (10%)	0/24 (0%)	3/55 (5%)
TOTAL	14/68 (20%)	9/57 (16%)	23/125 (18%)

[a] Includes 5 water samples collected from northern Tanzania.

[b] Environmental samples collected from rivers, streams, lakes, ponds, harbors, marshes, and standing water.

[c] Water supply samples were collected from wells, hot and cold municipal supplies, showers, and standpipes.

Table 2. Recovery of MAC from water collected from patient care facilities (clinics and hospitals).

	No. of MAC positive samples/No. of samples (%)		
SITE	**HOT WATER**	**COLD WATER**	**TOTAL**
New Hampshire	1/3 (33%)	0/3 (0%)	1/6 (17%)
Boston	1/3 (33%)	0/4 (0%)	1/7 (14%)
Finland	2/5 (20%)	3/5 (60%)	5/10 (50%)
Zaire	0/1 (0%)	0/2 (0%)	0/3 (0%)
Kenya	0/3 (0%)	0/8 (0%)	0/11 (0%)
TOTAL	4/15 (27%)	3/22 (14%)	7/37 (19%)

DISCUSSION

Although *M. avium* has previously been shown to be present in environmental samples from Africa and the United States (Falkinham *et al.*, 1980; Morrissey *et al.*, 1992), this survey is the first to compare isolation rates of *M. avium* from such diverse geographical areas. Isolatation frequencies of *M. avium* were higher in environmental samples from developed countries (10/34) than in developing countries (4/34) which can be attributed to the higher numbers of positive samples from piped water supply systems (i.e., water used for drinking and bathing). The exposure of humans to piped water is expected to be higher than the exposure to water from rivers, lakes and other environmental sources. Therefore, the higher isolation rates of *M. avium* from piped water supplies in the United States and Finland compared to lower rates in African waters is especially significant since many patients with late stage HIV from developed countries are more likely to be hospitalized and exposed to hospital water. The use of galvanized pipes made with zinc alloys by some hospitals may explain the higher numbers of *M. avium* since *M. avium* numbers correlate with higher zinc concentrations of <0.75 mg/L (Kirshner *et al.*, 1992).

In this study, we found that *M. avium* were most often recovered from rivers and streams than from standing water sources. Quite possibly these types of environmental sources are higher in certain elements (i.e., zinc) which are more conducive to the growth

of *M. avium* (Kirshner *et al.*, 1992). Thus, *M. avium* infection in humans could also be the result of the recreational use of natural waters as well as exposure to aerosols from these environmental waters. These water supplies could also serve as the source for municipal water supplies.

Chapter 3

Characterization of *Mycobacterium avium* complex isolates recovered from AIDS patients and their environments

INTRODUCTION

Disseminated infection caused by *M. avium* occurs in 25-50% of patients with late stage HIV infections in the United States (Hawkins *et al.*, 1986). These organisms are found throughout the environment (Falkinham *et al.*, 1980), and have been recovered in high numbers from waters (Falkinham *et al.*, 1980; Kirschner *et al.*, 1992) and soils (Brooks *et al.*, 1984; Kirschner *et al.*, 1992). Although the environment is thought to be the source of infection (Wolinsky, 1979), it has not been proven that *M. avium* isolated from the environment are directly responsible for infection in humans.

In order to prove that environmental *M. avium* are capable of human infection, it is important to compare characteristics of *M. avium* recovered from AIDS patients with *M. avium* isolates recovered from the immediate environment of the patient. Epidemiologic markers are specific characteristics (i.e., growth, physiologic, or genetic) that can be used to compare organisms. Thus, one can determine if two isolates are related. Previous studies comparing physiologic characteristics of clinical non-AIDS and environmental *M. avium* isolates determined that, because of the high frequency of clinical isolates that were able to grow at 43°C, and were resistant to cadmium and streptomycin, these characteristics would be useful as epidemiologic markers for *M. avium* and *M. intracellulare* (Fry *et al.*, 1986).

Characterization

Meissner and Falkinham (1986) determined that the plasmid DNA content of *M. avium* complex isolates could serve as useful epidemiologic markers because plasmids are common in *M. avium* clinical isolates and are stable during culture (Meissner and Falkinham, 1986). *M. avium* small plasmids pVT2 (Jucker and Falkinham, 1992) and pLR7 (Crawford and Bates, 1984) have been cloned previously and have been shown to hybridize with plasmids from clinical AIDS and non-AIDS *M. avium* isolates and environmental *M. avium* isolates (Crawford and Bates, 1984; Jucker and Falkinham, 1992). In this study, resistance to cadmium (Cd) and streptomycin (Sm), the ability to grow at 43°C and the presence of plasmids were used as epidemiologic markers to compare environmental and clinical *M. avium* isolates recovered from AIDS patients.

MATERIALS AND METHODS

Mycobacterial isolates - Environmental isolates used in this study were isolated from environmental water and soil samples collected by collaborating laboratories, shipped to Virginia Tech and processed as previously described (Falkinham *et al.*, 1980; Brooks *et al.*, 1984).

Growth at 43°C, and Cd and Sm susceptiblity - The ability of each isolate to grow at 43°C, in the presence of streptomycin and cadmium, and on Middlebrook and Cohn 7H9 agar plates without enrichment was measured as previously described (Fry *et al.*, 1986). One loop, containing 0.01 ml culture was streaked on the surface of Middlebrook and Cohn 7H10 agar (M7H10; BBL Microbiology Systems, Cockeysville, MD) medium containing 10% oleic acid albumin (OAA) enrichment in duplicate. One plate was incubated at 43°C and one at 37°C for 2 wk. Streptomycin resistance was measured on M7H10 agar medium containing a final concentration of antibiotic at 10 μg/ml and incubated at 37°C for 2 wk. Cadmium resistance of each isolate was determined on M7H10 agar medium plates containing 10^{-4} M $CdCl_2 \cdot 2\ 1/2\ H_2O$ and incubated at 37°C for 5 wk. M7H9 plates without enrichment were prepared and growth at 37°C was measured after 2 wk. Minimal growth only at the point of inoculation without isolated colonies was scored as no growth.

Characterization

Plasmid DNA hybridization - Plasmids pLR7, cloned and described by Crawford and Bates (1984), and pVT2, cloned and described by Jucker and Falkinham (1992), were radiolabeled with ^{35}S-dCTP using the Random-Primer DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN) according to manufacturers instructions.

Isolates were analyzed by dot blot as described by Via (1993). Cells were grown in 10 ml M7H9 broth containing 10% OAA enrichment to a density of 5 X 10^7 cells per ml and harvested by centrifugation (5,000 x *g* for 10 min at room temperature and suspended in 1 ml TE buffer. To the cell suspension was added 0.3 ml 2N NaOH and 1 gram of 0.1 mm dia glass beads and cells broken by 90-sec agitation in a Mini-Bead Beater (Bio-Spec Products, Baitersville, OK). The suspension was centrifuged at 5,000 x *g* for 15 min and the DNA in 0.4 ml of the supernatant liquid was loaded into each well of a Dot Blot apparatus (Bio Rad Laboratories, Richmond, CA) and impacted onto the surface of a Zeta-Probe (Bio-Rad, La Jolla, CA) membrane wetted with 2X SSC (Maniatis *et al.*, 1982) by vacuum. The membrane was removed and placed on a piece of 0.4 N NaOH-soaked filter paper for 5 min. The membrane was then rinsed once in 2X SSC and allowed to dry completely. The membrane was incubated in a hybridization mix containing 50% formamide, 0.5% Blotto, 4X SSPE, and 1% SDS at 50°C for at least 24 hr (Sambrook *et al.*, 1989). Denatured probe was added and allowed to hybridize to the membrane-bound DNA at 50°C for 24 hr. Following a series of washes in (i.e., 2X SSC in 0.1% SDS, 1X SSC in 0.1% SDS, 0.5X SSC in 0.1% SDS, 0.1X

SSC in 0.1% SDS, and 0.1X SSC in 1% SDS (Maniatis *et al.*, 1982), the membrane was dried and exposed to autoradiography film (Amersham) for 3-5 days.

