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Bias of Culture Techniques for Diagnosing Mixed *Mycobacterium genavense* and *Mycobacterium avium* Infection in AIDS

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Disseminated *Mycobacterium avium* infection is a common complication in late-stage AIDS. We describe a patient seropositive for human immunodeficiency virus with a disseminated infection caused by mycobacteria. Acid-fast rods were visible by microscopy in stool and bone marrow and in specimens from skin, liver, spleen, lung, and lymph nodes. Using molecular biology techniques and standard culture techniques, we found evidence of a mixed mycobacterial infection with *M. genavense* and *M. avium*. We suggest that the prevalence of *M. genavense* infection in patients with AIDS is underestimated because of the bias toward *M. avium* when using standard techniques for the detection and identification of mycobacteria.

Disseminated infections with nontuberculous mycobacteria, in particular, the *M. avium* complex (MAC), are seen in 15 to 40% of patients with late-stage AIDS and contribute significantly to both increased illness and death (10). Through effective chemotherapy and prophylaxis of opportunistic infections, patients with AIDS survive longer and more of them live to a point at which they have fewer than 100 $CD4^+$ cells per mm^3, when disseminated infections with nontuberculous mycobacteria usually occur (17). Probably as a consequence of this and of the extended availability of mycobacterial blood cultures, these infections are increasingly recognized (10).

Recently, a previously unrecognized mycobacterium which causes disseminated infections in patients with AIDS was identified. Isolates of this mycobacterium, named *M. genavense*, show limited growth in broth medium (3, 4, 6–9). Because of its inability to grow on the solid media used routinely in the mycobacteriology laboratory, *M. genavense* cannot be identified by standard procedures; appropriate diagnosis of *M. genavense* infection requires amplification and analysis of bacterial nucleic acids (2, 12). Therefore, despite the more than now 40 recognized cases of *M. genavense* infection on three continents, the frequency of this infection in human immunodeficiency virus (HIV)-positive patients remains unclear (3, 4, 7, 11, 16, 21).

A 30-year-old homosexual man presented with an 8-week history of profound watery diarrhea, fever, weight loss, and increasing weakness. In 1988, HIV type 1 infection was diagnosed. Except for *Candida* esophagitis 9 months before admission, no other opportunistic infection or Kaposi's sarcoma was documented. Physical examination revealed hepatosplenomegaly, fever, watery diarrhea, abdominal pain, a 12-kg weight loss during the last 3 months, and severe weakness. Laboratory findings demonstrated pancytopenia. The hemoglobin level was 6.5 g/dl, the platelet count was 3.9×10^{10}, and the leukocyte count was 1×10^9 per liter, with 6% lymphocytes. The $CD4^+$ cell count was $40/mm^3$. Therapy with rifampin, ethambutol, clarithromycin, and cefotaxime was initiated; however, the patient died of septic shock 11 days after admission.

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Tissue specimens were processed by standard techniques (18) and were inoculated directly into BACTEC 12B culture bottles (Becton-Dickinson Diagnostic Instruments Systems, Sparks, Md.). For mycobacterial blood cultures, lysis-centrifugation blood culture tubes (Isolator-10; Wampole Laboratories, Cranbury, N.J.) were used according to the instructions of the manufacturer and their contents were inoculated into BACTEC 13A bottles. Stool and urine samples were decontaminated by standard techniques. Samples were analyzed by the BACTEC radiometric method, following the recommendations of the manufacturer. In addition to radiometric culturing, Löwenstein-Jensen slants were inoculated (one per sample). Fecal specimens revealed no enteropathogenic bacteria, fungi, or parasites. In a culture of urine, 10^4 colonies of *Enterococcus faecalis* per ml were found. Routine cultures for bacteria, fungi, and viruses from tissue and blood specimens remained negative.

