Primary Isolation of *Mycobacterium avium* Complex-Serotype 6 on Blood Agar

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*Mycobacterium avium* complex-serotype 6 was isolated in pure culture on blood agar plates from inocula taken from the heart blood, lungs, liver, kidneys, and spleen of a naturally infected captive female opossum (*Didelphis marsupialis virginiana*). Repeat cultures from stored tissues and transfer of colonies from original blood agar plates revealed that the mycobacterium grew on tryptose, brilliant green, eosin-methylene blue, Sabouraud glucose, and mycobiotic agar plates and in Fletcher leptospira medium. The cultural, biochemical, and serological characteristics of the test isolate were compared with other mycobacteria. This is the first report to describe the primary isolation of a serotype from the *M. avium* complex from an animal species on blood agar or in Fletcher broth. In addition, this is the second documented report describing the isolation and identification of a mycobacterial species from the American opossum.

Atypical or opportunistic mycobacteria are widespread in nature and remain viable in soil and excreta for years (5). Infections due to these mycobacteria are becoming increasingly important in humans (2, 7); they are common in domestic animals (5, 14, 15) and are infrequently reported in wildlife (6, 11). Man and animals can be infected with the same serotypes (14). Epidemiologically, the majority of tuberculin skin reactions less than 10 mm in diameter in humans in the United States are caused by latent infections with atypical mycobacteria (7). Their prevalence is greatest in the southeastern states, where more than 50% of the population appears to have been exposed to one or more of these organisms.

Atypical mycobacteria from Runyon's group IV (12) that infect humans have been isolated on cyclohexamide agar (3), Sabouraud glucose (SAB) agar (10), blood agar (1), and from peptone broth (4).

The purposes of this report are (i) to describe the primary isolation of *Mycobacterium avium* complex-serotype 6 from a naturally infected opossum on blood agar and in Fletcher leptospira medium, (ii) to compare its cultural and biochemical characteristics with other mycobacteria, and (iii) to discuss the significance of these findings.

**MATERIALS AND METHODS**

Specimens of heart blood, lungs, liver, kidneys, spleen, and ileum were collected aseptically at necropsy from a captive female opossum. The lung, liver, kidney, and spleen were seared with a heated spatula and opened with a sterile scalpel, and inocula were collected with sterile swabs and plated directly onto 5% sheep blood agar plates (BAP; Trypticase soy base), phenylethyl alcohol (PEA), and MacConkey (MAC) agar plates (BBL-Bioquest, Cockeysville, Md.). Thioglycolate medium and selenite broth were inoculated as enrichment media for secondary culture. The heart blood was inoculated as described above. Inoculum from the ileum was plated directly on eosin-methylene blue (EMB), MAC, and brilliant green (BG) agar plates and inoculated into selenite broth and incubated at 35 to 37°C. All excess tissue specimens including the blood serum were then frozen for future use.

Two days later, inocula from the liver, kidney, and serum were placed in Fletcher medium base containing leptospira enrichment (Difco Laboratories, Detroit, Mich.) and incubated at 23 to 25°C for leptospira isolation attempts. The serum was tested for leptospiral antibodies to the following antigens: *Leptospira australis*, *L. ballum*, *L. canicola*, *L. griptoyphosa*, *L. hardjo*, *L. icterohemorrhagiae*, *L. georgia*, *L. pomona*, *L. sejroe*, and *L. wolfii*.

Eleven days later, inocula from all thawed specimens except the ileum were plated on the following media: Lowenstein-Jensen (L-J) medium (Difco), BAP, PEA, MAC, EMB, BG, tomato juice, SAB, tryptose, mycobiotic, and pseudomonas isolation agars; in Fletcher, thioglycolate and Trypticase soy broths. Cultural characteristics were determined at 23 to 25°C, at 35 to 37°C, and at 42°C. Biochemical tests for the production of catalase, nitrate reduction, arylsulfatase activity, synthesis of niacin, and hydrolysis of Tween 80 were determined as described by Kubica (8).

Drug sensitivity tests were performed on L-J medium (Difco) containing the following antibiotic lev-
els (µg/ml): streptomycin, 1, 10, 100, and 500; dihydrostreptomycin, 100; iso-nicotinic acid hydrazide, 1, 5, 10, and 100; para-aminosalicylic acid, 1, 50, and 100; nalidixic acid, 30; and a combination of iso-nicotinic acid hydrazide (5 µg) and para-aminosalicylic acid (100 µg).

RESULTS

Isolation. A mycobacterium identified as "serotype 6" of the M. avium complex was isolated in pure culture from the heart blood, lungs, liver, kidneys, and spleen on initial plating on BAP. The Fletcher broth was turbid at 7 days, and subculture of the broth to L-J and BAP revealed characteristic mycobacterial colonies in pure culture. The serum was negative for antibodies to all of the leptospira antigens tested, and the liver, kidney, and serum were culture negative for leptospira. Citrobacter diversus, Escherichia coli, Proteus mirabilis, and Enterobacter species were isolated from the ileum.

Cultural and biochemical characteristics. Pinpoint, smooth, round, white colonies were observed on the BAP at 10 days after initial inoculation. Gram-variable, beaded, and branched pleomorphic rods similar to those of Nocardia and mycobacterial species were observed after Gram staining. The Kinyoun acid-fast stain was positive with colonies from each BAP, and transfer of these colonies to L-J media confirmed the presence of mycobacteria. Cultural and biochemical characteristics that distinguished the opossum isolate as a member of the M. avium complex are presented in Table 1.