RESULTS

Phenotypic characteristics of *M. avium* isolates. Listed in Table 1 are the phenotypic characteristics of environmental *M. avium* complex isolates recovered from Boston, Massachusetts; Dartmouth, New Hampshire; Helsinki, Finland; Nairobi, Kenya and Kinsasha, Zaire. Eighty-two percent of the isolates were able to grow at 43°C, 68% were resistant to 1 mM cadmium, and 96% were resistant to 10 μg streptomycin/ml (Table 1). Although environmental isolates in developed countries (i.e., United States and Finland) seemed to have phenotypic characteristics similar to those of environmental isolates from developing countries (i.e., Kenya and Zaire), this may be due to the small sample size of isolates from Kenya and Zaire.

Listed in Table 2 are the phenotypic characteristics of clinical *M. avium* isolates recovered from AIDS patients in Boston, Massachusetts; Dartmouth, New Hampshire; Helsinki, Finland; and Nairobi, Kenya. No clinical isolates were recovered from AIDS patients in Kinsasha, Zaire. Ninety-seven percent of the clinical isolates were able to grow at 43°C, 50% were resistant to 1 mM cadmium, and 61% were resistant to 10 μg streptomycin/ml (Table 2). Although there was no difference in the ability of *M. avium* isolates from the United States and Finland to grow at 43°C compared with Kenyan clinical *M. avium* isolates, a significant difference was observed in the isolates from the

United States that were resistant to cadmium (60%) compared to cadmium-resistant isolates from developing countries (0%) (Table 2). However, Finnish and Kenyan clinical isolates were similar because all were susceptible to cadmium. In addition, the percentage of streptomycin-resistant isolates was higher among isolates from developed countries (84%) compared to the percentage of streptomycin-resistant colonies from developing countries (28%). However, a similar percentage of Dartmouth clinical isolates (29%) were resistant to streptomycin compared to clinical isolates from Kenya (28%). Ninety percent of isolates from the United States and Finland were able to grow on M7H10 agar plates without oleic acid albumin enrichment (OAA) compared to 57% of the Kenyan *M. avium* isolates (Table 2).

Plasmids present in *M. avium* isolates. Listed in Table 3 are the numbers of environmental *M. avium* isolates that had plasmids able to hybridize to *M. avium* plasmid homology groups I and II. Among the 28 strains that were screened, 9 (32%) had a group I-related plasmid and 13 (46%) had a group II-related plasmid (Table 3). Eight (29%) strains had representative plasmids from both groups I and II. However, 14 (50%) of the environmental isolates hybridized to neither plasmid homology group. There was only a slight difference in the number of isolates containing plasmids from the United States (58%) compared to the number of plasmid containing isolates from developing countries (50%) (Table 3). However, the small sample size from Kenya and Zaire could

be a factor in the percentages of plasmid containing isolates observed. Environmental isolates from Finland were unusual in that there were no group I-related plasmids, only 22% hybridized to group II-related plasmids, and 78% of the isolates did not contain a plasmid that hybridized to either group (Table 3).

Listed in Table 4 are the plasmids related to homology groups I and II that were present in clinical *M. avium* isolates recovered from AIDS patients. Plasmids from groups I and II were found in 75 of the 161 (47%) isolates tested from United States AIDS patients (Table 4). Plasmids related to groups I and II were rare in Finnish clinical isolates (2 of 33, (6%)) and Kenya clinical isolates (2 of 9, (22%)). Although clinical *M. avium* isolates from developing countries carried group I- and II-related plasmids, the percentage of isolates that hybridized to group II (22%) was lower than group II-related plasmids in clinical isolates from developed countries (36%) (Table 4). Group I-related plasmids present in clinical *M. avium* isolates in developed and developing countries was not significantly different (Table 4).

Table 1. Phenotypic characteristics of environmental *M. avium* complex isolates recovered from each study site.

SITE	TOTAL	PHENOTYPE			
		$43°C^{R\,a}$ # (%)	$Cd^{R\,b}$ # (%)	$Sm^{R\,c}$ # (%)	OAA [d] # (%)
Boston	12	9 (75%)	7 (58%)	12 (100%)	8 (67%)
Dartmouth	3	2 (67%)	1 (33%)	2 (67%)	3 (100%)
Finland	9	9 (100%)	8 (89%)	9 (100%)	6 (67%)
Kenya	2	2 (100%)	2 (100%)	2 (100%)	0 (0%)
Zaire	2	1 (50%)	1 (50%)	2 (100%)	2 (100%)
TOTAL	28	23 (82%)	19 (68%)	27 (96%)	19 (68%)

[a] Growth at 43°C

[b] Growth in 1mM Cd^{2+}

[c] Growth in 10 μg streptomycin/ml

[d] Growth in M7H10 + glycerol

Table 2. Phenotypic characteristics of clinical *M. avium* isolates recovered from AIDS patients at each study site.

SITE	TOTAL	PHENOTYPE			
		43°C[a] # (%)	Cd^{R} [b] # (%)	Sm^{R} [c] # (%)	No OAA[d] # (%)
Boston	108	103 (95%)	61 (56%)	78 (72%)	94 (87%)
Dartmouth	51	50 (98%)	34 (66%)	15 (29%)	47 (92%)
Finland	23	23 (100%)	0 (0%)	21 (91%)	23 (100%)
Kenya	7	7 (100%)	0 (0%)	2 (28%)	4 (57%)
Zaire	0	-	-	-	-
TOTAL	189	183 (97%)	95 (50%)	116 (61%)	168 (89%)

[a] Growth at 43°C

[b] Growth in 1mM Cd^{2+}

[c] Growth in 10 μg streptomycin/ml

[d] Growth in M7H10 + glycerol

Table 3. Plasmids present in environmental *M. avium* complex isolates.

SITE	TOTAL	PLASMIDS			
		pVT2[a] # (%)	pLR7[b] # (%)	BOTH # (%)	NEITHER # (%)
Boston	12	5 (42%)	7 (58%)	5 (42%)	5 (42%)
Dartmouth	3	2 (67%)	2 (67%)	1 (33%)	0 (0%)
Finland	9	0 (0%)	2 (22%)	0 (0%)	7 (78%)
Kenya	2	1 (50%)	1 (50%)	1 (50%)	1 (50%)
Zaire	2	1 (50%)	1 (50%)	1 (50%)	1 (50%)
TOTAL	28	9 (32%)	13 (46%)	8 (29%)	14 (50%)

[a] Representative Group I plasmid cloned by Jucker and Falkinham, 1992.

[b] Representative Group II plasmid cloned by Crawford and Bates, 1984.

Table 4. Plasmids present in clinical *M. avium* isolates.

SITE	TOTAL	PLASMIDS			
		pVT2[a] # (%)	pLR7[b] # (%)	BOTH # (%)	NEITHER # (%)
Boston	110	25 (23%)	46 (49%)	19 (17%)	58 (53%)
Dartmouth	51	9 (18%)	21 (41%)	7 (14%)	28 (55%)
Finland	33	0 (0%)	2 (6%)	0 (0%)	31 (94%)
Kenya	9	2 (22%)	2 (22%)	2 (22%)	7 (78%)
Zaire	0	-	-	-	-
TOTAL	203	36 (18%)	71 (35%)	28 (14%)	124 (61%)

[a] Representative Group I plasmid cloned by Jucker and Falkinham, 1992.

[b] Representative Group II plasmid cloned by Crawford and Bates, 1984.