DNA extraction, amplification, and sequencing were performed as described previously (2, 12, 13). Nucleic acids from BACTEC cultures were extracted by simple mechanical lysis of bacterial cells (12, 13). A 0.5-ml culture sample was centrifuged for 10 min in a microcentrifuge at maximum speed. The supernatant was discarded, and 100 μl of TE (10 mM Tris [pH 7.4], 1 mM EDTA) and a loopful of acid-washed glass beads with a diameter of 100 μm (Sigma, Munich, Germany) were added. The sample was placed in a tissue disintegrator (Mickle Laboratory, Gomshall, United Kingdom) for 2 min to disrupt the cells and was centrifuged at maximum speed for 2 min. A 5-μl aliquot of the supernatant was used in PCR. For extraction of nucleic acids from tissue samples, the sample was dissolved in guanidium isothiocyanate lysis buffer (12). An equal volume of buffered phenol-chloroform-isoamyl alcohol and a loopful of acid-washed glass beads were added. After disrupting the cells in a tissue disintegrator for 2 min, the phases were separated by a short centrifugation (5 min at maximum speed) and the upper phase was again extracted with phenol-chloroform-isoamyl alcohol. Nucleic acids were precipitated with an equal volume of isopropanol at −20°C and were dissolved in 50 μl of sterile water; a 5-μl aliquot was used in PCR.

Amplification of a mycobacterial 16S rRNA gene fragment by PCR was performed by using primer 264 (5′-TGC ACA

TABLE 1. Samples investigated, in vitro growth indices, and PCR identification results

Material	Culture date[a]	Ziehl-Neelsen staining result	PCR result[b]	Results of radiometric cultivation			
				Growth in vitro	Growth index	Duration of in vitro culture (days)[c]	Result of identification[d]
Bone marrow	1	Positive	*M. genavense*	+	0	1	*M. genavense*
				+	137	5	*M. genavense*
				+	>999	37	Mixed population of *M. genavense* and *M. avium*
Blood	1	Not done	Not done	+	69	36	*M. genavense*
Blood	8	Not done	Not done	+	>999	32	*M. avium*
Stool	1	Positive	*M. genavense*	+	0	1	*M. genavense*
				+	600	24	*M. avium*
Stool	4	Positive	Not done	−	0	60	*M. genavense*
Stool	8	Positive	Not done	−	0	60	*M. genavense*
Urine	4	Negative	Not done	−	0	60	Not done
Kidney	Postmortem	Negative	Not done	−	0	64	Not done
Lung	Postmortem	Positive	*M. genavense*	−	0	1	*M. genavense*
					0	48	*M. genavense*
Rectum tissue	Postmortem	Positive	*M. genavense*	+	0	1	*M. genavense*
				+	>999	24	*M. avium*
Liver	Postmortem	Positive	*M. genavense*	−	0	48	*M. genavense*
Skin	Postmortem	Positive	*M. genavense*	−	0	48	*M. genavense*
Lymph node	Postmortem	Positive	*M. genavense*	−	0	48	*M. genavense*
Spleen	Postmortem	Positive	*M. genavense*	+	0	1	*M. genavense*
				+	>999	20	Mixed population of *M. genavense* and *M. avium*
				+	>999	69	*M. avium*

[a] Day after patient's admission.
[b] These specimens were analyzed by PCR by using DNA extracted directly from tissue specimens by guanidium isothiocyanate-phenol extraction.
[c] At the time of PCR analysis.
[d] Determined by amplification and direct sequencing.

CAG GCC ACA AGG GA-3′) in combination with biotinylated primer 285 (5′-GAG AGT TTG ATC CTG GCT CAG-3′). This primer combination preferentially amplifies mycobacterial 16S rRNA gene fragments (2). Successful amplification of the 1.1-kb fragment was controlled by agarose gel electrophoresis. The single-stranded DNA template was prepared by using Dynabeads M-280–streptavidin (Dynal, Hamburg, Germany), as described by the manufacturer, and was resuspended in 20 μl of H_2O. Sequencing was performed by using 2 μl of the obtained Dynabead single-stranded DNA solution, 2 pmol of sequencing primer 244 (5′-CCC ACT GCT GCC TCC CGT AG-3′) per reaction, 0.5 μCi of [α-^{32}P]dCTP at 3,000 Ci/mmol (Amersham Buchler, Braunschweig, Germany), and Sequenase version 2.0 (USB, Bad Homburg, Germany) by standard procedures (12; USB and Dynal manuals). The 16S rRNA sequences within the hypervariable region were aligned manually and were compared with previously published mycobacterial sequences (12, 13).

