Isolation and characteristics of Mycobacterium avium complex from water and soil samples in Uganda

T. Eaton*, J. O. Falkinham*, T. O. Aisu†, T. M. Daniel‡

*Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, †Ministry of Health, Tuberculosis Control Programme, Makerere University Kampala, Uganda, ‡Department of Medicine, Case Western Reserve University, Cleveland, Ohio, USA

SUMMARY. Setting: Mycobacterium avium complex organisms have not been isolated from late stage AIDS patients in Uganda. This could possibly be due to the absence of M. avium complex in the Uganda environment.

Objective and Design: Determine whether M. avium complex organisms could be isolated from water and soils collected in the living environment of Ugandan AIDS patients.

Results: Representatives of the M. avium complex were isolated from 3 of 7 (43%) water and 3 of 7 (43%) soil samples collected in Kampala, Uganda. The average number of colony-forming units per ml water was 3.3 and average colony-forming units per gram of soil was 7825. In terms of growth characteristics, antimicrobial susceptibility patterns, and the presence or absence of plasmids and IS901, Ugandan M. avium complex isolates were similar to those isolated from the US and European AIDS patients and their environment.

Conclusion: M. avium complex organisms sharing genetic and physiological characteristics of M. avium complex isolates recovered from patients with AIDS can be isolated from water and soil samples in Uganda.

RESUMÉ. Cadre: Des organismes du complexe Mycobacterium avium n'ont pas été isolés à partir de malades au stade final du SIDA en Ouganda. Ceci pouvait être dû à l'absence du complexe M. avium dans l'environnement ougandais.

Objet et Méthode: Déterminer si des organismes du complexe M. avium peuvent être isolés à partir d'échantillons d'eau et de terre recueillis dans l'environnement de sidéens ougandais.

Résultats: Des représentants du complexe M. avium ont été isolés à partir de 3 des 7 (43%) échantillons d'eau et également de 3 des 7 (43%) échantillons de terre recueillis à Kampala, Ouganda. Le nombre moyen d'unités formant colonies (UFC) par ml d'eau était de 3,3 et par grammes de terre il était de 7825. En termes de particularités de croissance, de profils de susceptibilité antimicrobienne et de présence ou d'absence de plasmides et de IS901, les isolats ougandais étaient similaires à ceux recueillis à partir de sidéens américains et européens et de leur environnement.

Conclusion: Des organismes du complexe M. avium qui partagent les particularités génétiques et physiologiques des isolats du même complexe récupérés à partir des sidéens peuvent être isolés à partir d'échantillons d'eau et de terre en Ouganda.

RESUMEN. Marco de referencia: En Uganda, no se han aislado microorganismos del complejo Mycobacterium avium en los pacientes con SIDA en estado final. Posiblemente esto es debido a la ausencia del complejo M. avium en el medio ambiente de Uganda.

Objetivo y Método: Determinar si microorganismos del complejo M. avium pueden ser aislados de muestras de agua y tierra recolectadas del medio ambiente donde viven los pacientes con SIDA de Uganda.

Resultados: Microorganismos del complejo M. avium fueron aislados en 3 de 7 (43%) muestras de agua y en 3 de 7 (43%) muestras de tierra recolectadas en Kampala, Uganda. El promedio de unidades que forman colonias (UFC) por ml de agua fue de 3,3 y el promedio de unidades que forman colonias por gr de tierra fue de 7825. En base a las características de crecimiento, patrones de sensibilidad antimicrobiana y presencia...
INTRODUCTION

Although 25–50% of US acquired immune deficiency syndrome (AIDS) patients have disseminated *Mycobacterium avium* complex infection, it has not been detected in late stage Ugandan AIDS patients. Because representatives of the *M. avium* complex are opportunistic human pathogens whose source of infection is the environment, one possible explanation for the absence of *M. avium* complex infection in late stage Ugandan AIDS patients is that the organism is absent in the Ugandan environment.

Although *M. avium* complex isolates have been recovered from non-HIV-infected patients in Rhodesia and from water and soil samples collected in Kenya and Zaire, the physicochemical characteristics of soils and waters in Uganda, which have been shown to influence *M. avium* complex numbers, may prevent *M. avium* complex growth and survival.