DISCUSSION

Previous work has shown that 90% of clinical non-AIDS isolates were able to grow at 43°C, 54% were resistant to cadmium, 83% were resistant to streptomycin and 95% were able to grow without enrichment. In the same study, 30% of environmental *M. avium* isolates tested were able to grow at 43°C, 21% were cadmium resistant, 35% were resistant to streptomycin, and 41% were able to grow without enrichment (Fry *et al.*, 1986). In the study conducted by Fry, a significantly higher percentage of environmental isolates was able to grow at 43°C and was resistant to cadmium and streptomycin than was observed previously (Fry *et al.*, 1986) and these percentages were similar to those for clinical AIDS isolates that were tested for the same characters. It would be expected that the environmental isolates in the present study would share physiologic characteristics similar to the clinical AIDS isolates tested because these environmental isolates were recovered from samples to which the AIDS patients may have exposed to (i.e., hospital water systems, municipal water, local rivers, ponds, lakes and streams). Physiologic characteristics were similar among environmental *M. avium* isolates from all 5 study sites. However, clinical isolates from Kenya were less resistant to cadmium and streptomycin than isolates from the United States and Finland. This number may be due to the small sample size of clinical isolates from developing

countries. Finnish environmental and clinical AIDS isolates shared similar characteristics.

Isolates were also analyzed for the presence of plasmids related to *M. avium* plasmid homology groups one and two. Plasmids related to plasmid homology group II were more commonly found among the environmental and clinical isolates screened than group I related plasmids. These results are consistant with those reported previously (Jucker and Falkinham, 1992). Plasmid contents were not significantly different among any of the isolates from the United States and Africa; however, a high percentage of clinical and environmental isolates from Finland carried neither plasmid groups I or II. Mercury-resistance in *M. scrofulaceum* has been associated with plasmid DNA (Meissner and Falkinham, 1986) and Fry *et al.* (1986) speculated that resistance to cadmium and the ability to grow without enrichment (OADC) may be plasmid-encoded. Chi-square analysis (with Yates' correction for continuity) was used to determine whether any epidemiologic markers (i.e., growth at 43 °C, cadmium- and streptomycin-resistance) may correlated with plasmid DNA. Cadmium resistance in clinical *M. avium* isolates was observed to be associated with the presence of either a group I plasmid or group II plasmid. No other association between plasmid DNA and the epidemiologic markers was observed.

Chapter 4

Isolation and Characteristics of *Mycobacterium avium* complex from Water and Soil Samples in Uganda

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Uganda

INTRODUCTION

M. avium complex organisms are opportunistic human pathogens whose source of infection is the environment (Wolinsky, 1979). Though disseminated *M. avium* infection occurs in 25-50% of United States AIDS patients (Hawkins *et al.*, 1986), disseminated *M. avium* infection is absent in Ugandan AIDS patients (Okello *et al.*, 1990; Morrissey *et al.*, 1992). This could be due to (1) an absence of *M. avium* in the Ugandan environment, (2) the absence of a route of infection of Ugandan AIDS patients, (3) differences in Ugandan AIDS patients that make them resistant to *M. avium* infection or (4) factors which lead to death of Ugandan AIDS patients before *M. avium* infection. To test the hypothesis that *M. avium* are absent in the Ugandan environment, we attempted to recover *M. avium* complex isolates from water and soil samples collected in Kampala, Uganda. The methods of *M. avium* recovery used in this study have been successfully used to recover isolates from water and soil in the United States (Falkinham *et al.*, 1980; Brooks *et al.*, 1984; Kirschner *et al.*, 1992) and we were able to obtain a limit of detection of <0.02 cfu/ml of sample.

Further, to determine if Ugandan environmental *M. avium* complex isolates were different than AIDS related *M. avium* isolates from the United States, the isolates were characterized with regard to biotype (i.e., ability of isolates to grow in the presence of

streptomycin and cadmium and at 43°C) and the presence of plasmids and IS901 (Kunze *et al.*, 1992). Previous studies have demonstrated that the frequency of clinical non-AIDS *M. avium* isolates that were able to grow at 43°C and were cadmium- and streptomycin-resistant was higher than in environmental *M. avium* isolates (Fry *et al.*, 1986) and that clincial isolates more frequently carried small plasmids (Meissner and Falkinham, 1986). The presence of IS901 was screened for because it has only been found in *M. avium* isolates recovered from animals (Kunze *et al.*, 1992) and is not expected to be found in these isolates.

Although 28 serovars are known for *M. avium* and *M. intracellulare* (Wolinsky, 1992), only a few serotypes predominate in human disease. A predominance of serotypes 1, 4, 8, and 9 are seen among strains isolated from AIDS and non-AIDS patients (Wolinsky, 1992). Because of the wide use of the seroagglutination test as an epidemiological marker and the association of certain serotypes with human disease, we determined the serotypes of the Ugandan environmental *M. avium* isolates.

METHODS

Collection of water and soil samples - Samples were collected in 200 ml plastic tubes at ambient temperature, and shipped to Virginia Tech for *M. avium* complex isolation.

Processing of water samples - Twenty-five ml samples were centrifuged at 5,000 x *g* for 30 min at 22°C in sterile 50 ml screw cap centrifuge tubes. The supernatant was decanted and its pH measured. The pellet was suspended in 1 ml of sterile distilled water and 0.1 ml of the liquid was spread to dryness onto TTC agar medium (George and Falkinham, 1986) in triplicate. The plates were sealed with Parafilm (American Can Co., Greenwich, CT) and incubated at 37°C for up to 6 weeks.

Processing of soil samples - Five grams of each soil sample were suspended in 25 ml sterile, 0.1X Nutrient Broth (Difco Laboratories, Detroit, MI) in a sterile screw cap 50 ml centrifuge tube. The suspensions were shaken for 30 min at 120 oscillations/min and sonicated (60 sec) immediately after suspension and at 10 min intervals in a Branson Ultrasonic Cleaner (Branson Cleaning Equipment Co., Shelton, CT). Following the final sonication, the suspensions were centrifuged at 1,000 x *g* for 10 min at room temperature, and 20 ml of the supernatant was transferred to a sterile 50 ml screw cap centrifuge tube

and centrifuged at 5,000 x *g* for 30 min at room temperature. The supernatant solution was discarded and the pellet suspended in 1.0 ml sterile distilled water by vortexing. One tenth ml was spread onto TTC agar medium in triplicate and incubated at 37°C for up to 6 weeks.

Determination of soil pH - Five gm wet weight of soil was suspended in 5 ml distilled water and shaken at 120 oscillations/min at room temperature. The pH of the slurry was measured.

Identification of Mycobacteria - Acid-fast colonies were identified by Ziehl-Neelson staining. Those acid-fast colonies of the same morphology were counted and representatives streaked on Lowenstein-Jenson (BBL Microbiology Systems, Cockeysville, MD) agar slants and incubated at 37°C. Growth, as judged by the appearance of colonies, was followed daily for up to 60 days. Rapidly growing mycobacteria (i.e., growth occurring by 7 days) were discarded. Slowly growing mycobacteria (i.e., growth in more than 7 days) were kept for identification. Pigmentation was noted and isolates were then grown in 10 ml of Middlebrook and Cohn 7H9 broth medium (M7H9; BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid albumin (OAA) for identification by DNA probe. Isolates were also streaked on Middlebrook and Cohn 7H10 medium (M7H10; BBL Microbiology Systems,

Cockeysville, MD) plates containing 10% OAA to insure a pure culture.

Putative *M. avium* complex isolates were identified using the SNAP DNA Probe following the manufacturers instructions (Digene Diagnostics INC., Silver Spring, MD).

Growth at 43°C, and Cd and Sm susceptibility - The ability of each isolate to grow at 43°C, and in the presence of 10 μg streptomycin/ml and 1 mM cadmium was measured as previously described (Fry *et al.*, 1986).

Plasmid DNA hybridization - Cloned *M. avium* plasmid pLR7 (Crawford and Brooks, 1984), the cloned fragments of *M. avium* plasmid pVT2 (Jucker and Falkinham, 1992) and the clone containing IS901 (Kunze *et al.*, 1992) were radiolabeled with ^{35}S-dCTP using the Random-Primer DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN) according to manufacturers instructions and then denatured (Maniatis *et al.*, 1982).

