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Use of a Multiplex PCR To Detect and Identify *Mycobacterium avium* and *M. intracellulare* in Blood Culture Fluids of AIDS Patients

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The presence of mycobacteria in blood culture fluids (BACTEC) of AIDS patients with positive growth indices (GIs, >20 U) was investigated by using a multiplex PCR to detect and identify members of the genus *Mycobacterium*, *M. avium*, *M. intracellulare*, and *M. tuberculosis*. Three different methods of extracting mycobacterial DNA from blood culture fluid were compared for use with the multiplex PCR. Mycobacterial cells were pelleted from a small aliquot of blood culture fluid by centrifugation, and the DNA was extracted from cells by heat lysis or a sodium iodide-isopropanol or a phenol-chloroform method. DNAs of different sizes were amplified from a region of the MPB70 gene of *M. tuberculosis* (372 bp) and from a region of the 16S rRNA gene of members of the genus *Mycobacterium* (1,030 bp), *M. intracellulare* (850 bp), or *M. avium* (180 bp) as a multiplex PCR in a single tube. The amplified DNA products were detected by agarose gel electrophoresis and ethidium bromide staining in all 41 (100%) positive cultures after sodium iodide-isopropanol extraction, in 18 (44%) after heat lysis, and in 5 (12%) after phenol-chloroform extraction. Of the 41 positive cultures, 38 were identified as *M. avium* and 2 were identified as *M. intracellulare* by both routine methods and multiplex PCR. The remaining mycobacterium was identified as *M. intracellulare* by routine methods and as *M. avium* by the multiplex PCR. Another six blood cultures that were negative for the presence of acid-fast bacilli after Ziehl-Neelson staining were also negative by PCR. The study shows that the multiplex PCR is a useful method for the detection and identification of either *M. avium* or *M. intracellulare* in small samples of cultured BACTEC 13A fluid with positive GIs ranging from 21 to 999 U. The average time to a positive GI was 18 days (median, 13 days) and ranged between 8 and 42 days. The multiplex PCR may permit cultured mycobacteria to be identified at an earlier stage than the routine methods which have been adapted for use with the BACTEC system. The results also show that the method selected for extracting mycobacterial DNA from blood culture fluids is crucial for providing sensitive and accurate PCR results.

The *Mycobacterium avium* complex (MAC) consists of two closely related species, *M. avium* and *M. intracellulare* (30). Human infection with MAC is rare in immunocompetent individuals but is prevalent in human immunodeficiency virus (HIV)-infected patients, often occurring late in the course of AIDS, and is associated with profound immunodeficiency, dissemination, and a poor prognosis (12, 18). The incidence of MAC bacteremia in HIV-positive patients followed at one institution over a 3-year period from the day of AIDS diagnosis was 21% at 1 year and 43% at 2 years (19). The detection rate of MAC at autopsy has been as high as 53% (7).

Rapid identification of disseminated MAC infections in AIDS patients is important for the early initiation of triple or quadruple drug therapy to improve patient outcome (12, 13, 18). Laboratory diagnosis of MAC infection in AIDS patients is usually made by the BACTEC blood culture-fluid system which radiometrically detects bacterial growth. The presence of acid-fast bacilli (AFB) is confirmed by Ziehl-Neelson (ZN) staining (18, 25). Once sufficient growth has occurred, nucleic acid probes (2, 6, 8) and conventional microbiological methods (27, 29) are used to identify the organisms. However, because of the slow growth of mycobacteria and the variable numbers of organisms present in the blood, a delay from a few days to 6 weeks may occur before growth is detected, and the delay may be longer before the organism is fully identified. In addition, identification of the MAC organisms in positive blood culture bottles by ZN staining lacks sensitivity and species specificity (33), gene probing is expensive (2, 6, 8), and differentiation between *M. avium* and *M. intracellulare* by heat tolerance is slow and lacks reliability (27).