Disseminated mycobacterial infection in our patient was suspected shortly after admission to the hospital because of the presence of massive quantities of acid-fast bacilli (AFB) in stool and bone marrow biopsy specimens. In the 11-day period before the patient's death, samples from bone marrow, blood, urine, and stool were obtained. On postmortem examination, numerous AFB were observed in the liver, spleen, lymph nodes, lung, and skin; kidney and muscle tissue showed no AFB. The samples obtained were cultured for mycobacteria in BACTEC 12B and 13A broth media (Table 1). Nucleic acids were extracted directly from AFB-positive samples and were used for amplification of a 16S rRNA gene fragment. In addition, aliquots from the cultures were examined by PCR at different intervals. Some cultures were examined regardless of their growth index shortly after inoculation. This procedure was chosen to enable detection of mycobacteria, which do not readily grow in culture. Mycobacterial nucleic acids were amplified from AFB-positive samples and from culture bottles 1 day after inoculation with samples from bone marrow, stool, spleen, lung, and rectum tissue (Table 1). All of these PCR-positive cultures had a growth index of zero at the time of investigation (Table 1). The amplified gene fragments were analyzed and revealed the characteristic sequence of *M. genavense*. Controls included DNAs extracted from samples of different origin from patients without AIDS, from the buffers and enzymes used for PCR, and from the culture medium; in all of these, no amplified DNA was observed.

Surprisingly, retesting of the spleen and bone marrow cultures with a growth index of 999 after 20 and 37 days of in vitro incubation, respectively, resulted in sequences with some ambiguous nucleotide positions characteristic of a mixed population. Analysis of the sequences revealed mixed sequences of *M. genavense* and *M. avium*. At positions which showed sequence differences between *M. genavense* and *M. avium*, both nucleotides were seen simultaneously (Fig. 1). After incubation for 3 to 9 weeks (growth index, 600 to >999), an unambiguous sequence of *M. avium* was obtained from cultures of spleen, rectum, and stool samples, which originally showed the presence of *M. genavense* only (Fig. 1; Table 1). One blood culture revealed *M. avium* after incubation for 32 days, whereas another blood culture did show some growth of *M. genavense* after 36 days. Upon subcultivation of BACTEC vials on Löwenstein-Jensen slants, those with a mixed bacterial population showed colonies of *M. avium* (data not shown).

The proportion of different mycobacterial 16S rRNA genes in amplified DNA fragments is dependent on their relation prior to amplification, since all mycobacterial 16S rRNA genes are amplified with the same efficiency with the primers used (2,

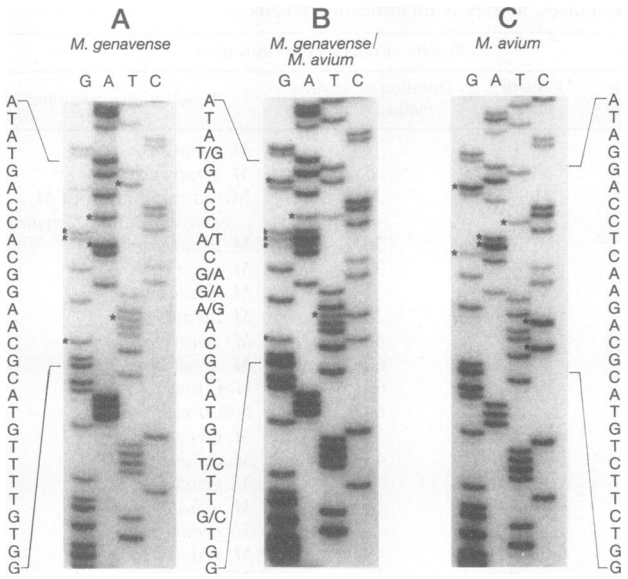

FIG. 1. Mixed mycobacterial infection revealed by sequence determination of a 16S rRNA gene fragment amplified from a spleen sample undergoing in vitro cultivation. Nucleic acids were extracted after 1 day of incubation (growth index, 0) (A), 20 days of incubation (growth index, >999) (B), and 69 days of incubation (growth index, >999) (C). The nucleotides that were different between *M. genavense* and *M. avium* are indicated by asterisks. While analysis of the sample after 1 and 69 days of incubation gave unambiguous sequences for *M. genavense* (A) and *M. avium* (C), respectively, analysis of the culture after 20 days of incubation revealed several sequence ambiguities characteristic of a mixed population (the sequences shown correspond to *Escherichia coli* positions 178 to 205).