Dot-Blot Hybridization - Isolates were analyzed by dot blot as described (Via, 1993). Cells were grown in 10 ml Middlebrook and Cohn 7H9 broth (M7H9; BBL Microbiology Systems, Cockeysville, MD) medium containing 10% OAA to early log phase (5 X 10^7 colony forming units/ml) and harvested by centrifugation (5,000 x *g* for 10 min at room temperature) and suspended in 1 ml TE buffer (Maniatis *et al.*, 1982). To the cell suspension was added 0.3 ml 2N NaOH and 1 gram of 0.1 mm dia. glass beads and

broken by 90 sec agitation in a Bio Spec Mini-Bead Beater (BioSpec Products, Bartlesville, OK). The suspension was centrifuged at 5,000 x *g* for 15 min and the DNA in 0.4 ml of the supernatant liquid was loaded into each well of a Dot Blot Apparatus (Bio Rad Laboratories, Inc., Richmond, CA) and impacted onto the surface of a Zeta-Probe membrane wet with 2X SSC (Maniatis *et al.*, 1982) by vacuum. The membrane was removed and placed on a piece of 0.4 N NaOH-soaked filter paper for 5 min. The membrane was then rinsed once in 2X SSC and allowed to dry completely. The membrane was incubated in a hybridization mix containing 50% formamide, 0.5% Blotto, 4X SSPE, and 1% SDS (Maniatis *et al.*, 1982) at 50°C for at least 24 hr. Denatured probe was added and allowed to hybridize to the membrane-bound DNA at 50°C for 24 hr. Following a series of washes (i.e., 2X SSC in 0.1% SDS, 1X SSC in 0.1% SDS, 0.5X SSC in 0.1% SDS, 0.1X SSC in 0.1% SDS, 0.1X SSC in 1.0% SDS (Maniatis *et al.*, 1982) , the membrane was dried and exposed to autoradiography film (Amersham Life Science, Arlington Height, IL) for 3-5 days.

Serotyping - *M. avium* positive samples were serotyped using standard procedures by Mitchell Yakrus and colleages at the Centers for Disease Control (CDC), Atlanta, Georgia.

RESULTS

Recovery of *M. avium* complex isolates. Listed in Table 1 are the sample sites in Kampala, Uganda from which soil and water samples were collected. Six of 14 (42.8%) environmental samples yielded *M. avium* (Table 1). *M. avium* was found in 3 of 7 (42.8%) water and 3 of 7 (42.8%) soil samples (Table 1). An average of 3.3 colony forming units (cfu)/ml water with a range of 1-6 cfu/ml was recovered (Table 1). Soil samples yielded an average of 7,825 colony forming units (cfu)/gm soil with a range of 50-25,000 cfu/ml *M. avium* isolates recovered (Table 1).

Effects of pH on the recovery of *M. avium* complex. *M. avium* was recovered in higher numbers of soil samples that had a pH of >7.0 (Table 1) compared to soil samples of higher or lower pH. Water pH was not appreciably associated with the cfu/ml of *M. avium* in water (Table 1).

Characteristics of *M. avium* complex isolates. Listed in Table 2 are the phenotypic characteristics of the 8 Ugandan environmental *M. avium* complex isolates. Four of 7 (57%) were able to grow at 43°C, 5 of 7 (71%) were resistant to 1 mM cadmium (Cd) and 5 of 7 (71%) were resistant to 10 μg streptomycin (Sm)/ml (Table 2).

Uganda

Plasmids which hybridized to pVT2 (Jucker and Falkinham, 1990) were found in 5 of 7 (71%) of Ugandan environmental isolates and 6 of 7 (86%) of the isolates carried plasmids which hybridized to pLR7 (Crawford and Bates, 1984) (Table 3). IS901, which is only found in *M. avium* isolates recovered from animals (Kunze et al., 1992), was not found in any of the isolates (Table 3).

Serotypes of *M. avium* complex isolates. Listed in Table 3 are the serotypes of *M. avium* complex isolates from Uganda. Three of 7 isolates were serotype 9, one was serotype 4 and one was serotype 8. Two of the isolates were untypeable.

Table 1. Recovery of *Mycobacterium avium* complex isolates from Kampala, Uganda.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE NUMBER	CFU/ML
Logogo Stream	water	7.1	none	<0.2/ml
Mulago Hosp.	water	7.3	UW4-6	1/ml
Gayaza Road	water	6.1	none	<0.2/ml
Gayaza Spring	water	5.4	UW7-8	2/ml
Lake Nabugaba	water	6.0	UW9-16T	6/ml
Nabugaba puddle	water	6.8	none	<0.2/ml
Kololo	water	6.6	none	<0.2/ml
SUBTOTAL WATER				3.3/ml
Logogo	soil	6.3	none	<0.2/gm
Mulago Hosp,	soil	5.8	US3-9	50/gm
Gayaza Road	soil	6.6	none	<0.2/gm
Gayaza Spring	soil	6.1	US8-24	1,250/gm
Lake Nubugaba	soil	5.9	none	<0.2/gm
Nubugaba	soil	4.5	none	<0.2/gm
Kololo	soil	7.0	US14-13	5,000/gm
			US14-14	25,000/gm
SUBTOTAL SOIL				7,825/gm

Uganda

Table 2. Phenotypic characteristics of *M. avium* complex isolates from Uganda.

	PHENOTYPE		
ISOLATE	**43°C**[a]	**Cd^R** [b]	**Sm^R** [c]
UW4-6	+	+	+
UW4-14T	-	-	-
UW7-8	+	+	+
UW9-16T	+	+	+
US8-24	-	-	-
US3-9	-	+	+
US14-13	+	+	+
US14-14			

[a] growth at 43°C

[b] growth in 1mM Cd^{2+}

[c] growth in 10 μg streptomycin/ml

Table 3. Small plasmids and IS901 present in Ugandan *M. avium* complex isolates and the serotype of each isolate.

ISOLATE	SPECIES	SEROTYPE	PLASMID		
			pVT2[a]	pLR7[b]	IS901[c]
UW4-6	*M. avium*	8	+	+	-
UW4-14T		NEG.	+	+	-
UW7-8		NEG.	+	+	-
UW9-16T		9	+	+	-
US8-24		9	-	-	-
US3-9		4	+	+	-
US14-13		9	-	+	-
US14-14					

[a] Group I representative plasmid cloned by Jucker and Falkinham, 1992.

[b] Group II representative plasmid cloned by Crawford and Bates, 1984.

[c] Insertion sequence cloned by Kunze *et al.*, 1992.

DISCUSSION

The objective of this study was to determine if *M. avium* complex organisms exist in the African environment. Assuming that the environment of Kampala, Uganda is representative of the African environment, the results of this study demonstrate that *M. avium* is present in water and soil in Africa and is present in numbers that are similar to concentrations of *M. avium* found in the environment of the southeastern United States (Falkinham *et al.*, 1980; Kirschner *et al.*, 1992; Brooks *et al.*, 1984). However, concentrations of *M. avium* complex found in Ugandan soils were significantly higher with 7,825 cfu/ml recovered (Table 1). Compared to concentrations of *M. avium* found in the soils of the southeastern United States, the numbers of *M. avium* in Ugandan soils is at least one order of magnitude higher (Brooks *et al.*, 1984). Therefore, it seems unlikely that the lack of *M. avium* infection in Ugandan AIDS patients (Morrissey *et al.*, 1992) is not due to the absence of *M. avium* organisms in the environment.

Our earlier studies had shown that higher numbers of *M. avium* were found in waters and soils of low pH (Falkinham *et al.*, 1980; Kirschner *et al.*, 1992), but this was not observed in the present study (Table 1). Possibly, the sample size here was too small to be truly representative of the actual numbers of *M. avium* present in the Ugandan environment.

