Epidemiology of Infection by Nontuberculous Mycobacteria

*Mycobacterium avium, Mycobacterium intracellulare, and Mycobacterium scrofulaceum* in Acid, Brown-Water Swamps of the Southeastern United States and Their Association with Environmental Variables

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**Introduction**

Members of the *Mycobacterium avium, Mycobacterium intracellulare,* and *Mycobacterium scrofulaceum* (MAIS) group are opportunistic pathogens that cause pulmonary infections in humans (1). An estimated 2,000 or more U.S. residents are infected annually by these organisms (2, 3), and recently it has been shown that a substantial proportion of patients with the acquired immunodeficiency syndrome have disseminated MAIS infections (4, 5). In the absence of any evidence for a human-to-human transfer of MAIS organisms (1), searches for one or more environmental sources for MAIS have ensued.

Higher frequencies of persons reacting to either purified protein derivative (PPD)-B (6) or PPD-G (7) and of MAIS isolates from patient samples (2, 3) correlated with the higher numbers of MAIS recovered from water (8) and soil (9) samples collected in the southeastern U.S. coastal plain. Furthermore, the fact that a high proportion of MAIS aerosol isolates from this region (10) shared characteristics in common with clinical isolates (11, 12) supports the hypothesis that at least one source of MAIS organisms is in the southeastern U.S. environment.

Based on the observation that MAIS organisms were recovered more frequently and in higher numbers from acidic soils and those high in organic matter (9), that they grow optimally at pH 5.0 to 5.5 (13), and that most clinical MAIS isolates grow at 43°C (12) but eventually die or grow slowly below 15.5°C (14), we hypothesized that the warm, acid, brown, low oxygen-containing swamp waters and associated soils would have high MAIS numbers. We sought to test this hypothesis by recovering, enumerating, and identifying MAIS from waters, soils, dusts, and aerosols collected from coastal swamps (Okefenokee Swamp, GA, and Great Dismal Swamp, VA) in regions of high frequency of PPD-B and PPD-G (6, 7) reactors (as an index of MAIS infection). By contrast, and as potentially negative control MAIS habitats with low frequency of PPD-B and PPD-G reactors, we selected Claytor Lake, VA, and Cranberry Glades, WV. In parallel with the enumeration of MAIS from these four habitats, we sought to identify physiochemical variables (e.g., pH, temperature, dissolved oxygen, zinc, humic and fulvic acid content, etc.) that correlated with MAIS numbers.

**Methods**

**Sampling and Isolation**

Four sampling locations (figure 1) were chosen because of differences in frequency of PPD-B and PPD-G reactors (6, 7). Four types of samples were collected at each location, as described previously, namely water (8), soil (9), aerosols (10), and ejected water droplets (15).

MAIS organisms were isolated from waters and soils using at least five replicate spread plates of Middlebrook and Cohn 7H10 medium (M7H10; BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol after NaOH-decontamination treatment with HICL neutralization (15) and tsukamura Tween 80 cycloheximide- (TTC) selective agar medium (13) without decontamination. TTC agar medium was also used for collection and isolation of the aerosol and ejected water droplet samples. Ejected droplet sizes (150 to 450 µm) of waters from the four locations overlapped the size range reported by Falkinham and coworkers (16) for natural aquatic aerosols. In both cases, plates were spread. After samples were processed or plated in the laboratory, plates were sealed with parafilm and incubated at 37°C in candle jars. Checks for colonies were done at 7-day intervals, and plates were removed from the incubator after sufficient incubation time to allow colony development (28 to 42 days).

**MAIS Identification**

Acid-fast (Ziehl-Neelsen stain) colonies were picked and transferred to Lowenstein-Jensen (LJ) slants (BBL Microbiology Systems). Mycobacteria were identified as previously described (8). Acid-fast isolates failing to grow on LJ slants were transferred to M7H10 plates.

**Summary**

*Mycobacterium avium, Mycobacterium intracellulare, and Mycobacterium scrofulaceum* (MAIS) organisms were isolated and identified from waters, soils, aerosols, and droplets ejected from water collected from four geographically separate aquatic environments (Okefenokee Swamp, GA; Dismal Swamp, VA; Claytor Lake, VA; and Cranberry Glades, WV) during several seasons. Recovery of MAIS was significantly higher from waters, soils, and aerosols collected from the two acid, brown-water swamps located in the southeastern coastal plain. High MAIS numbers correlated with warmer temperature, low pH, low dissolved oxygen, high soluble zinc, high humic acid, and high fulvic acid. This research, in relation to previous findings for the geographic distribution and physiologic ecology of MAIS, supports the conclusion that waters, soils, and aerosols of the acid, brown-water swamps of the southeastern United States coastal plain represent major environmental sources likely connected with the higher incidence of human infection in this region.