DNA amplification by PCR is a rapid analytical technique for the detection and identification of various bacteria (32) and has the potential to reduce the time taken for the identification and species determination of *M. avium* and *M. intracellulare* to a few days. Various approaches have been described for the identification of MAC by PCR. Hance et al. (11) amplified a gene encoding a 65-kDa mycobacterial antigen and identified the amplification product by hybridization to oligonucleotide probes, one of which is specific for MAC. Fries et al. (9, 10) used primers and probes to detect and identify a specific sequence of *M. avium* after in vitro culture of the organism. Boddinghaus et al. (1) amplified a 16S rRNA gene of mycobacteria at the genus level and identified the species of MAC by hybridizing the amplified product with species-specific oligonucleotide probes. By comparison, Wilton and Cousins (31) described a sensitive multiplex PCR which specifically amplifies DNA fragments of different sizes from the MPB70 gene of *M. tuberculosis* and from the 16S rRNA gene of members of the genus *Mycobacterium*, *M. avium*, and *M. intracellulare* in a single-tube assay without using a hybridization step.

We have chosen the multiplex PCR (31) as a simple nonradioactive method to detect and identify members of the genus *Mycobacterium*, *M. tuberculosis*, *M. avium*, and *M. intracellulare*

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TABLE 1. Analysis of 143 bacterial isolates by multiplex PCR for the detection of members of the genus *Mycobacterium*, *M. intracellulare*, *M. tuberculosis*, and *M. avium*

Bacterial species	No. of isolates	No. testing positive by multiplex PCR[a]			
		Mycobacterium genus (1,030 bp)	*M. intracellulare* (850 bp)	*M. avium* (180 bp)	*M. tuberculosis* (372 bp)
Escherichia coli	1				
Actinomyces pyogenes	1				
Nocardia asteroides	1				
Nocardia brasiliensis	1				
Rhodococcus equi	1				
M. bovis	4	4			4
M. bovis BCG	3	3			3
M. chelonae	3	3			
M. fortuitum	1	1			
M. gordonae	5	5			
M. kansasii	3	3			
M. scrofulaceum	1	1			
M. smegmatis	2	2			
M. terrae	3	3			
M. tuberculosis	24	24			24
M. avium	55	55	5	50	
M. intracellulare	32	32	30	2	
M. marinum	2	2			
Total	143	138	35	52	31

[a] Values in parentheses are multiplex PCR product size.

cultured in BACTEC growth fluids. The use of this multiplex PCR has not been previously reported for the detection and identification of mycobacteria in blood culture fluids obtained from AIDS patients. In previous studies, mycobacterial DNA for PCR was usually isolated by enzymatic lysis and phenol-chloroform extraction (1, 3, 11). In the present study, we compared phenol-chloroform extraction (PC) with heat lysis (HL) (5, 10) and sodium iodide-isopropanol extraction (NI) (15) in order to simplify the DNA extraction method for routine laboratory practice.

MATERIALS AND METHODS

Bacteria used to assess and standardize PCR. A collection of 143 isolates including reference strains of mycobacteria, clinical isolates of mycobacteria, and other bacteria obtained from the routine diagnostic laboratory were used to assess and standardize the multiplex PCR (Table 1). Many of the mycobacterial strains described by Wilton and Cousins (31) were obtained from the Mycobacterium Reference Laboratory, State Health Laboratories (Perth, Australia). These included *M. bovis*, *M. bovis* BCG, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. scrofulaceum*, *M. smegmatis*, *M. terrae*, and *M. tuberculosis*. A collection of 55 different patient isolates of *M. avium* (including serotypes 1 to 4, 8, 10, and 21 and nontypeable) and 32 different isolates of *M. intracellulare* (including serotypes 12, 14, 16, and 17 and nontypeable) were also tested. The nonmycobacterial strains tested included *Actinomyces pyogenes*, *Nocardia asteroides*, *Nocardia brasiliensis*, and *Rhodococcus equi.*

All mycobacterial strains were grown by standard methods on egg-based media (29), and the other bacteria were grown by standard bacteriological procedures. Colony growth from each strain was mixed in 0.5 ml of sterile distilled water to obtain a suspension equivalent to a no. 5 McFarland opacity standard (Difco). A 10-fold dilution in water was heated at 95°C for 10 min, and the samples were stored frozen at −20°C until they were either tested by PCR or further extracted with phenol-chloroform (3, 4) or by a NaI-isopropanol cell lysis and DNA isolation method (15) and were then tested by PCR.