Uganda

Previous studies demonstrated that a significantly higher number of clinical *M. avium* isolates were able to grow at 43°C and in the presence of cadmium and streptomycin than water, soil, and aerosol isolates from the United States (Fry *et al.*, 1986). Ugandan *M. avium* isolates also shared characteristics that are found in clinical *M. avium* isolates recovered from non-AIDS patients in the United States (Fry *et al.*, 1986). The majority of Ugandan *M. avium* isolates were able to grow at 43°C and were cadmium- and streptomycin-resistant. Small plasmids, which are characteristically found in AIDS isolates (Crawford and Bates, 1986; Jucker and Falkinham, 1990), were also found in both soil and water *M. avium* isolates. Differences in characteristics (i.e., the ability to grow at 43°C, resistance to cadmium and streptomycin, and the presence of pVT2 and pLR7 related plasmids) were not observed between soil and water isolates in this study. There was also no correlation between the ability to grow at 43°C, cadmium and streptomycin resistance and the presence of plasmids. Two of the isolates were serovars 4 and 8 which are the most commonly found *M. avium* serovars found in AIDS patients. However, 3 of the isolates were *M. avium* serovar 9 which is not commonly found in *M. avium* infected AIDS patients. Based on phenotypic and genetic characteristics, and results of seroagglutination, *M. avium* isolates from the Ugandan environment are similar to those recovered from United States AIDS and non-AIDS patients.

As demonstrated here, the lack of *M. avium* infections among Ugandan AIDS

patients is not due to the absence of *M. avium* organisms in the Ugandan environment. There may be other factors affecting the virulence of the organism that have not been explored here. Also, because of the higher incidence of infection by other pathogens (i.e., *M. tuberculosis*), the patient may die first of other infections. Finally, the numbers of *M. avium* that are present in municipal water systems (although not included in this study) may be too low to cause infection.

Chapter 5

Recovery of *Mycobacterium avium* from Cigarettes

INTRODUCTION

Disseminated *M. avium* infections are the most common systemic bacterial infection among AIDS patients in the U.S. (Ellner, 1991). Previous studies have proposed that the environment is the source of infection (Wolinsky, 1979), and *M. avium* has been isolated from natural water and soils (Falkinham *et al.*, 1980; Brooks *et al.*, 1984). However, a specific source of *M. avium* or environmental risk factors which would increase the likelihood of *M. avium* infection have not yet been identified.

Preliminary results from a prospective, multi-site study of the epidemiology of *M. avium* infection in AIDS patients has identified cigarette smoking as a risk factor (C.F. von Reyn *et al.*, in preparation). Accordingly, we sought to determine if *M. avium* could be recovered from cigarettes.

MATERIALS AND METHODS

Processing of cigarettes - *Mycobacterium avium* complex isolates were recovered from three brands of cigarettes: Marlboro Light's (Philip Morris Inc., Richmond, Va), Benson and Hedges 100 Light's (Benson and Hedges, Richmond, Va), and Winston (RJ Reynolds Tobacco Co., Winston-Salem, NC). Packages were opened with gloves to ensure that *M. avium* in the laboratory did not contaminate the cigarettes. Some cigarettes were also smoked employing a sterilized vacuum apparatus.

Using a sterilized razor blade, the cigarettes were divided into the following fractions: (1) filter, (2) paper enclosing filter, (3) tobacco and (4) paper enclosing tobacco. The filter, filter paper, and tobacco paper were suspended in 5 ml sterile 0.068M phosphate buffer containing 0.85% (wt/vol) NaCl, 0.01% (wt/vol) gelatin, and 0.05% (vol/vol) Tween 80. The tobacco was weighed and suspended in 10 ml of the same buffer in a sterile screw cap centrifuge tube. Using a sterile glass rod, the fractions were pulverized and then shaken at 120 oscillations/min for 2 hours at room temperature. The cigarette extract was separated from the material by filtration through a sterilized, sintered glass-filter holder (Millipore Corporation, Bedford, MA), collected in sterile plastic centrifuge tubes and the volume measured. The filtrate was centrifuged at 5,000 x *g* for 20 min at 4°C, the supernatant liquid discarded, and the pellet suspended in 1 ml

of the extraction buffer. One-tenth ml of each cigarette extract was spread to dryness on TTC agar medium (George and Falkinham, 1986) (5 plates), and plate count agar medium (Difco, Detroit, MI) in duplicate.

To measure the possible inhibition of *M. avium* colony formation by tobacco, paper, or filter extracts, 0.1 ml of each extract was spread with 0.1 ml *M. avium* strain LR25 suspension diluted to yield 200 colonies/0.1 ml.

All media were incubated at 37°C and examined for at least 30 days for colony formation.

Identification of Mycobacteria - Acid-fast colonies were identified by Ziehl-Neelson staining and those acid-fast colonies of the same morphology were counted and representative single colonies streaked on Middlebrook and Cohn 7H10 medium slants (M7H10; BBL Microbiology Systems, Cockeysville, MD) and incubated at 37°C. Growth, as judged by the appearance of colonies, was followed for up to 60 days. Rapidly growing mycobacteria (i.e., growth in less than 7 days) were discarded. Slowly growing mycobacteria (i.e., growth in more than 7 days) were kept for identification. Pigmentation was noted and isolates were then grown in 5 ml Middlebrook and Cohn 7H9 broth (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% glycerol and 10% oleic acid albumin (OAA) for identification by DNA probe. Isolates were also streaked on Middlebrook and Cohn 7H10 agar medium (M7H10; BBL Microbiology Systems,

Cockeysville, MD) plates containing 10% OAA to insure a pure culture. *Mycobacterium avium* complex isolates were identified using DNA probes following the manufacturers' instructions (Gen-Probe Inc., San Diego, CA or Syngene Inc., San Diego, CA).

RESULTS

The data in Table 1 demonstrate that *M. avium* can be recovered from cigarettes. *M. avium* was found in filters from Winston cigarettes at a concentration of 333.3 colony-forming units (cfu)/ml and in tobacco paper extracts from Benson and Hedges (0.5 cfu/ml) and Marlboro Lights (1.0 cfu/ml). There were no *M. avium* isolates recovered from filter paper or tobacco extracts from any of the brands of cigarettes tested (Table 1). Although some of the isolates recovered from TTC medium were spread with *M. avium* strain LR25, those isolates were different strains judging by colony morphology and pigmentation. They were confirmed as distinct isolates by biochemical and cultural characteristics and plasmid DNA profiles (data not shown).

Table 1. Recovery of *M. avium* isolates from cigarettes.

CIGARETTE	FRACTION	ISOLATE	CFU/ml EXTRACT	SPECIES
Benson & Hedges	tobacco	none	<0.4	
	tobacco paper	BH1-TP-1	0.5	*M. avium*
	filter	none	<0.6	
Marlboro	tobacco	none	<0.3	
	tobacco paper	M1-TP-1	1.0	*M. avium*
	filter	none	<0.6	
Winston	tobacco	none	<0.3	
	tobacco paper	none	<0.5	
	filter	W1-F+-3	333.3	*M. avium*

DISCUSSION

The numbers of *M. avium* found in the different extracts may be underestimates of the actual numbers present. *M. avium* strain LR25 spread on TTC agar medium with some cigarette extracts had fewer colonies than the unexposed control. However, because the extracts were not sterilized, it is impossible to report exact values for survival. On TTC agar medium spread with the LR25 suspension and Benson and Hedges tobacco extract, only 5 colonies appeared. By contrast 280 colonies were on the control. However, on plates spread with LR25 and filter, paper and tobacco extracts from Marlboro Lights and Winston, more than 280 colonies grew.

