

Isolation of a Fastidious *Mycobacterium* Species from Two AIDS Patients

KATHY JACKSON,* AINA SIEVERS, BRUCE C. ROSS, AND BRIAN DWYER

*Clinical Pathology, Fairfield Hospital, Yarra Bend Road,
Fairfield, Victoria, Australia 3078*

Received 29 May 1992/Accepted 26 August 1992

Two strains of fastidious mycobacteria were isolated from two patients with AIDS and clinical disease suggestive of *Mycobacterium avium* complex infection. Acid-fast bacilli were isolated from blood and bone marrow of both patients in BACTEC 12B and/or 13A media. The acid-fast bacilli failed to grow on subculture to routine Löwenstein-Jensen medium containing pyruvate and egg yolk agar. After several attempts, the strain from one patient was finally cultured on Middlebrook 7H9 medium with agar, charcoal, and yeast extract 13 months after the initial specimens were received in the laboratory. The second patient's strain was cultured on the same medium 6 weeks postinoculation with fresh BACTEC fluid and 5 months after specimen collection. Routine biochemical and growth tests were performed on these isolates but failed to give definitive identifications. 16S rRNA gene sequencing suggested that the organisms share at least 98.9% homology with *M. simiae*. Even greater homology (99.86%) was found with the recently described species "*M. genavense*." Recognition of the fastidious nature of some mycobacteria that infect AIDS patients is important in the treatment of infections in these patients and in understanding the epidemiology of atypical mycobacterial infections. It is suggested that a liquid culture medium such as BACTEC be employed for primary isolation of mycobacteria from AIDS patients and that subculture to the charcoal medium described here be carried out for those organisms that fail to grow on subculture to routine media.

Disseminated mycobacterial infection is a well-documented opportunistic infection of patients with AIDS. *Mycobacterium avium* complex (MAC) organisms are responsible for most of these infections (5, 7), although other species have been implicated, including *M. tuberculosis*, *M. kansasii*, *M. haemophilum*, and *M. simiae* (2, 4, 7, 11, 14). Less commonly, *M. gordonae*, *M. fortuitum*, and *M. chelonae* have also been reported as causing disseminated mycobacterial infections in AIDS patients (7). Patients with disseminated MAC infections may experience fever, malaise, weight loss, and other nonspecific symptoms and often fail to clear the organism despite antimycobacterial therapy (5).

Hirschel et al. recently reported an infection with a novel unidentified organism resembling *M. simiae* which failed to grow on solid media but showed limited multiplication in BACTEC liquid medium (6). This patient had clinical signs and symptoms consistent with MAC infection. Böttger et al. (1) also described disseminated infections in 18 patients infected with an organism that could not be grown on solid media. These strains had a unique 16S rRNA gene sequence and have been tentatively named "*M. genavense*." Here we report on the isolation of two strains of fastidious mycobacteria from two AIDS patients who also had clinical disease suggestive of MAC infection. These strains were both isolated in BACTEC broth cultures (Becton Dickinson, Sydney, Australia) and initially failed to grow on routine solid media. After several attempts, the organisms were cultured on Middlebrook 7H9 medium (Difco, Detroit, Mich.) containing agar, charcoal, and yeast extract.

CASE REPORTS

Patient 1. Patient 1 was a 38-year-old homosexual man who was found to have antibodies to the human immunode-

fiency virus in May 1987, when he reported to a physician with esophageal candidosis. In August 1987, the patient developed fever and other symptoms thought to be consistent with MAC septicemia. Three blood cultures were collected in BACTEC 13A bottles over a 2-month period, and a bone marrow specimen was taken and sent to the laboratory. Upon detection of acid-fast bacilli (AFB) in the BACTEC broths of these specimens, antimycobacterial therapy was commenced. The patient showed obvious clinical improvement at the time but later contracted other opportunistic infections associated with AIDS, including an episode of *Pneumocystis carinii* pneumonia in May 1988. The patient died in January 1989 after deterioration of his mental state. An autopsy revealed a cerebral lymphoma. Several autopsy specimens were sent for mycobacterial culture; however, AFB were not detected by microscopy or culture.