12). Therefore, direct sequencing of PCR fragments reveals results which, in the case of mixed populations, also reflect the proportion of the different species in the sample. *M. genavense* is a fastidiously growing mycobacterium which grows to only a limited extent in liquid media (3). Thus, incubation of a mixed population of *M. genavense* and *M. avium* is expected to result in an overgrowth of the cultures with *M. avium*. As a consequence, the relation between the two species will be shifted toward overgrowth of *M. avium*. We estimate that for a mixed infection to be recognized by PCR-mediated sequence analysis, a proportion of an organism of at least 5 to 10% within the total bacterial population is necessary. In our patient, *M. avium* seemed to be present in the samples in small numbers and was detectable only after prolonged in vitro culture.

An issue which needs to be addressed is a possible cross-contamination with *M. avium*, in particular, via the BACTEC system (20). This possibility, however, seems extremely unlikely given that (i) five independent samples (bone marrow, blood, stool, rectum tissue, and spleen) showed the presence of *M. avium*, and four of these samples had mixed mycobacterial infections, (ii) these samples were obtained on different occasions, (iii) in two samples (bone marrow and spleen), growth of *M. avium* was observed on the Löwenstein-Jensen slants used for primary culture (data not shown), (iv) the culture bottles were separated from each other by at least 6 to 20 bottles, and (v) no other positive *M. avium* samples were processed on the same day of inoculation for samples from spleen, rectum tissue, and bone marrow.

Mixed infections with *M. avium* and *M. simiae* (14, 19), *M. avium* and *M. intracellulare* (5), *M. avium* and *M. kansasii* (15), and different strains of *M. avium* (1) have been reported previously in patients with AIDS. However, quite often the pathogenic significance of each species could not clearly be determined. In retrospect, the mixed infection of *M. genavense* and *M. avium* reported here did not seem unexpected given the high prevalence of MAC infections in patients with AIDS (10, 17). MAC bacteremia was observed over a period of 1 year in 25, 32, and 44% of patients with AIDS with $CD4^+$ cell counts of 20 to 49, 10 to 19, and less than 10/mm^3, respectively (17). Most patients with disseminated *M. genavense* infection described so far have $CD4^+$ cell counts of less than 50/mm^3 (4). Hence, both types of infections are found in patients with advanced stages of HIV infection with severe immunodeficiency. By conventional culture techniques, the disseminated mycobacterial infection reported here would have been attributed to *M. avium*. PCR analysis of various tissues, however, allowed us to demonstrate that the huge masses of AFB seen in almost all organs by microscopy were predominantly due to *M. genavense*. Only after prolonged incubation some cultures also eventually yielded *M. avium*. Because *M. genavense* was repeatedly detected in blood and bone marrow samples and because of the unbalanced ratio of *M. genavense* and *M. avium* in clinical samples in vivo, we consider that *M. genavense* more likely accounts for the patient's disease.

We hypothesize that mixed infections with *M. avium* and *M. genavense* may be more frequent than demonstrated by this one case report because of the biased recognition of culture-based detection techniques toward *M. avium*.