To test the hypothesis that *M. avium* complex is absent in the Ugandan environment, water and soil samples were collected in Kampala, Uganda and an attempt was made to isolate *M. avium* complex organisms. Because of the possibility that *M. avium* complex organisms recovered from Ugandan water and soil samples are different from those recovered from the US or European AIDS patients and their environment, each isolate was characterized. The objective was to demonstrate that Ugandan *M. avium* complex isolates shared the same characteristics unique to the US *M. avium* isolates recovered from patients. Those include: growth at 43°C, resistance to cadmium and streptomycin, the presence of small plasmids, and the absence of IS901.

In addition, the serotype of each isolate was determined.

METHODS

**Collection of water and soil samples**

Samples were collected in sterile 50 ml plastic tubes at ambient temperature and shipped to Virginia Tech for processing. Only cold, unchlorinated water samples were collected. Samples were collected from sites where persons could be exposed through their activities (e.g. drinking, bathing or gardening), and were processed within 2 weeks of collection. *M. avium* complex can survive for months in water and soil samples.

**Processing water samples**

Twenty-five ml samples were centrifuged at 5000 × g for 30 min at room temperature 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 on TTC agar medium in triplicate. The plates were sealed with Parafilm (American Can Co., Greenwick CT) and incubated at 37°C for up to 6 weeks.

**Processing soil samples**

Five grams of a soil sample were suspended in 25 ml sterile, one-tenth strength Nutrient Broth (Difco Laboratories, Detroit, MI) in a sterile screw cap 50 ml centrifuge tube and centrifuged at 5000 × g for 30 min at room temperature. The supernatant was transferred to a sterile, 50 ml screw-cap centrifuge tube and centrifuged at 5000 × 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 up to 6 weeks.

**Determination of soil pH**

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

**Identification of Mycobacteria**

Acid-fast colonies were identified by Ziehl-Neelson staining. Those acid-fast colonies of the same morphology were counted and representative single colonies streaked on Lowenstein-Jensen medium slants (BBL Microbiology Systems, Cockeysville, MD) 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 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 10 ml Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, MD) for identification by DNA probe. Isolates were also streaked on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, MD) to ensure a pure culture. *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 streptomycin and cadmium susceptibility

The ability of each isolate to grow at 43°C and in the presence of 10 μg streptomycin/ml or 0.1 mM cadmium as CdCl₂ on Middlebrook 7H10 agar medium was measured as previously described.¹¹

Plasmid DNA hybridization

A clone of the M. avium plasmid pLR7,¹² the cloned fragments of the M. avium plasmid pVT²,¹³ and the clone containing IS901¹⁴ were radiolabeled with ³⁵S-dCTP using the Random-Primer DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN) following the manufacturers’ instructions, and denatured.¹⁷

Dot-Blot hybridization

Cells were grown in 10 ml Middlebrook 7H9 broth to early log phase (i.e. 5 × 10⁷ colony-forming units/ml) and harvested by centrifugation (5000 × g for 10 min at room temperature). The cells were suspended in 1 ml TE buffer¹⁷ and 0.3 ml 2 N NaOH and 1 gram of 0.1 mm diameter glass beads added and the cells broken by 90 s agitation in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK). The suspension was centrifuged at 5000 × g for 10 min at room temperature to pellet the glass beads. The DNA in 0.25 ml of the supernatant liquid was loaded into the wells of a Bio Dot Blot Apparatus (Bio-Rad Laboratories, Inc., Richmond, CA) and drawn by vacuum on the surface of a Zeta-Probe membrane (Bio-Rad Laboratories, Inc. Richmond, CA) wet with 2X SSC.¹⁷ The membrane was removed and placed on a piece of 0.4 N NaOH-soaked filter paper for 5 min, then rinsed once in 2X SSC and allowed to completely dry, before being incubated in a hybridization mix containing 50% formamide, 0.5% Blotto, 4X SSPE, and 1% SDS at 50°C for at least 24 h. Denatured probe was added and allowed to hybridize to the membrane-bound DNA at 50°C for 24 h. Following a series of 5 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; 17), the membrane was dried and exposed to autoradiography film (Amersham Life Science, Arlington Heights, IL).