Patient 2. Patient 2 was a 35-year-old homosexual man in whom human immunodeficiency virus antibodies were detected in 1985. On 12 November 1990, the patient reported to a physician with fatigue, occasional diarrhea, and a temperature of 38°C. A blood culture for mycobacteria was collected at this time, and five more were taken over the following months. Two weeks later, the patient was admitted with left-side abdominal pain, nausea, vomiting, diarrhea, and a slightly elevated temperature of 37.8°C. The fever and abdominal pain persisted for several days. A bone marrow biopsy was taken and sent for mycobacterial culture and histology. Histological examination showed granulomatous infiltrates consistent with MAC infection. A computerized tomography scan demonstrated widespread mediastinal and retroperitoneal lymphadenopathy also suggestive of atypical mycobacterial infection. Rifabutin, isoniazid, ethambutol, and clofazamine therapy was commenced empirically. Three months later, the patient's abdominal lymphadenopathy was markedly decreased and the fevers had abated. The quadruple therapy was continued.

* Corresponding author.

MATERIALS AND METHODS

Culture methods. Blood samples were collected in 5-ml volumes from each of these two patients and inoculated into BACTEC 13A bottles. Enrichment supplement (0.5 ml) containing 15% bovine serum albumin (Becton Dickinson) was aseptically added to each bottle. The bone marrow specimens were lysed with sterile distilled water and centrifuged at $2,500 \times g$ for 20 min. The deposits (0.5 ml) were inoculated into BACTEC 12B broths (Becton Dickinson) supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA; Becton Dickinson). The deposits (0.2 ml) were also cultured onto Löwenstein-Jensen medium containing 0.4% sodium pyruvate (LJP) and egg yolk agar (EYA), and a smear was prepared for Ziehl-Neelsen staining. The slopes were incubated at 31 and 36°C in air for 12 weeks and examined weekly. The BACTEC bottles were incubated at 36°C and read weekly by the BACTEC 460 TB instrument (Becton Dickinson) for 6 weeks and then again at 12 weeks. Cultures displaying positive growth readings were subcultured onto LJP and EYA, which were incubated at 36°C in air indefinitely. Smears were prepared for Ziehl-Neelsen staining, and NAP TB differentiation tests (Becton Dickinson) were performed to differentiate *M. tuberculosis* complex organisms from other mycobacteria.

As the AFB from both patients' specimens failed to grow on LJP and EYA, the BACTEC broths from patient 1 were subcultured at regular intervals onto a variety of media which were incubated at 36°C in a 5% carbon dioxide atmosphere. These included LJP, EYA, Herrold's medium containing mycobactin J, Middlebrook 7H10 medium (Difco) with 1.3% hemin, chocolate agar, Kirschner's broth, Middlebrook 7H10 medium supplemented with an autoclaved suspension of *M. phlei*, and Middlebrook 7H9 medium (Difco) containing 1.3% agar, 0.2% charcoal, and 1% yeast extract (7H9/CYE) (7H9/CYE can also be prepared by adding 2.5 g of *Legionella* CYE agar base [Oxoid] to 90 ml of Middlebrook 7H9 broth supplemented with 10 ml of ADC enrichment [Difco]). The organism was kept viable by subculturing in BACTEC 12B broths. The isolate from patient 2 was subsequently subcultured onto LJP, EYA, Löwenstein-Jensen medium-1% ferric ammonium citrate, and 7H9/CYE medium and incubated at 36°C in a 5% CO₂ atmosphere.

Identification procedures. The AFB were identified by standard biochemical and growth tests (10). A niacin test was performed with BACTEC 12B medium by using niacin test strips. The organisms were also probed with commercial DNA probes specific for *M. tuberculosis* complex and MAC organisms (Genprobe Inc., San Diego, Calif.). Drug susceptibility tests were performed by using MICs on Middlebrook 7H10 agar (3) after adaptation to growth on this medium.

Sequencing of 16S rRNA genes. The mycobacterial strains were grown in 20 ml of Middlebrook 7H9 broth, and the DNA was extracted as previously described (16). The DNA pellets were suspended in sterile distilled water. The 16S rRNA genes were then amplified by polymerase chain reaction using oligonucleotide primers rRNA1 and rRNA2 (Table 1) (13). The DNA from each sample was amplified in a buffer supplied by the *Taq* polymerase manufacturer (Promega, Madison, Wis.) in four 50- μ l reactions containing 50 ng of DNA, 1 μ M primers, 1 U of *Taq* polymerase, 100 μ M dATP, 100 μ M dCTP, 100 μ M dGTP, and 100 μ M dTTP. The reactions were performed by using an automated thermal cycler (Thermal Reactor; Hybaid). After an initial denaturation step of 94°C for 4 min, the reactions underwent 35