Mycobacteria have been found to adhere to cellulose acetate reverse osmosis membranes used for the treatment of water in municipal systems (Ridgeway *et al.*, 1984). Cigarette filters, which are subjected to a washing process prior to the assembly of the cigarettes, would be able to collect mycobacteria from the water since *M. avium* has been found in both natural (Falkinham *et al.*, 1980) and municipal water (duMoulin and Stottmeier, 1988) systems.

Because of the increased risk for pulmonary infections of HIV-infected patients, cigarette smoking poses a significant health hazard (Polsky *et al.*, 1986). Cigarette smoking not only impaires pulmonary defenses but also damamges lung parenchyma

(Burack, 1992) providing a portal of entry for *M. avium* organisms. Patients with AIDS often present with respiratory infection and it is quite possible that *M. avium* present in cigarettes is the source of the infection. Mycobacteria present in any part of the cigarette, but especially the cigarette filter, can be inhaled during smoking and enter the repiratory tract. Therefore, cigarette smoking is a possible risk factor for AIDS patients.

Chapter 6

Summary

Summary

Although the presence *M. avium* complex in the environment has been well established (Falkinham *et al.*, 1980; Brooks *et al.*, 1984), there has not been a study which focuses on the immediate environment of the AIDS patient. One of the main objectives of this study was to recover *M. avium* complex isolates from the local environments of AIDS patients and compare the characteristics of these isolates with *M. avium* complex isolates recovered from patients with AIDS. Because the prevalence of disseminated *M. avium* infection varies in different areas of the world, we sought to establish this variation by comparing the frequency of recovery and the numbers of *M. avium* complex from 5 geographically diverse locations. We also wanted to demonstrate that *M. avium* complex isolates recovered from areas where the disease is less prevalent among AIDS patients (i.e., Africa) share characteristics similar to those of environmental isolates in other areas of the world. Although the frequency of recovery and the numbers of *M. avium* complex were much lower in Africa, we were able to isolate *M. avium* complex organisms from the African environment. Thus, the low incidence of disseminated *M. avium* infection among African AIDS patients is not due to its absence in African water and soil. It is especially noteworthy that *M. avium* complex was not recovered from hospital and patients care facilities in Africa in contrast to the frequent isolation of *M. avium* complex from these municipal sources in the United States and Finland. Because of the severe immunosuppression of the AIDS patient during prolonged

hospital admission in developed countries, it is possible that *M. avium* is acquired from the hospital environment. Methods of municipal water treatment in Africa and types of water pipes used in African hospitals may offer an explanation to the absence of *M. avium* complex in hospital water systems in Africa.

Through the utilization of epidemiologic markers, we were able to show that the environmental isolates tested here resemble clinical AIDS *M. avium* isolates in their physiologic characteristics (i.e., ability to grow at 43°C, and resistance to streptomycin and cadmium) and plasmid DNA content. This provides further evidence for the hypothesis that the source of *M. avium* infection is the environment. Further, we demonstrated that isolates recovered from areas where the disease is less prevalent (i.e., Kenya and Zaire) are similar to those from areas where disseminated *M. avium* is most prevalent (i.e., United States and Finland).

A second objective was to isolate *M. avium* complex from the Ugandan environment. Because there have been no Ugandan AIDS patients identified with disseminated *M. avium* infection (Okello *et al.*, 1990; Morresey *et al.*, 1992), it was necessary to characterize Ugandan environmental isolates and compare them with environmental isolates recovered from locations in which *M. avium* disease in AIDS patients is more prevalent. The results of this comparison indicated that *M. avium* complex isolates recovered from the Ugandan environment are similar to those environmental isolates found in the United States and Finland. Further studies should be

initiated to determine why AIDS patients in Africa are not infected by *M. avium*. It is not known if the characters used as epidemiologic markers in this study (i.e., ability to grow at 43°C, streptomycin- and cadmium-resistance, and the presence of plasmids from homology groups I and II) are are associated with virulence. An increase in the knowledge of *M. avium* virulence mechanisms and the identification of virulence associated characters would enable us to determine why African AIDS patients are not infected by *M. avium* present in the African environment. Also, information on the differences in the African AIDS patients themselves or in their lifestyles may also provide the necessary clues.

The identification of risk factors is important in the prevention of infection by *M. avium*. In addition to hospital and municipal water systems and several environmental sources, cigarette smoking has also been identified as a risk factor. A survey of the *M. avium* positive AIDS patients revealed that most smoked cigarettes. Although *M. avium* complex isolates found in cigarettes have not yet been directly linked to *M. avium* isolates found in AIDS patients, the cessation of smoking may be neccesary to decrease the risk for disseminated *M. avium* infection.

The development of a rapid and sensitive technique for the detection of *M. avium* complex in the environment of the AIDS patient is needed. Methods used here for the detection and enumeration of environmental *M. avium* complex can take up to 4 weeks because of the slow growth of *M. avium* and our limit of detection is <0.02 colony

forming units (cfu)/ml. The polymerase chain reaction (PCR) has previously been used for the direct detection of mycobacteria from patient samples (Hance *et al.*, 1989). PCR has been used to amplify a portion of the 65-kD heat shock protein gene (Rodrigo *et al.*, 1992; Bollet *et al.*, 1992) and by cleaving these amplified products with specific restriction endonucleases, specific species of mycobacteria can be identified. *M. avium* can be detected in water samples with a considerably shorter time using this method (Hensley and Shumann, unpublished).

It is also important to determine a specific number of mycobacteria present in an environmental sample which is significant to cause infection in immunocomprimised patients. With standard methods of enumerating *M. avium* from environmental samples we were able to attain a limit of detection of <0.2 cfu/ml. However, it is not clear whether these numbers are capable of establishing infection in these patients. To do this, HIV positive *M. avium* negative patients would have to be observed in a closed environment in which controlled numbers of mycobacteria were present.

A recent report in which patterns of large restriction fragments (LRF) of genomic DNA were compared, revealed that 2 of 13 AIDS patients with disseminated *M. avium* complex infection were infected with multiple *M. avium* isolates (Arbeit *et al.*, 1993). While analyzing clinical strains for the presence of plasmid groups I and II, we observed polyclonal infections among 41% of our clinical isolates. Detection of plasmid DNA by dot blot is a rapid and repoducible way of determining if individual patients are infected

with more that one *M. avium* isolate. Because of the difficulty in successfully treating *M. avium* infection, the knowledge of polyclonal infection could be used to select for the most effective antibiotics for chemoprophylaxis.

Pulsed field gel electrophoresis (PFGE) based analysis was used by our colleagues to further compare the clinical and environmental *M. avium* isolates (von Reyn *et al.*, in preparation). Analysis of the large restriction fragment profiles resolved by PFGE of several patients and environmental isolates revealed an exact match therefore identifying an exact source of *M. avium* infection for the patient (von Reyn *et al.*, in preparation). These findings are significant for several reasons: (1) the identification of specific sources of *M. avium* infection may permit the modification of behavior in order to reduce the risk of infection by AIDS patients (i.e., avoidance of exposure to specific environmental sources known previously to infect AIDS patients), (2) there may also be implications for the treatment of *M. avium* infection (i.e., determination of antibiotic susceptibilities on a specific type of *M. avium* isolate causing infection in AIDS patients would aid in choosing more effective chemotherapeutic drugs).