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or slants containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin enrichment (OAA) (17) and incubated at 37°C. Isolates that grew in the broth media were transferred to LJ slants for identification. Acid-fast colonies that failed to grow under all conditions were not counted in the study.

**Physicochemical Variables**

During dust and aerosol collections, air temperature, wind velocity and direction, relative humidity, and light intensity were measured. Water pH, temperature, and transparency (Secchi disc depth) were measured in the field, and modified-Winkler determinations of dissolved oxygen (18) were completed in the laboratory. Soil sample pH, temperature, organic matter content, and moisture content were measured as described by Brooks and colleagues (9). Various cationic elements and heavy metals, including zinc, were determined in filtered waters or soil extracts using Inductively Coupled Plasma spectral analysis (ICAP-9000 Plasma Spectrometer; Jarrell-Ash, Inc., Waltham, MA) (18).

Humic and fulvic acids were extracted from soils using the method outlined by Schnitzer (19) except for our use of 1 N NH₄OH in methanol as the primary extractant. Humic and fulvic acid fractions were isolated from water samples and concentrations measured using the procedure of Carber and colleagues (20). Following several purification steps, humic and fulvic acid fractions from both soil and water were dried at 37°C for 24 h, then weighed.

**Statistical Analysis**

Because of the high percentage of samples that yielded no MAIS, nonparametric one-way analysis procedure using the Kruskal-Wallis test (chi-square approximation) of the Statistical Analysis Systems (SAS) program (21) was used to compare the numbers of MAIS between the four locations. The General Linear Model (GLM) procedure of SAS (21) was performed to compare MAIS recovery at each sampling site in the four locations with the location's water, soil, and aerosol physicochemical characteristics. The influence of both individual characteristics and multifactorial interactions between the characteristics were compared. For significance, a confidence interval of 95% was selected for both procedures.

**Results**

**MAIS Recovery**

Table 1 summarizes the frequency of recovery and number of MAIS from the four sampling locations. Values for water or soil samples reflect the average number of MAIS organisms recovered from both TTC medium and M7H10 medium following NaOH decontamination. Higher numbers per milliliter of water were recovered on TTC compared with M7H10 after NaOH decontamination, whereas higher numbers of MAIS per gram of soil were recovered on M7H10 after NaOH decontamination than on TTC (data not shown). MAIS were recovered from 13 of 20 (65%) water samples and 12 of 20 (60%) soil samples collected over the 2-year period from the two coastal swamps. In contrast, none of nine (<11%) water samples and only two of 18 (11%) soil samples collected from Claytor Lake and Cranberry Glades yielded MAIS. MAIS numbers were different in those soil samples collected at different times of the years at the same site. Higher numbers of MAIS were typically recovered during warmer seasons. MAIS were also recovered from aerosols (seven of 24 [29%]) and ejected droplets (six of 10 [60%]) collected at the Okefenokee and Dismal Swamps, whereas no Claytor Lake or Cranberry Glades aerosol (0 of 20 [<5%]) or ejected droplet (0 of 10 [<10%]) samples yielded MAIS. When MAIS were recovered from the two swamp locations, their numbers were significantly higher (p < 0.05) for all four of the sample types compared with the two Appalachian locations (table 1). MAIS were recovered more frequently from soils of the Okefenoke Swamp, whereas waters from the Dismal Swamp yielded more MAIS than any of the other locations.

**MAIS Recovery and Site Characteristics**

MAIS colony-forming units (cfu)/ml wa-
ter and some selected physiochemical characteristics and MAIS cfu/g dried soil and selected physiochemical variables were correlated (figure 2, table 2). Water samples in the Okefenokee and Dismal Swamps that yielded high MAIS numbers had lower pH and dissolved oxygen and higher zinc, humic acid, and fulvic acid contents, as compared with the Appalachian waters (figure 2). More MAIS were recovered from relatively warmer waters (figure 2). Similarly, soil samples that were lower in pH and higher in zinc, organic matter, humic acid, and fulvic acid contents (abundant within the Okefenokee and Dismal Swamps) yielded more MAIS than soils of Claytor Lake and Cranberry Glades (figure 2). Water samples had higher concentrations of fulvic than humic acid because some humic acid may have been insoluble and precipitated at the low pH of the waters (19, 22). However, soils had about equal concentrations of both humic and fulvic acids (figure 2).