TABLE 1. Primers used for both amplification of the 16S rRNA genes and sequencing

Name	Sequence	Positions ^a
rRNA1	5' AGA GTT TGA TCC TGG CTC AG 3'	10-29
rRNA2	5' AAG GAG GTG ATC CAG CCG CA 3'	1532-1513
MR1	5' AGT CCC AGT GTG GCC GG 3'	321-305
MR2	5' TGC AGG TGG TGC ATG GC 3'	1033-1049
MR3	5' CCT ACG AGC TCT TTA CG 3'	575-559
MR4	5' CTT TGA GTT TTA GCC TT 3'	908-892
MR5	5' TGA GAC AAG GGT TGC GCT CG 3'	1112-1093
MR6	5' GAT CTG CGA TTA CTA GCG 3'	1350-1333

^a Positions in *M. bovis* BCG 16S rRNA (GenBank accession number, M20940).

cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Following amplification, the polymerase chain reaction products were purified by high-performance liquid chromatography (15) and then sequenced by using the direct incorporation protocol of the *fmol* DNA sequencing system (Promega) in accordance with the manufacturer's instructions. The sequencing primers MR1 to MR6 and rRNA2 (Table 1) were designed to read overlapping regions of the whole 16S rRNA gene. The 1,422 bases sequenced were then compared with known mycobacterial sequences by searching the GenBank data base (release 71).

RESULTS

The bone marrow specimens from both patients were negative for AFB on direct microscopy and on primary culture on solid media. The three blood cultures and the bone marrow BACTEC 12B broth culture from patient 1 showed positive growth readings of between 22 and 570 after 5 to 12 weeks of incubation. The bone marrow BACTEC 12B broth culture from patient 2 yielded a positive growth reading of 25 at week 6, and two of six blood cultures gave positive growth index values of 75 and 169 after 12 weeks of incubation. AFB were seen in smears from all of these BACTEC cultures, and NAP TB differentiation tests gave results indicative of *M. tuberculosis* complex organisms. The AFB from all BACTEC cultures however, failed to grow on subculture to routine solid media (LJP and EYA). The AFB were kept viable by serial subculturing in BACTEC broths. Over a 7-month period, a variety of different media were inoculated with fresh BACTEC cultures and incubated in a 5% CO₂ atmosphere. The strain from patient 1 was first cultured on solid medium (7H9/CYE) after 6 months of incubation at 36°C in a CO₂-enriched environment. The time from specimen receipt to growth on solid medium was 13 months. The organism also showed limited growth on Middlebrook 7H10 medium supplemented with an autoclaved suspension of *M. phlei* and on Herrold's medium containing mycobactin J at this time. However, in contrast to the results obtained with 7H9/CYE medium, the organism could not be easily subcultured on these media. The organism now grows readily on 7H9/CYE, Middlebrook 7H10, and Löwenstein-Jensen medium containing 1% ferric ammonium citrate but grows poorly or not at all on EYA and LJP.

The isolates from patient 2 were cultured on solid (7H9/CYE) medium 6 weeks after inoculation with a BACTEC broth culture. This strain later adapted to growth on Löwenstein-Jensen medium containing 1% ferric ammonium citrate and on Middlebrook 7H10 medium.

The colony morphology of strains from both patients was

TABLE 2. Base changes in 16S rRNA between patient strains and *M. simiae*

Position ^a	Change
40	C→T
47	A insertion
137	G→T
144	T→G
145	T→G
146	G→A
147	G→A
154	C→T
155	C→T
186	Deletion
399	C→T
412	C→G/T
906	A→G
1122	G→A
1182	G→A
1204	C→T

^a Positions in *M. simiae* 16S rRNA (GenBank accession number, X52931).

smooth and nonpigmented. The results of the biochemical and growth tests for both patients' strains were identical. The isolates demonstrated ability to grow at 25°C (weakly), 31°C, and 36°C but not at 43°C. Negative results were obtained with the following tests: niacin secretion, nitrate reduction, Tween hydrolysis, aryl sulfatase production, and tellurite reduction. It is unknown whether these strains gave negative results in the latter four tests, owing to inability to grow in the biochemical media or whether they truly lacked the appropriate enzymes. However, the biochemical tests were heavily inoculated and it is assumed that if the appropriate enzymes had been available positive results would have been obtained. The organisms both showed pyrazinimidease and urease activities and gave negative results with both *M. tuberculosis* complex- and MAC-specific DNA probes.

