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Detection of Untreated Mycobacteria by Using Polymerase Chain Reaction and Specific DNA Probes

JOCHEN W. U. FRIES,[1]† RUBINA J. PATEL,[2] WILLY F. PIESSENS,[1] AND DYANN F. WIRTH[1]*

Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115,[1] and American Registry of Pathology, Department of Infectious and Parasitic Disease Pathology, Armed Forces Institute of Pathology, Washington, D.C. 20306-6000[2]

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A method for specific identification of mycobacteria by using the polymerase chain reaction on organisms taken from liquid cultures, frozen suspensions, or colonies grown on Lowenstein-Jensen slants is presented. This direct detection of mycobacterial organisms has important implications for strain typing and diagnosis.

Besides *Mycobacterium tuberculosis*, *Mycobacterium avium* is the most frequently encountered opportunistic mycobacterial pathogen in immunocompromised patients (10, 15, 25). While the overall incidence of tuberculosis in the United States is decreasing, individuals suffering from AIDS are becoming reservoirs for both *M. avium* and *M. tuberculosis* infections in increasing numbers. The differential diagnosis of these pathogens is essential. Although successful treatment of tuberculosis is possible even for patients with AIDS, treatment for *M. avium* infections is usually disappointing (15, 25). Specific and rapid detection of *M. avium*, directly from body fluids or after initial isolation, would help to limit unnecessary treatment with tuberculostatic drugs. While infection with *M. avium* and some other mycobacteria is usually related to immunosuppression, *M. tuberculosis* is a potential pathogen for all human beings. Rapid diagnosis in mixed infections may prevent transmission of tuberculosis and aid in early therapeutic intervention.

In recent years, several groups have proposed methods to shorten the time necessary for specific identification of mycobacteria, including differentiation based on microscopy (1, 16), antigen and/or antibody assays (13, 22, 23, 28), restriction fragment length polymorphism (4, 5, 10, 14), and hybridization with DNA probes with or without amplification of target DNA (2, 3, 6, 7, 9–12, 14, 16, 17, 20, 21, 24, 26, 27, 29–31). While these approaches have shown promise, their routine application has been hampered by one or more of the following limitations: (i) low specificity and/or sensitivity, (ii) the necessity to culture the organisms, and (iii) elaborate methods of extracting genomic DNA. Recently, we described methods to circumvent these problems (8, 18, 19). We showed that 10 fg of DNA, the equivalent of two mycobacteria, could be amplified sufficiently to visualize distinct bands in agarose gels. In preliminary studies with the polymerase chain reaction (PCR), we were able to amplify target sequences in both genomic DNA and intact, frozen

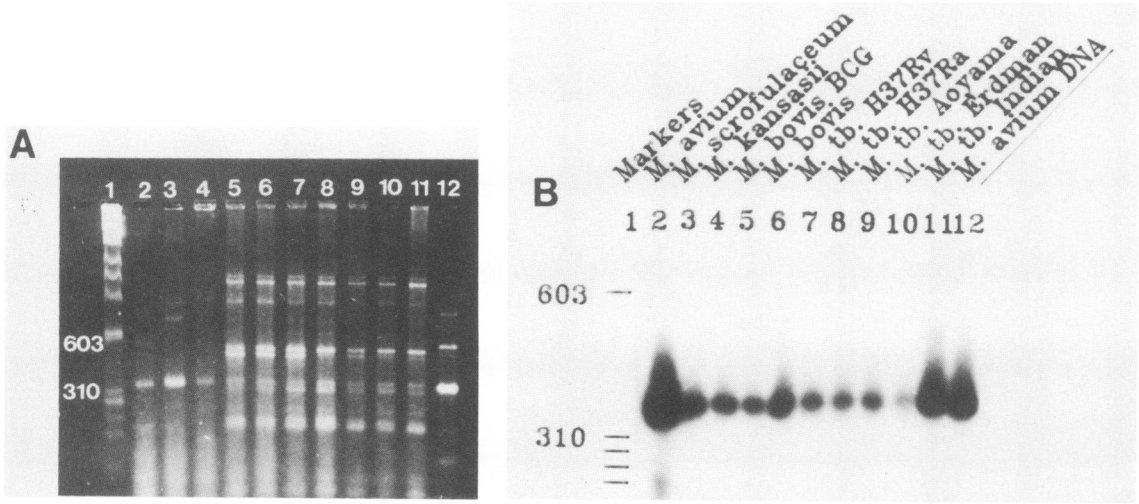

FIG. 1. (A) Electrophoresis of PCR products through a 1.8% agarose gel by using frozen mycobacteria as a template and genus-specific primers Mav17A and Mav17B (8). (B) Hybridization with the genus-specific probe pMav17 (8). Lanes in the gel correspond to those on the autoradiogram. Note the multiple bands in all lanes, particularly lanes 5 through 11, which contain members of the tuberculosis complex.

* Corresponding author.
† Present address: Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115.