The cultural characteristics of the opossum isolate were compared with other atypical mycobacteria that were isolated from humans on simple laboratory media (Table 2). The M. avium complex isolate grew on a variety of commonly used media upon subculture, and growth was slower at room temperature and at 42°C, compared with 35 to 37°C. Colonies were pinpoint on the following plate media as described: cream colored and pitted in SAB agar (without streptomycin), dry with an irregular border on mycobiotic agar, white and oval on tryptose agar, dry and flat with a reddish tinge around colonies on BG, and clear and oval on EM agar. The mycobacterium did not grow on MAC, PEA, tomato juice, or pseudomonas isolation agars or in thioglycolate and Trypticase soy broths. Colonies on L-J medium were larger than those described on the conventional media. They were smooth, round, and cream colored at 7 to 10 days; however, upon incubation, a definite buff color developed and did not change from dark to light exposure.

The opossum isolate was resistant to all the antibiotics tested except streptomycin (100 and 500 µg/ml) and the dihydrostreptomycin (100 µg/ml). The agent did not grow on the SAB agar containing streptomycin or any other medium that contained the above antibiotics at these levels.

DISCUSSION

Clinical history and postmortem examination of this opossum suggested salmonellosis. After 2 days of negative bacterial growth, routine cultures were prolonged, and leptospirosis was considered as a possible etiology. While Leptospira cultures were in progress, bacterial-like growth was detected on the initial BAP(s). Gram and acid-fast stains revealed an unexpected agent. Subculture of colonies from the BAP(s) to L-J medium and subsequent biochemical tests resulted in the correct diagnosis. Our findings were made possible by holding initially inoculated plates for 10 days before discarding as negative. This is especially important when utilizing exotic laboratory animals, which may harbor rare or unexpected infectious agents.

The understaffed laboratory or the laboratory with an excessive caseload must specialize in diagnosing certain diseases, depending on the animals one is working with and the economic and epidemic importance of the agent. In addition, it is usually impractical for the small microbiology laboratory to routinely inoculate all the necessary media and incubate in the proper environment to isolate all the agents involved in animal diseases. Therefore, the choice of media to be inoculated initially de-

<table>
<thead>
<tr>
<th>Test or substrate</th>
<th>Test isolate M. avium complex-serotype 6</th>
<th>M. avium complex</th>
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</thead>
<tbody>
<tr>
<td>Catalase at 68°C/20 min</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tellurite reduction (3 days)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Niacin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arylsulfatase (3 days)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tween 80 hydrolysis (10 days)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MacConkey</td>
<td>V³</td>
<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>Slow</td>
<td>Slow</td>
</tr>
</tbody>
</table>

³ Serologically typed by the Schaefer method (13) and identified as serotype 6 of the M. avium complex (16).
TABLE 2. Summary of the cultural characteristics of atypical mycobacteria initially isolated on commonly used laboratory media

<table>
<thead>
<tr>
<th>Mycobacterial species (reference)</th>
<th>Species and source of isolate</th>
<th>Classification</th>
<th>Primary isolation medium</th>
<th>Cultural characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mycobacterium avium</strong> complex-serotype 6</td>
<td>Opossum blood, lung, liver, kidney, and spleen</td>
<td><em>M. avium</em> complex</td>
<td>5% BAP</td>
<td>Pinpoint, round, smooth, and white in 7–10 days</td>
</tr>
<tr>
<td><strong>M. chelonei</strong> subsp. abscessus (4)</td>
<td>Human thigh—abcess</td>
<td>Runyon group IV</td>
<td>Peptone broth</td>
<td>Turbid in 4 days</td>
</tr>
<tr>
<td><strong>M. chelonei</strong> (1)</td>
<td>Human arm—thigh abscesses</td>
<td>Runyon group IV</td>
<td>5% sheep blood agar</td>
<td>Tiny, whitish growth in 4 days</td>
</tr>
<tr>
<td>Atypical Mycobacterium (3)</td>
<td>Human subcutaneous thigh abscess</td>
<td>Runyon group IV</td>
<td>Cyclohexamide agar</td>
<td>Grayish, mucoid in 4 days</td>
</tr>
<tr>
<td><strong>M. abscessus</strong> (10)</td>
<td>Human subcutaneous knee abscess</td>
<td>Runyon group IV</td>
<td>Sabouraud glucose agar</td>
<td>Smooth, moist, shiny, light cream in color in 4 days</td>
</tr>
</tbody>
</table>

PENDS on the preliminary diagnosis. The isolation of atypical mycobacteria on conventional media will be missed if one does not suspect them or is not aware that some of these mycobacteria will grow on simple media. Sixteen to forty-nine percent of the *M. avium* complex mycobacteria isolated from humans grow on MacConkey agar (9). The atypical mycobacteria that may be less selective in their growth requirements and grow on commonly used laboratory media may have been previously missed for diphtheroids or other contaminants or completely overlooked (4).

The primary isolation of a mycobacterium from the *M. avium* complex from an animal species on blood agar or in Fletcher broth has not been reported. In addition, this is only the second documented report describing the isolation and identification of a mycobacterial species from the American opossum. Earlier, *M. intracellulare-Howell* (11), now recognized as *M. avium* complex-serotype 12 (16), was isolated from the American opossum. Inocula from this animal were plated on blood agar; however, the authors did not report any growth on the blood agar plate, nor did they say how long the plates were observed.

These findings indicate that some of the atypical mycobacteria from the *M. avium* complex which infect domestic animals, wildlife, and humans may be isolated on routine laboratory media and that the microbiologist processing specimens from exotic laboratory animals should emphasize the need to store excess specimens and to retain all culture plates, especially the negative ones, as long as feasible or until a correct diagnosis is obtained.

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LITERATURE CITED