The isolate from patient 1 was resistant to streptomycin, isoniazid, ethambutol, rifampin, rifabutin, and ciprofloxacin and susceptible to amikacin. The strain from patient 2 demonstrated resistance to isoniazid, ethambutol, and ciprofloxacin and susceptibility to rifampin, rifabutin, and amikacin.

The 16S rRNA sequences of the strains from both patients 1 and 2 are identical and are listed in GenBank (accession number, M95488). Results of a search through the GenBank data base confirmed that this organism is a *Mycobacterium* species and shares greatest homology (at least 98.9%) with *M. simiae*. The 1.1% variation in the sequence between *M. simiae* and the patients' strains is represented by 14 base substitutions, one deletion, and one insertion, and 13 bases were considered unreadable. Table 2 indicates the positions of these divergences. Comparison of the "*M. genavense*" 16S rRNA sequence listed in the EMBL data base (accession number, X60070) with that of the patient strains, however, demonstrated even greater homology (99.86%). The divergence from "*M. genavense*" is represented by a G deletion at position 186 and a T-for-G substitution at position 399 (position numbers are those found in the *M. simiae* sequence [GenBank accession number, X52931]), and 13 bases were unreadable.

DISCUSSION

The fastidious nature of some mycobacteria isolated from AIDS patients has been previously noted (6, 8, 9). In 1990,

we isolated a fastidious *M. tuberculosis* strain from an AIDS patient. The strain demonstrated a preference for broth culture and glycerophobicity when it was finally cultured on solid media (8). We have also encountered a fastidious *M. avium* strain which was isolated from the blood of an AIDS patient. The organism grew proficiently in BACTEC 12B broth and was only cultured on a solid medium, Herrold's medium containing mycobactin J, 6 months after primary isolation (9). Hirschel et al. (6) reported the isolation of a fastidious *Mycobacterium* sp. from an AIDS patient which was cultivated in congenitally athymic nude mice and showed limited multiplication in BACTEC 13A medium. The AFB, however, could not be subcultured to solid media. The mycolic acid pattern of this strain, together with gas chromatography results, suggested a relationship to *M. simiae*. The *Mycobacterium* species reported in this study also resembled *M. simiae* biochemically, and the 16S rRNA genes share at least 98.9% homology with *M. simiae*.

The 16S rRNA sequence, however, was almost identical to that of the recently described new species of mycobacteria tentatively named "*M. genavense*" (1). Böttger et al. described 18 cases of infection with this organism in patients with AIDS. The clinical course in these patients was not unlike that of cases 1 and 2, with predominant symptoms of fever, diarrhea, and weight loss. These strains were also unable to grow on a range of solid media. The researchers proposed that these organisms are a new species on the basis of a unique 16S rRNA sequence. The 0.14% variation between the "*M. genavense*" sequence and the one reported here may be due to errors caused by *Taq* DNA polymerase and/or reading of the sequence. If that is the case, it is highly probable that the organisms isolated from cases 1 and 2 are "*M. genavense*". Unfortunately, biochemical and drug susceptibility tests could not be performed on the strains described by Böttger et al., owing to lack of growth on solid media.

M. simiae is an organism which has been found to be difficult to characterize fully, owing to poor reproducibility in standardized tests (11). It may therefore be premature to draw conclusions about the definitive identity of the organism described in this study and whether it should be classified as a new species or subspecies of mycobacterium. Further characterization is required, with particular reference to the methods described by Lévy-Frébault and Portals (12), and this work is currently being undertaken.

We were fortunate that, unlike the strains described by Böttger et al. (1) and Hirschel et al. (6), the organisms reported here were culturable on solid media after extended incubation. The usefulness of incorporating charcoal to absorb toxic substances and yeast extract to provide additional nutrients in media for fastidious mycobacteria should be understated. It is possible that the strain from patient 1 adapted to laboratory growth after more than 1 year of repeated subculture in broth. The strain from patient 2, however, was cultured well before the strain from patient 1 (6 weeks postinoculation) on 7H9/CYE agar after initial failure to grow on routine LJP and EYA.

The two strains were later cultured on media with and without charcoal and yeast extract to determine whether one or both of these additives enhance growth. However, by this time, both strains had adapted to growth on Middlebrook 7H10 without charcoal and yeast extract. It is therefore recommended that both ingredients be included in the medium for isolation.