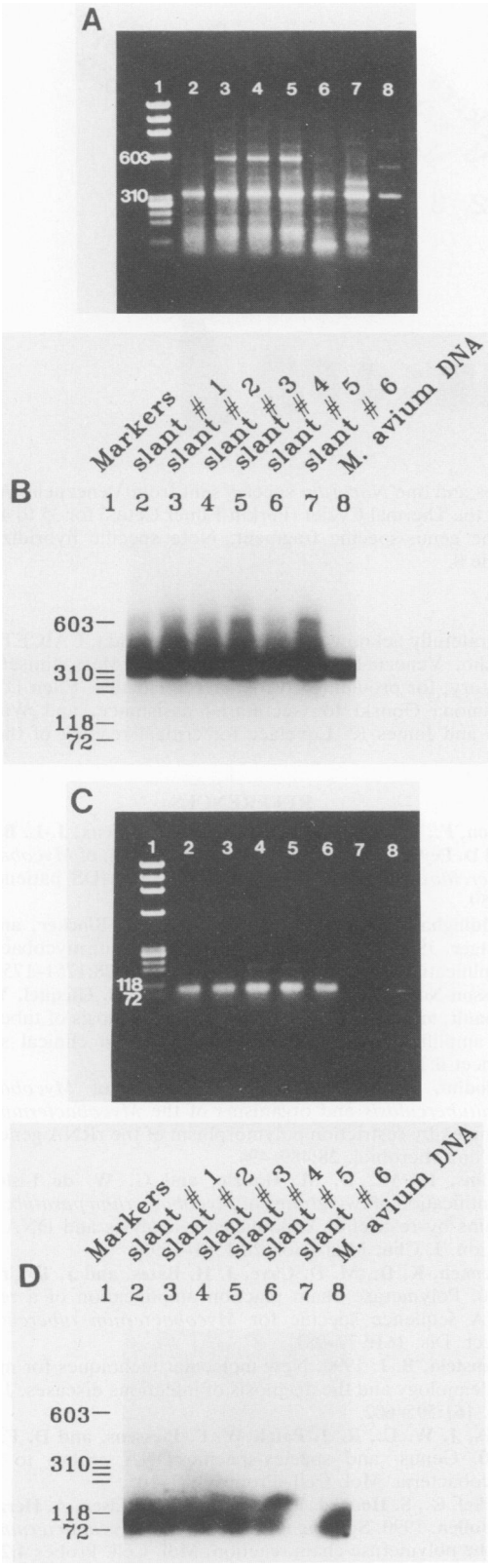

FIG. 2. (A) Agarose gel of genus-specific PCR products obtained from single loopfuls of *M. avium* grown on Lowenstein-Jensen slants. (B) Autoradiogram showing hybridization of a ^{32}P-labeled, 321-bp insert from pMAv17 (8). (C) Agarose electrophoresis of PCR products from isolates from the same patient with *M. avium*-specific primers Mav22A and Mav22B (8). (D) Autoradiogram of hybridization with the radiolabeled *M. avium* subfragment Mav22s (91-bp PCR product). Note that isolate no. 6 (lane 7) hybridizes with the genus-specific but not the *M. avium*-specific probe.

bacilli for the identification of *M. tuberculosis* (14). Here, we report the use of genus- and *M. avium*-specific DNA probes to detect and identify mycobacteria from frozen stocks and log-phase liquid cultures and those grown on Lowenstein-Jensen slants.

Stock cultures which had been frozen at −80°C in 1-ml aliquots containing approximately 10^7 or 10^8 organisms were thawed in a 37°C water bath. The cell suspensions were centrifuged, washed twice with sterile, deionized, distilled water, washed once with 1× PCR buffer (8), and resuspended in 100 μl of buffer. Ten microliters of this suspension was used in each reaction. From 250-ml log-phase cultures of *M. avium* 5- to 10-ml samples were removed, the bacterial pellets were washed as described above, and 10 μl of the resulting suspensions were used in PCRs. A loopful of bacilli from a colony grown on Lowenstein-Jensen slants was suspended in 1 ml of sterile, deionized, distilled water. After centrifugation for 15 min at 4°C, the supernatant was discarded and the bacterial pellet was processed as described above. In an attempt to determine the lowest number of organisms to be detected, 1:100 dilutions were made in sterile, deionized, distilled water containing 0.05% Tween 80 (Sigma Chemical Company, St. Louis, Mo.), and 10 μl from each dilution was used in triplicate PCRs with genus- and species-specific primer pairs. Details of primers and conditions of amplification reactions have been provided elsewhere (8, 18).

Reaction products were analyzed by electrophoresis through 1.8% agarose gels in 1× TBE buffer (18) and either transferred to nitrocellulose filters by the method of Southern or dot blotted (8) onto a nylon membrane (GeneScreen Plus; Dupont, Boston, Mass.). The genus-specific probe was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.) by random priming (8); the *M. avium* probe was labeled by adding [α-^{32}P]dCTP to a PCR mixture, followed by five cycles of amplification.