Appendix

Appendix

TABLE A. Recovery of *Mycobacterium avium* isolates from Boston, Massachusetts water samples.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Boston City Hospital, Med 2, cold	WS	6.7	none	<0.2
Boston City Hospital, Med 2, hot	WS	6.7	none	<0.2
Charles River	E	6.5	E1503-2	1.4
			E1503-4	1.4
Jamaica Pond	E	6.7	none	<0.2
Puddle	E	7.0	none	<0.2
Boston Harber	E	7.2	none	<0.2
Boston City Hospital, clinic cold	WS	6.7	none	<0.2
Boston City Hospital, clinic hot	WS	6.5	E1508-1	2.4
			E1508-2	0.8
			E1508-3	1.0
Homeless Shelter	WS	7.0	E1509-1	3.6
			E1509-2	0.2

Table A. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
			E1509-4	0.4
Charles River	E	7.0	E1510-2	0.8
Boston Harbor	E	8.2	none	<0.2
Squantum Marsh	E	7.4	none	<0.2
Jamaica Pond	E	8.5	none	<0.2
Provincetown Harbor	E	7.3	none	<0.2
Provincetown Municipal	WS	6.6	none	<0.2
AIDS Hospice cold	WS	6.7	none	<0.2
AIDS Hospice hot	WS	6.5	none	<0.2
Charles River	E	6.8	E1518-4	0.8
Boston Harbor	E	7.2	none	<0.2
Jamaica Pond	E	7.8	E1520-2	0.2
Fenway Marsh	E	6.9	E1521-4	20
Boston YMCA Pool	P	7.2	none	<0.2
L Street Baths	P	6.9	none	<0.2
South Block Pool	P	7.7	none	<0.2
Boston YWCA Pool	P	7.8	none	<0.2

Table A. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
S. Block Pool	P	7.3	none	<0.2
S. Block Pool scum	P	7.0	none	<0.2
S. Block Pool shower	MS	7.1	none	<0.2
S. Block Pool fauwcet	MS	7.0	none	<0.2
S. Block Pool drain	P	7.4	none	<0.2
L Street Shower	MS	7.2	none	<0.2
L Street Shower	MS	7.2	none	<0.2
L Street Scum	P	6.0	none	<0.2
YMCA Pool surface	P	7.6	none	<0.2
YMCA Pool scum	MS	6.7	none	<0.2
YMCA shower cold	MS	7.2	none	<0.2
YMCA shower hot	MS	7.2	none	<0.2
YWCA Pool surface	P	7.7	none	<0.2
YWCA scum	P	6.7	none	<0.2
YWCA shower cold	MS	7.0	none	<0.2
YWCA shower hot	MS	6.9	none	<0.2

Appendix

Table B. Recovery of *Mycobacterium avium* complex isolates from Hanover, New Hampshire samples.

SAMPLE SITE	SAMPLE TYPE	pH	ISOLATE NUMBER	CFU/ML
Old DHMC cold	MS	6.5	none	<0.1
Old DHMC hot	MS	7.0	E5502-1	5.2
Conn River	E	6.6	E5503-1	0.4
Occom Pond	E	6.4	none	<0.1
Vermont Roadside	E	6.9	none	<0.1
Vermont Bog	E	7.3	none	<0.1
Well Water cold	MS	6.7	none	<0.1
Pond Hartland VT	E	7.0	E5508-1 E5508-2	30 0.8
White River	E	7.3	none	<0.1
Municipal cold	MS	7.6	none	<0.1
New DHMC cold	MS	7.6	none	<0.1
New DHMC hot	MS	7.5	none	<0.1

Table B. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Fntn Youth Pool	P	7.6	none	<0.2
Dartmouth Pool	P	7.4	none	<0.2
CCB Pool	P	7.5	none	<0.2
CCB Hot Tub	P	7.5	none	<0.2
CCB Pool Scum	P	7.1	none	<0.2
Ftn Youth Scum	P	8.1	none	<0.2
Woodstock scum	P	7.4	none	<0.2
Dartmouth scum	P	7.0	none	<0.2
CCB Pool water	P	7.7	none	<0.2
Fnt Youth Pool	P	7.4	none	<0.2
Woodstock Pool	P	8.0	none	<0.2
Dartmouth Pool	P	7.8	none	<0.2
Puddle Hartland	E	7.4	none	<0.2
Pond Hartland	E	7.3	none	<0.2
Well	MS	7.3	none	<0.2
White River	E	8.0	none	<0.2

Table B. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
AIDS DHMC cold	MS	8.0	none	<0.2
AIDS DHMC hot	MS	7.8	none	<0.2
Vermont Bog	E	7.3	none	<0.2
Occum Pond	E	7.1	none	<0.2
Conn River	E	7.6	none	<0.2
Conn river Scum	E	5.3	none	<0.2

Appendix

Table C. Recovery of *Mycobacterium avium* complex isolates from Helsinki, Finland water samples.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Helsinki Hosp cold	MS	lost	none	<0.2
Helsinki hosp hot	MS	6.7	none	<0.2
Kerava River	E	lost	none	<0.2
Espoo Lake	E	6.5	none	<0.2
Laajasalo Pond	E	5.4	none	<0.2
Baltic Sea	E	7.2	none	<0.2
AIDS Ward cold	MS	7.4	none	<0.2
AIDS Ward hot	MS	7.4	none	<0.2
Tammerkoski Falls	E	6.4	E4509-1-1	7.6
Iidesjarvi	E	6.3	E4510-1	1,000
Viinikka	E	6.0	E4511-2	2×10^7
Well Tahmela	MS	6.6	none	<0.2
Sauna	E	6.9	E4513-1	1,000
Ward 4 Rm 1 cold	MS	6.9	none	<0.2
Wrd 4 Rm 2 hot	MS	6.9	none	<0.2

Table C. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Wrd 4 Rm 2 shw hot	MS	7.0	E4516-2 E4516-3	17 6.8
Wrd 4 Rm 2 Dk cold	MS	6.9	E4517-1	3.4
Pdl melted snow	E	6.7	E4518-2-1 E4518-2-2	40
OP Pentam cold	MS	7.0	E4519-1	3.8
OP Pentam hot	MS	7.1	E4520-1	4.0
OP WC cold	MS	7.0	E4521-1	2.8

Appendix

Table D. Recovery of *Mycobacterium avium* complex isolates from Nairobi, Kenya samples.

SAMPLE SITE	SAMPLE TYPE	pH	ISOLATE NUMBER	CFU/ML
Clinic Centre cold	WS	6.7	none	<0.2
Clinic Centre hot	WS	6.8	none	<0.2
Athi River	E	6.8	none	<0.2
Nairobi Park Pool	E	6.6	none	<0.2
Kenyatta Hospital Stream	E	6.5	E2505-1	6.2
Kenyatta Hospital Tower	E	6.7	none	<0.2
Pumwani-Dangorani	E	7.6	none	<0.2
Pumwani Junction	E	7.7	none	<0.2
Kenyatta Hospital Puddle	E	7.3	E2509-5	20

Table D. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Kenyatta AIDS hot	WS	7.0	none	<0.2
Kenyatta AIDS cold	WS	7.2	none	<0.2
Kiambu Dist well	WS	6.9	none	<0.2
Infect. dis. blk 1	WS	6.9	none	<0.2
Infect. Dis. blk 2	WS	6.9	none	<0.2
Jarabony Lily Pond	E	7.7	none	<0.2
Lutzangany Stream	E	7.7	none	<0.2
Lutzangany Lake	E	7.4	none	<0.2
NG-ombai Puddle	E	7.6	none	<0.2
Lily Pond	E	7.8	none	<0.2

Table D. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Tank Water Supply	WS	8.0	none	<0.2
Main Water Supply	WS	8.2	none	<0.2
Rain Water Tank	WS	8.2	none	<0.2
Jimba River	E	7.4	none	<0.2
TB Lab Tap	WS	7.0	none	<0.2
Lab cold	WS	6.7	none	<0.2
Lab hot	WS	6.9	none	<0.2
Nyakate River	E	7.3	none	<0.2
Lake Victoria	E	7.1	none	<0.2
Standpipe supply	WS	7.3	none	<0.2
Paddy Sanga Buye	E	6.6	none	<0.2
Rice Paddy Mwanza	E	8.0	none	<0.2

Table D. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Nyahururu Stream	E	7.5	none	<0.2
Nyabururu weeds	E	7.5	none	<0.2
Nyahururu weeds	E	7.1	none	<0.2
Joro Orok River	E	7.1	none	<0.2
Nwea Rice paddy	E	7.1	none	<0.2
Forest Lily Pond	E	6.4	none	<0.2
Rice Paddy calm	E	7.4	none	<0.2
Rice Paddy flow	E	7.3	none	<0.2
WT Lab hot	WS	6.8	none	<0.2
WT Lab cold	WS	7.3	none	<0.2
Kenyatta National Hos. 42 cold	WS	7.1	none	<0.2

Table D. con't.