It is expected that we will continue to encounter new or fastidious strains of mycobacteria in AIDS patients. Immune

suppression predisposes these patients to infection with organisms that may be less virulent or unable to cause disease in normal, healthy people. The AFB isolated from both patients in this study were implicated as the cause of the patients' symptoms, and both patients were treated with antimycobacterial therapy. Unlike many patients with MAC infections, the two patients reported here demonstrated clinical improvement with therapy and had apparent bacteriological clearance of the AFB. Patient 1 died 18 months after infection of unrelated causes, and patient 2 is still living.

We suggest employment of a liquid culture medium such as BACTEC for primary isolation of mycobacteria from AIDS patients and subculture to 7H9/CYE medium for those strains that initially fail to grow on solid media. It may also be necessary to incubate these slopes in a carbon dioxide-enriched environment and for extended periods. Isolation and characterization of these unusual mycobacteria are important in understanding the epidemiology of atypical mycobacterial infections in AIDS patients.

REFERENCES

1. Böttger, E. C., A. Teske, P. Kirschner, S. Bost, H. R. Chang, V. Beer, and B. Hirschel. 1992. Disseminated "*Mycobacterium genavense*" infection in patients with AIDS. *Lancet* 340:76-80.
2. Chaisson, R. E., G. F. Schecter, C. P. Theuer, G. W. Rutherford, D. F. Echenberg, and P. C. Hopewell. 1987. Tuberculosis in patients with the acquired immunodeficiency syndrome. Clinical features, response to therapy, and survival. *Am. Rev. Respir. Dis.* 136:570-574.
3. Dwyer, B., et al. Unpublished procedure on file at the Mycobacterium Reference Laboratory, Fairfield Hospital, Fairfield, Victoria, Australia.
4. Festenstein, F., and J. M. Grange. 1991. A review, tuberculosis and the acquired immune deficiency syndrome. *J. Appl. Bacteriol.* 71:19-30.
5. Hawkins, C. C., J. W. M. Gold, E. Whimbrey, T. E. Kiehn, P. Brannon, R. Cammarata, A. E. Brown, and D. Armstrong. 1986. *Mycobacterium avium* complex infections in patients with the acquired immunodeficiency syndrome. *Ann. Intern. Med.* 105:184-188.
6. Hirschel, B., H. R. Chang, N. Mach, P. Pigué, M. T. Silva, L. Larsson, P. R. Klatser, J. E. R. Thole, L. Rigouts, and F. Portaels. 1990. Fatal infection with a novel unidentified mycobacterium in a man with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 323:109-113.
7. Horsburgh, C. R., Jr., and R. M. Selik. 1989. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). *Am. Rev. Respir. Dis.* 139:4-7.
8. Jackson, K., B. Ross, A. Sievers, and B. Dwyer. 1992. A fastidious strain of *Mycobacterium tuberculosis* in a patient with AIDS. *N. Engl. J. Med.* 326:1434-1435. (Letter.)
9. Jackson, K. M., A. S. Sievers, and B. W. Dwyer. Unpublished data.
10. Kent, P. T., and G. P. Kubica. 1985. Public health mycobacteriology. A guide for the level 3 laboratory. Centers for Disease Control, Atlanta, Ga.
11. Lévy-Frédault, V., B. Pangon, A. Buré, C. Katlama, C. Marche, and H. L. David. 1987. *Mycobacterium simiae* and *Mycobacterium avium-M. intracellulare* mixed infection in acquired immune deficiency syndrome. *J. Clin. Microbiol.* 25:154-157.
12. Lévy-Frédault, V. V., and F. Portaels. 1992. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *Int. J. Syst. Bacteriol.* 42:315-323.
13. Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger. 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int. J. Syst. Bacteriol.* 40:323-330.
14. Rogers, P. L., R. E. Walker, H. Clifford Lane, F. G. Witebsky, J. A. Kovacs, J. E. Parrillo, and H. Masur. 1988. Disseminated *Mycobacterium haemophilum* infection in two patients with the acquired immunodeficiency syndrome. *Am. J. Med.* 84:640-642.
15. Ross, B. C., K. Raios, K. Jackson, and B. Dwyer. 1992. Molecular cloning of a highly repeated DNA element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. *J. Clin. Microbiol.* 30:942-946.
16. Ross, B. C., K. Raios, K. Jackson, A. Sievers, and B. Dwyer. 1991. Differentiation of *Mycobacterium tuberculosis* strains by use of a nonradioactive Southern blot hybridization method. *J. Infect. Dis.* 163:904-907.