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REFERENCES

1. **Arbeit, R. D., A. Slutsky, T. W. Barber, J. N. Maslow, S. Niemczyk, J. O. Falkingham III, G. T. O'Connor, and C. F. von Reyn.** 1993. Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. J. Infect. Dis. **167:**1384–1390.
2. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
3. **Böttger, E. C., B. Hirschel, and M. B. Coyle.** 1993. *Mycobacterium genavense* sp. nov. Int. J. Syst. Bacteriol. **43:**841–843.
4. **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.
5. **Conville, P. S., J. F. Keiser, and F. G. Witebsky.** 1989. Mycobacteremia caused by simultaneous infection with *Mycobacterium avium* and *Mycobacterium intracellulare* detected by analysis of a BACTEC 13A bottle with the Gen-Probe kit. Diagn. Microbiol. Infect. Dis. **12:**217–219.
6. **Coyle, M. B., L. C. Carlson, C. K. Wallis, R. B. Leonard, V. A. Raisys, J. D. Kilburn, M. Samadpour and E. C. Böttger.** 1992. Laboratory aspects of *Mycobacterium genavense*, a proposed species from AIDS patients. J. Clin. Microbiol. **30:**3206–3212.
7. **Heiken, H., P. Kirschner, M. Stoll, E. C. Böttger, and R. E. Schmidt.** 1993. Mykobacterium genavense Infektion bei AIDS-Patienten. Deutsch. Med. Wochenschr. **118:**296–300.
8. **Hirschel, B., H. R. Chang, N. Mach, P.-F. Piguet, J. Cox, J.-D. Piguet, M. T. Silva, L. Larsson, P. R. Klatser, J. E. R. Thole, C. Rigonts, 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.
9. **Hoop, R. K., E. C. Böttger, P. Ossent, and M. Salfinger.** 1993.

Mycobacteriosis due to *Mycobacterium genavense* in six pet birds. J. Clin. Microbiol. **31:**990–993.

10. **Horsburgh, C. R.** 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:** 1332–1338.
11. **Jackson, K., A. Sievers, B. C. Ross, and B. Dwyer.** 1992. Isolation of a fastidious *Mycobacterium* species from two AIDS patients. J. Clin. Microbiol. **30:**2934–2937.
12. **Kirschner, P., A. Meier, and E. C. Böttger.** 1993. Genotypic identification and detection of mycobacteria: facing novel and uncultured pathogens, p. 173–190. *In* D. T. Persing, T. D. White, F. C. Tenover, and T. F. Smith (ed.), Diagnostic molecular microbiology. American Society for Microbiology, Washington, D.C.
13. **Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. Bange, and E. C. Böttger.** 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. **31:**2882–2889.
14. **Levy-Frebault, V., B. Pangon, A. Bure, C. Katalama, 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.
15. **Massenkell, G., M. Opravil, M. Salfinger, A. von Graevenitz, and R. Lüthy.** 1992. Disseminated coinfection with *Mycobacterium avium* complex and *Mycobacterium kansasii* in a patient with AIDS and liver abscess. Clin. Infect. Dis. **14:**618–619.
16. **Nadal, D., R. Cadulff, P. Kraft, M. Salfinger, T. Bodmer, P. Kirschner, E. C. Böttger, and U. B. Schaad.** 1993. Invasive infection with *Mycobacterium genavense* in children with the acquired immunodeficiency syndrome. Eur. J. Clin. Microbiol. Infect. Dis. **12:**5–11.
17. **Nightingale, S. D., L. T. Byrd, P. M. Southern, J. D. Jockusch, S. X. Cal, and B. A. Wynne.** 1992. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. J. Infect. Dis. **165:**1082–1085.
18. **Roberts, G. D., E. W. Koneman, and Y. K. Kim.** 1991. *Mycobacterium*, p. 304–339. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
19. **Torres, R. A., J. Nord, B. Feldmann, V. La Bombardi, and M. Barr.** 1991. Disseminated mixed *Mycobacterium simiae-Mycobacterium avium* complex infection in acquired immunodeficiency syndrome. J. Infect. Dis. **164:**432–433.
20. **Vannier, A. M., J. J. Tarrand, and P. B. Murray.** 1988. Mycobacterial cross-contamination during radiometric culturing. J. Clin. Microbiol. **26:**1867–1868.
21. **Wald, A., M. B. Coyle, L. C. Carlson, R. L. Thompson, and T. M. Hooton.** 1992. Infection with a fastidious mycobacterium resembling *Mycobacterium simiae* in seven patients with AIDS. Ann. Intern. Med. **117:**586–589.