As seen in Fig. 1A, gel electrophoresis of products from genus-specific primers and several species of mycobacteria resulted in typical ladderlike banding patterns. Hybridization with radiolabeled, genus-specific probe yielded bands of approximately 350 bp in all mycobacterial species (Fig. 1B). Figure 2A shows results of PCRs with organisms from six patient isolates identified at the Massachusetts State Laboratory as *M. avium*. Although the same banding pattern was not seen as with purified DNA, hybridization resulted in positive signals in all lanes (Fig. 2B). When the experiment was repeated with *M. avium*-specific primers (Fig. 2C), amplified bands were seen in all lanes except lane 7. Hybridization with the *M. avium*-specific probe also showed no signal in lane 7 (Fig. 2D). This isolate was later reclassified as *Mycobacterium kansasii*.

Similarly, patient isolates sent from Venezuela showed successful amplification (Fig. 3A) and hybridization (Fig. 3B). All mycobacterial samples gave positive results with both genus-specific primers and probes, but no hybridization was seen with *Nocardia* spp. (Fig. 3B, lane 8). When repeated with the *M. avium*-specific primers, amplification and hybridization were seen only in lanes containing *M. avium* bacilli or purified DNA (data not shown). These

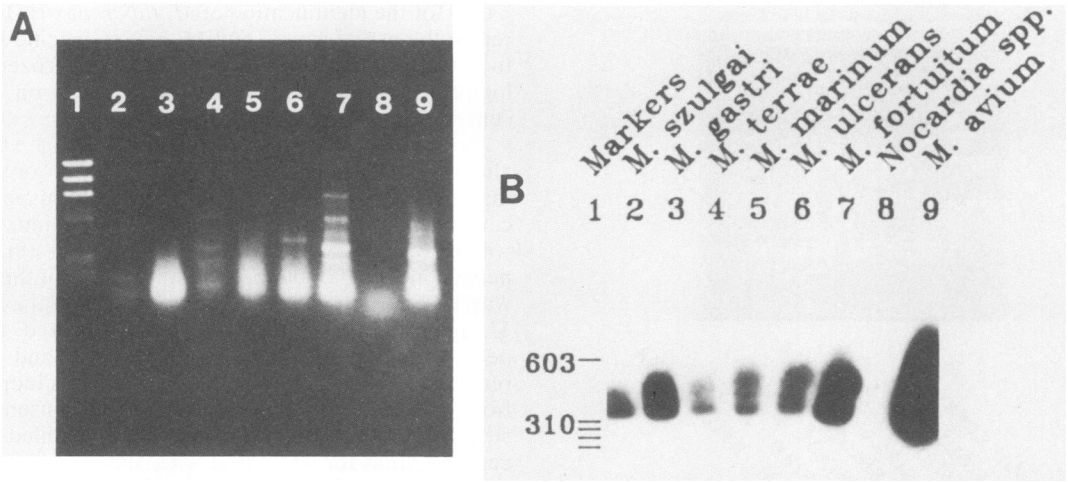

FIG. 3. (A) Amplification of DNA from several mycobacterial patient isolates and one *Nocardia* species sent from Venezuela. A loopful of bacteria was added to each PCR, and the tubes were loaded immediately onto the Thermal Cycler (Perkin-Elmer Cetus) for 35 to 40 cycles of amplification. (B) Autoradiogram of a nitrocellulose filter probed with the genus-specific fragment. Note specific hybridization in mycobacterial lanes and the absence of hybridization with *Nocardia* spp. in lane 8.

results demonstrate that mycobacterial DNA can be amplified and specifically identified even after the organisms have been transported over long distances and stored at room temperature and even if they have not been subjected to extensive lysis procedures.

At different times during culture and subculture of *M. avium*, growth was monitored by acid-fast staining with a Kinyoun staining kit (Fisher Scientific, Boston, Mass.). Since staining occurred with a considerable degree of variability, aliquots of the culture were used in PCRs with *M. avium*-specific primers. As expected, the PCRs produced single specific bands of about 90 bp (data not shown). These bands hybridized specifically with the radiolabeled *M. avium* probes. Dilutions of cultures were also made, but after several attempts at obtaining uniform suspensions, small clumps of bacilli were still seen upon microscopic examination. Mycobacteria could no longer be detected microscopically from the tube containing approximately 10^3 mycobacteria per ml, but gel electrophoresis of triplicate PCRs with both genus- and species-specific primer pairs resulted in characteristic bands as seen before. After Southern transfer and hybridization, all samples containing aliquots from the dilutions gave positive signals, whereas no hybridization was seen in the control lanes containing no DNA template.

In this article, we demonstrate the specific identification of whole mycobacteria grown under different culture conditions by using PCR. Furthermore, amplification of target DNA was possible after merely washing bacilli and with no further attempt at lysis. We were able to detect mycobacteria grown in liquid cultures, from aliquots which had been frozen for over 5 years, and from colonies on Lowenstein-Jensen slants shipped from Venezuela at room temperature. While the genus-specific probe detected all mycobacterial species, the *M. avium*-specific probe recognized only *M. avium* bacilli or purified DNA. From serial dilutions of a mycobacterial culture, we concluded that no more than 10 mycobacteria are needed for specific detection by PCR.

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