SAMPLE SITE	SAMPLE TYPE	pH	MAC ISOLATE	CFU/ML
Kenyatta National Hos. 23 cold	WS	6.9	none	<0.2
Kenyatta National Hos. Cslty cold	WS	6.9	none	<0.2
KNH puddle	E	6.5	none	<0.2
WT puddle	E	6.5	E2546-1	0.2
KNH stream	E	6.1	E2547-2	0.8
Pumwani House	WS	6.5	none	<0.2
Pumwani House	WS	6.5	none	<0.2
Pumwani stream	E	6.9	E2550-2	<0.2
Pumwani stream	E	6.9	none	<0.2
Pumwani puddle	E	7.0	none	<0.2
Pumwani Pipe	WS	6.9	none	<0.2
Kemri Ward hot	WS	7.1	none	<0.2
Kemri Ward cold	WS	7.0	none	<0.2

Appendix

Table E. Recovery of *Mycobacterium avium* complex isolates from Kinshasa, Zaire samples.

SAMPLE SITE	SAMPLE TYPE	pH	ISOLATE NUMBER	CFU/ML
Hospital cold	WS	6.3	none	<0.2
Hospital hot	WS	6.3	none	<0.2
Congo River	E	5.6	E3503-1	0.4
Standing water	E	6.6	none	<0.2
Roadside water	E	6.4	none	<0.2
Well water	WS	6.6	none	<0.2
Binza Mnicipal	WS	6.5	E3508-1	1.8
Hospital cold	WS	6.8	none	<0.2

Appendix

Table F. Recovery of *Mycobacterium avium* complex from patients with AIDS from Boston, Massachusetts.

ISOLATE NUMBER	ISOLATE NUMBER
1009-1-BC[a]-1	1052-2-F-3
1009-1-BC-2	1052-2-R-1
1009-1-BC-3	1056-1-BC-1
1011-1-BC-1	1056-1-BC-2
1011-1-BC-2	1056-1-BC-3
1011-1-BC-3	1056-1-F-1
1011-1-F[b]-1	1060-2-R-3
1011-1-F-2	1056-1-F-2
1011-1-F-3	1056-1-F-3
1014-2-F-1	1056-1-R-1
1014-2-F-2	1056-1-R-2
1014-2-F-3	1056-1-R-3
1014-2-R[f]-1	1057-1-F-1
1014-2-R-2	1057-11-L-1
1014-2-R-3	1057-11-L-2

Table F. con't.

ISOLATE NUMBER	ISOLATE NUMBER
1025-1-BC-1	1057-11-L[c] -3
1025-1-BC-2	1060-2-F-1
1025-1-BC-3	1060-2-F-2
1028-1-BC-1	1060-2-F-3
1028-1-BC-2-T[i]	1060-2-R-1
1028-1-BC-2-X[h]	1060-2-R-2
1028-1-BC-3	1060-2-R-3
1028-1-BC-3-T	1061-1-BC-1
1028-1-BC-3-X	1061-1-BC-2
1031-1-BC-1	1061-1-BC-3
1031-1-BC-2	1062-1-F-1
1031-1-BC-3	1062-1-F-2
1031-1-F-1	1062-1-F-3
1031-1-F-2	1062-1-R-1
1031-1-F-3	1062-1-R-2
1031-1-F-3-T	1062-1-R-3
1031-1-F-3-X	1064-1-BC-1

Table F. con't.

ISOLATE NUMBER	ISOLATE NUMBER
1041-2-F-1	1064-1-BC-2
1041-2-F-2	1064-1-BC-3
1041-2-F-3	1064-1-F-1
1045-2-BC-1	1064-1-F-2
1045-2-BC-2	1064-1-F-3
1045-2-BC-3	1066-1-F-2
1045-2-F-1	1066-2-F-2
1045-2-F-2	1077-1-BC-1
1045-2-F-3	1077-1-BC-2
1045-3-BC-1	1077-1-BC-3
1045-3-BC-2	1077-1-BC-2
1047-1-BC-1	1085-1-BC-2
1047-1-BC-2	1085-1-BC-3
1047-1-BC-3	1085-1-R-1
1047-1-F-1	1086-1-R-1
1047-1-F-2	1086-1-R-2
1047-1-F-3	1086-1-R-3

Table F. con't.

ISOLATE NUMBER	ISOLATE NUMBER
1047-1-R-1	1093-1-BC-1
1047-1-R-2	1093-1-BC-2
1047-1-R-3	1093-1-BC-3
1052-2-F-1	1093-1-F-1
1052-2-F-2	

Appendix

Table G. Recovery of *Mycobacterium avium* complex isolates from AIDS patients in Dartmouth, New Hampshire.

ISOLATE NUMBER	ISOLATE NUMBER
5002-1-BC-2	5047-1-BC-2
5002-1-BC-3	5047-1-BC-3
5002-1-F-1	5047-1-F-1
5002-1-F-2	5047-1-F-2
5002-1-F-3	5047-1-F-3
5026-1-BC-1	5047-11-BC-1
5026-1-BC-2	5047-EG-1
5026-1-BC-3	5047-EH-1
5026-1-F-1	5049-1-F-1
5026-1-F-2	5049-1-F-2
5026-1-F-3	5049-1-F-3
5027-3-LN[d]-1	5058-1-F-1
5027-3-LN-2	5058-1-F-2
5027-3-LN-3	5058-1-F-3

Table G. con't.

ISOLATE NUMBER	ISOLATE NUMBER
5028-3-BC-1	5060-1-BC-1
5028-3-BC-2	5060-1-BC-2
5029-3-BC-1	5060-1-BC-3
5029-3-BC-2	5060-1-F-1
5029-3-BC-3	5060-1-F-2
5029-3-F-1	5060-1-F-3
5029-3-F-2	5060-1-M-1
5029-3-F-3	5060-1-M-2
5029-3-M^{e}-1	5060-1-M-3
5029-3-M-2	5060-1-R-1
5029-3-M-3	5060-1-R-2
5029-3-R-1	5060-1-R-3
5029-3-R-2	5040-1-R-1
5029-3-R-3	
5029-3-SK^{s}-1	
5029-3-SK-2	
5029-3-SK-3	

Appendix

Table H. Recovery of *Mycobacterium avium* complex isolates from AIDS patients in Helsinki, Finland.

ISOLATE NUMBER	ISOLATE NUMBER
4031-2-BC-1	4046-1-BC-1
4031-2-BC-2	4046-1-BC-2
4031-2-BC-3	4046-1-BC-3
4035-1-BC-1	4048-1-BC-1
4035-1-BC-2	4048-1-BC-2
4035-1-BC-3	4048-1-BC-3
4035-1-F-1	1905.1/90
4035-1-F-2	V124.6/90
4035-1-F-3	V4613./89
4035-1-R-1	M3835/87
4035-1-R-2	J385/85
4035-1-R-3	M3711/88
4039-2-BC-1	J204/88
4039-2-BC-2	M3024/87
4039-2-BC-3	1486.3/89
	M2984./84

Appendix

Table I. Recovery of *Mycobacterium avium* complex from AIDS patients in Nairobi, Kenya.

ISOLATE NUMBER
2032-00-F-1
2032-00-F-2
2032-00-F-3
2032-0-R-1
2032-0-R-2
2032-0-R-3
2032-01-F-1
2032-01-F-2
2032-01-F-3

[a] blood culture

[b] feces (stool)

[c] liver

[d] lymph node

[e] bone marrow

[f] respiratory (sputum)

[g] skin biopsy

[h] opaque colony morphology

[i] transparent colony morphology

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