RESULTS

Recovery of M. avium complex

Listed in Table 1 are the sample sites in Kampala, Uganda from which soil and water samples were collected. M. avium complex isolates were recovered from 3 of 7 (43%) water and 3 of 7 (43%) soil samples (Table 1). All isolates were identified by DNA probe as M. avium. The concentrations of M. avium were 1, 2, 6 and 50 colony-forming units (cfu/ml) water and 50, 1250, 5000, and 20 000 cfu/gm soil (Table 1). Other slow-growing acid-fast mycobacteria were recovered from all but the soil sample collected from Lake Nabugaba and the water sample collected from a nearby puddle. Those isolates were not identified.

Effect of pH on M. avium recovery

The frequency of recovery of M. avium was similar for samples of different pH (i.e. < 6.0, 6.0-6.9, and > 7.0) for both water and soil samples (Table 1). There were higher numbers of cfu/gm soil for the soil sample of pH 7, than for other soil samples (Table 1), but little difference in cfu/ml water for samples of different pH (Table 1). However, the small sample size limits the significance of any conclusions.

Characteristics of M. avium isolates

The characteristics of the M. avium complex isolates recovered from Uganda water and soil samples are listed in Table 2. One water isolate was serotype 8 and one soil isolate was serotype 4 (Table 2). None of the isolates carried IS901. Further, both water and soil isolates carried small plasmids (Table 2) which hybridized with Table 1. Recovery and identification of M. avium complex isolates from Uganda

<table>
<thead>
<tr>
<th>Site</th>
<th>Sample</th>
<th>pH</th>
<th>MAC isolate number</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logogo Stream water</td>
<td>7.07</td>
<td>none</td>
<td>&lt; 0.2/ml</td>
<td></td>
</tr>
<tr>
<td>Mulago Hospital water</td>
<td>7.30</td>
<td>UW4-6</td>
<td>1/ml</td>
<td></td>
</tr>
<tr>
<td>Gayaza Road water</td>
<td>6.06</td>
<td>UW4-14T</td>
<td>50/ml</td>
<td></td>
</tr>
<tr>
<td>Gayaza Spring water</td>
<td>5.40</td>
<td>UW7-8</td>
<td>&lt; 0.2/ml</td>
<td></td>
</tr>
<tr>
<td>Lake Nabugaba water</td>
<td>6.00</td>
<td>UW9-16T</td>
<td>60/ml</td>
<td></td>
</tr>
<tr>
<td>Nabugaba puddle water</td>
<td>6.80</td>
<td>none</td>
<td>&lt; 0.2/ml</td>
<td></td>
</tr>
<tr>
<td>Kololo water</td>
<td>6.60</td>
<td>none</td>
<td>&lt; 0.2/ml</td>
<td></td>
</tr>
<tr>
<td>Subtotal water</td>
<td></td>
<td>8.4/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logogo soil</td>
<td>6.30</td>
<td>none</td>
<td>&lt; 0.2/gm</td>
<td></td>
</tr>
<tr>
<td>Mulago Hospital soil</td>
<td>5.80</td>
<td>US3-9</td>
<td>50/gm</td>
<td></td>
</tr>
<tr>
<td>Gayaza Road soil</td>
<td>6.55</td>
<td>none</td>
<td>&lt; 0.2/gm</td>
<td></td>
</tr>
<tr>
<td>Gayaza Spring soil</td>
<td>6.05</td>
<td>US8-24</td>
<td>1250/gm</td>
<td></td>
</tr>
<tr>
<td>Lake Nabugaba soil</td>
<td>5.90</td>
<td>none</td>
<td>&lt; 0.2/gm</td>
<td></td>
</tr>
<tr>
<td>Nabugaba soil</td>
<td>4.50</td>
<td>none</td>
<td>&lt; 0.2/gm</td>
<td></td>
</tr>
<tr>
<td>Kololo soil</td>
<td>7.00</td>
<td>US14-13</td>
<td>5000/gm</td>
<td></td>
</tr>
<tr>
<td>Subtotal soil</td>
<td></td>
<td>7825/gm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Characteristics of Ugandan *M. avium* complex isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Serotype</th>
<th>Phenotype</th>
<th>Sm&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW4-6</td>
<td><em>M. avium</em></td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UW4-14T</td>
<td><em>M. avium</em></td>
<td>+</td>
<td>+</td>
<td>non-typable</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW7-8</td>
<td><em>M. avium</em></td>
<td>+</td>
<td>–</td>
<td>non-typable</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UW9-16T</td>
<td><em>M. avium</em></td>
<td>–</td>
<td>+</td>
<td>4</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>US3-9</td>
<td><em>M. avium</em></td>
<td>–</td>
<td>+</td>
<td>not done</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>US8-24</td>
<td><em>M. avium</em></td>
<td>–</td>
<td>+</td>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>US14-13</td>
<td><em>M. avium</em></td>
<td>+</td>
<td>+</td>
<td>not done</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>US14-14</td>
<td><em>M. avium</em></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>By SNAP DNA probe; <sup>b</sup>Plasmid by DNA hybridization; <sup>c</sup>Serotyping by (20); <sup>d</sup>Growth at 43°C; <sup>e</sup>Growth in 1 mM Cd<sup>2+</sup>; <sup>f</sup>Growth in 10 μg streptomycin/ml.