These physiochemical variables and interactions between them were then tested for correlation with MAIS cfu in both water (n = 29) and soil (n = 38) samples. The statistical significance of these correlations for the samples collected at the four locations is shown in table 2. Noteworthy are the highly significant correlations between high zinc and fulvic acid content and the interactions between these two variables for the water samples. Thus, any multivariable analysis involving zinc and/or fulvic acid with any other variables results in decreased p values, indicating a greater degree of significance. MAIS cfu/g soil was most significantly influenced by humic or fulvic acid concentrations or total organic matter contents of the soils and any interactions with other variables involving those three. There were only poor correlations for the aerosol samples between MAIS cfu/m³/h and light intensity, relative humidity, or air temperature; similarly, only poor correlations occurred for ejected droplet samples between MAIS cfu/cm²/h and water temperature (data not shown). Finally, poor correlations occurred between MAIS cfu/ml water and concentrations of Mn, Mg, Ca, P, K, Cu, Al, Fe, B, Na, and S or MAIS cfu/g soil concentrations of Mn, Mg, Ca, P, K, NO₃-N, and soluble salt (data not shown). Correlations between MAIS cfu and some heavy metals (e.g., Cd, Cr, Hg, Pb, Ni, and Tl) in water could not be determined because concentrations were below the limit of detection for all samples.

**Discussion**

The data on recovery of MAIS from the four sampling locations show that significantly higher numbers are recovered from water, soil, and aerosol samples of the Okefenokee and Dismal Swamps compared with Claytor Lake and Cranberry Glades (table 1). "Recovery" by our methods represents only a percentage of the total MAIS numbers present, but the different numbers recovered by the same methods from similar environmental compartments at different locations should reflect the comparative sizes of the resident MAIS communities. Therefore, our finding of higher MAIS recovery in the two coastal swamps agrees well with the geographic distribution of MAIS in water (8) and soil (9) samples and with the geographic incidence of skin sensitivity to PPD-B (6) and PPD-G (7) and clinical isolates from infected patients (2, 3).

Lower recovery of MAIS organisms from Okefenokee waters compared with Dismal Swamp waters (table 1) could be due to the fact that Okefenokee waters were of higher dissolved oxygen and lower humic and fulvic acid concentrations (figure 2). Okefenokee soils yielded more MAIS than did Dismal Swamp soils (table 1). This source of MAIS possibly results in the higher frequency of PPD-B (6) and PPD-G (7) reactors in the Okefenokee Swamp compared with the Dismal Swamp. Values for soil pH, organic matter, zinc, humic acid, and fulvic acid were similar (figure 2). Comparisons of these recovery data using our methods cannot be made with those of Falkinham and coworkers (8) for water and Brooks and colleagues for soil (9) because different media were used. However, numbers of MAIS recovered on TTC medium per millilitre of water in the Dismal Swamp were higher than those from nonswamp waters in Virginia (16).

Highly significant correlations for the data were apparent between high numbers of MAIS and high zinc and fulvic acid levels of water samples. In soils, significant correlations between high MAIS numbers and humic and fulvic acids and organic matter contents were also observed. Zinc probably was not limiting nor toxic for most organisms in any of the water samples whose zinc concentrations ranged from 10.3 to 159.2 µg/L, which is typical for many freshwaters sampled previously in the United States (23). It has been established that some mycobacteria have a high zinc requirement (24), that zinc is a common metal in many metalloenzymes (23), and that humic and fulvic acids complex with and chelate zinc (23). The high correlation between the recovery of MAIS and high zinc concentrations, therefore, may be connected to MAIS abundance in the environment. This finding also may explain

**TABLE 2**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Water</th>
<th>Soil</th>
</tr>
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<tr>
<td>pH, &lt; 5.5</td>
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<td>0.15</td>
</tr>
<tr>
<td>Dissolved oxygen, &lt; 2 mg/L water</td>
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<td>-</td>
</tr>
<tr>
<td>Zinc, &gt; 0.75 mg/L water; &gt; 4 mg/kg soil</td>
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<td>0.23</td>
</tr>
<tr>
<td>Temperature, &gt; 15° C</td>
<td>0.92</td>
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<tr>
<td>Organic matter, &gt; 15%</td>
<td>-</td>
<td>0.004</td>
</tr>
<tr>
<td>Humic acid, &gt; 20 mg/L water; &gt; 15 mg/g soil</td>
<td>0.13</td>
<td>0.004</td>
</tr>
<tr>
<td>Fulvic acid, &gt; 200 mg/L water; &gt; 15 mg/g soil</td>
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<td>0.006</td>
</tr>
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<td>0.12</td>
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<td>pH and organic matter</td>
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<td>0.02</td>
</tr>
<tr>
<td>pH and humic acid</td>
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<td>0.02</td>
</tr>
<tr>
<td>pH and fulvic acid</td>
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<td>0.02</td>
</tr>
<tr>
<td>Zinc and dissolved oxygen</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>Zinc and organic matter</td>
<td>-</td>
<td>0.02</td>
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<tr>
<td>Zinc and humic acid</td>
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<td>Zinc and fulvic acid</td>
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<tr>
<td>Organic matter and humic acid</td>
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<td>Organic matter and fulvic acid</td>
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<td>0.01</td>
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<td>Humic acid and dissolved oxygen</td>
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<td>-</td>
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<tr>
<td>Humic acid and fulvic acid</td>
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<td>0.01</td>
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<tr>
<td>Fulvic acid and dissolved oxygen</td>
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<tr>
<td>Organic matter, humic acid, and fulvic acid</td>
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<td>0.19</td>
</tr>
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</table>
high recoveries of MAIS from hospital water systems with galvanized pipes consisting of zinc alloys (25).

Higher recovery of MAIS from samples rich in humic and fulvic acids agrees with earlier work by Kazda (26), demonstrating M. intracellular growth stimulation inside dead storage cells of Sphagnum, a moss genus long recognized as a primary constituent of acid bogs and important in acid peat and humic acid production (27). Results also agree with
the findings of Brooks and colleagues (9), who demonstrated higher MAIS recoveries from acidic soils high in organic matter content. Humic and fulvic acids compose a significant portion of water’s and soil’s organic fractions (19, 22).

Most MAIS have also been shown to be microaerobic (Falkinham and Kirschner, unpublished data), thereby agreeing with our data suggesting higher recoveries of MAIS in less oxygenated waters. In fact, of 33 MAIS isolates tested, 23 (70%) isolates were classified as facultatively microaerobic, exhibiting growth both on the media surface and > 5 mm below the surface, whereas five (15%) isolates exhibited growth solely at > 5 mm below the media surface and were classified as obligately microaerobic (data not shown). Higher frequencies of isolation of MAIS from warmer waters also agrees with the work of George and coworkers (14), suggesting greater recoveries of MAIS in temperatures above 15.5 °C.

Our data suggest that MAIS organisms probably are much more abundant in the acid, brown swamp waters and associated soils of the southeastern coastal plain than those waters and soils found elsewhere. On a weight basis, somewhat higher MAIS numbers were recovered from water compared with soil. However, strong binding of MAIS to soil particles (15), suggests that swamp soils may harbor even higher numbers and be as important or more so than swamp water as a source of infection. In fact, if one corrects for the loss of MAIS after the decontamination procedure and subsequent platting on MTH10, our recovery numbers would be about 100-fold higher for water and 1,000-fold higher for soil (15). On a volume basis, the numbers in aerosols are still smaller, but the volume of air regularly inhaled by humans makes this third potential source of infection also important.

Probably the abundance of MAIS organisms in these swamps results from several interacting variables. The combination of higher temperatures, low oxygenated waters, and lower pH soils and waters higher in zinc and humic and fulvic acids most likely favor growth and survival of MAIS organisms in the environment. A single environmental source (soil, water, or aerosols) for members of the M. avium, M. intracellulare, and M. scrofulaceum group is neither necessary nor likely. The swamp soils, waters, and aerosols containing MAIS may all play a role in the epidemiology of MAIS infections in humans within this geographic region. Sources having higher numbers of infective units or sources with greater frequencies of human exposure can be more significant for human infection. Whereas MAIS organisms may be ubiquitous, their population size and proximity to routes of transfer to humans may be of key importance for explaining the epidemiology of infection. Apparently the acid, brown-water swamps of the coastal plain of the southeastern United States apply the epidemiologic model.

Acknowledgment

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References