both pLR7<sup>13</sup> and pVT2. Finally, a majority of water and soil isolates grew at 43°C and in the presence of either 1 mM Cd<sup>2+</sup> or 10 μg streptomycin (Sm)/ml.

**DISCUSSION**

The foregoing results demonstrate that *M. avium* complex organisms are present in the Ugandan environment, if Kampala is representative of the rest of the nation. The numbers of *M. avium* complex organisms in water are similar to those observed in the south-eastern US<sup>10,18</sup> while the numbers in soil are at least one order of magnitude higher than those in the south-eastern US.<sup>9</sup> Because *M. avium* complex numbers in both water and soil samples were similar to those reported in the south-eastern US,<sup>2-11</sup> the absence of *M. avium* bacteremia in Ugandan AIDS patients<sup>2</sup> cannot be due to an absence of *M. avium* in the Ugandan environment. Although samples were chosen from sites where persons could be exposed, Ugandans may not have been exposed to those types of sites.

Although earlier studies had shown that higher numbers of *M. avium* complex were found in waters and soils of low pH,<sup>3,10</sup> this was not observed in these Ugandan samples (Table 1). Quite possibly, the sample size here was too small.

Not only is *M. avium* present in the Ugandan environment, but the *M. avium* isolates recovered shared a set of unique characteristics found in *M. avium* complex isolates recovered from US and European patients.<sup>11-14</sup> The isolates had serotypes found in *M. avium* complex isolates recovered from US AIDS patients.<sup>1</sup> A significantly higher proportion of US human *M. avium* complex isolates were shown to grow at 43°C and were Cadmium and Streptomycin resistant compared to water, soil and aerosol US *M. avium* isolates.<sup>11</sup> Further, the Uganda *M. avium* isolates carried small plasmids found in *M. avium* isolates recovered from US AIDS patients.<sup>12,13</sup> Like European *M. avium* isolates from patients with AIDS, the Uganda *M. avium* isolates lacked IS901.<sup>14</sup> Thus, based on the genetic and physiologic tests used, it would appear that the *M. avium* isolates in Uganda are no different than those recovered from US and European AIDS patients.

In conclusion, an alternative hypothesis must be invoked to explain why *M. avium* bacteremia is not found in late stage Uganda AIDS patients. Quite possibly the patients die from other causes before *M. avium* disease can develop or fail to reach the CD4-counts associated with *M. avium* disease.<sup>1</sup> Additionally, it is possible that the Uganda *M. avium* isolates lack some characteristic that endows them with virulence in AIDS patients. It has been shown that the restriction fragment length polymorphism (RFLP) type of *M. avium* isolates recovered from African HIV-infected patients were unique and different from RFLP-types of *M. avium* recovered from US and European AIDS patients.<sup>19,20</sup> Ongoing studies will define the RFLP-type and measure the virulence of these Uganda isolates.

**Acknowledgements**

The authors acknowledge the support of the United States Public Health Service Grant AI-27482 from the National Institute of Allergy and Infectious Disease and Centers for Disease Control Collaborative Research agreement U52/CCU501180.

The authors wish to thank Dr Mitchell A. Yakrus of the Centers for Disease Control for serotyping the isolates.

**References**

8. von Reyn C F, Waddell R D, Eaton T et al. Isolation of Mycobacterium avium complex from water in the United States,