Collection and processing of blood cultures. Forty-seven blood specimens were collected for culture of mycobacteria in BACTEC bottles from 16 AIDS patients who were either suspected of or were known from previous analyses to have a disseminated *M. avium-M. intracellulare* infection (see Table 3). The AIDS patients were one woman and 15 men with HIV infection and with a median age of 39 years (range, between 30 and 60 years). The volume of blood used to inoculate 30 ml of growth medium in BACTEC 13A bottles (Middlebrook 7H12 medium; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) was 5 to 7 ml. All cultures were incubated at 37°C for 6 to 7 weeks, and the BACTEC 13A bottles were examined for growth with the BACTEC 460 radiometer (Becton Dickinson Diagnostic Instrument Systems) twice weekly during the first 2 weeks and then weekly thereafter. A BACTEC growth index (GI) of >20 U was considered positive, and a smear for detecting AFB by ZN staining was prepared by using a small portion of the broth from the BACTEC bottle.

A 5-ml sample was retained for PCR from the 43 blood cultures that had a GI of >20 U and 4 blood cultures that had a GI of <21 U. AFB were detected in only 41 of the 43 positive blood cultures by ZN staining. A *Cryptococcus* sp. was isolated from the two AFB-negative cultures with a GI of >20 U (see Table 3, patient 1). No organisms were isolated from the other four AFB-negative blood cultures which had GIs of <21 U. The average GI for the AFB-positive cultures was 208 U (median GI, 69 U), and the GI ranged between 21 and 999 U. The average time to a positive GI was 18 days (median, 13 days) and ranged between 8 and 42 days.

TABLE 2. Results of DNA hybridization between oligonucleotide probes and PCR products obtained by multiplex PCR of DNA of members of the genus *Mycobacterium*, *M. intracellulare*, *M. tuberculosis*, and *M. avium*

Hybridization oligonucleotide probes (code and sequence)	DNA hybridization result[a]			
	Mycobacterium genus (1,030 bp)	*M. intracellulare* (850 bp)	*M. avium* (180 bp)	*M. tuberculosis* (372 bp)
MAV-180Pr (region of 16S rRNA gene) (5′-CGA ACG GGT GAG TAA CAC GTG GGC AAT CTG-3′)	+	−	+	−
Pr338 (region of 16S rRNA gene) (5′-ACT CCT ACG GGA GGC AGC-3′)	+	+	−	−
MYCTBPr (region of MPB70 gene) (5′-TAC ACG GTG TTC GCA-3′)	−	−	−	+

[a] Values in parentheses are multiplex PCR product size.

The remainder of the blood culture fluid (30 ml) was sent to the State Health Laboratories (Perth) for identification and species determination of the organisms by their routine method of DNA hybridization and differential characterization by heat tolerance. The MAC DNA was identified by hybridization with a commercial probe (Gen-Probe Inc., San Diego, Calif.), and *M. avium* and *M. intracellulare* were differentiated from each other by their ability to grow at 45°C (heat tolerance method), assuming that the majority of *M. avium* isolates grow at this temperature, whereas *M. intracellulare* isolates do not (27).

Preparation of DNA from blood cultures for analysis by PCR. The following DNA extraction methods were used to prepare DNA for PCR.

(i) NI. A volume of 1.5 ml of blood culture fluid in an Eppendorf tube was centrifuged at 10,000 × *g* for 5 min to deposit the bacterial cells. The cell pellet was washed twice with distilled water and was resuspended with vigorous mixing in 400 μl of NaI solution which consisted of 6 M NaI, 13 mM EDTA, 26 mM Tris-HCl (pH 8), 0.5% sodium *N*-lauroylsarcosine, and 10 μg of glycogen per ml (15). After incubating the mixture at 60°C for 15 min, 400 μl of isopropanol (100%) was added and the mixture was mixed vigorously and incubated at room temperature for 15 min. The DNA was pelleted by centrifugation at 10,000 × *g* for 5 min and was washed with 1.0 ml of 40% isopropanol. After centrifugation, the DNA was dried with a vacuum desiccator and was redissolved in 100 μl of distilled water. An aliquot of 5 μl was used for PCR in a total reaction volume of 25 μl.

(ii) HL. A volume of 1.5 ml of blood culture fluid in an Eppendorf tube was centrifuged at 10,000 × *g* for 5 min to deposit the bacterial cells. The cell pellet was washed twice with distilled water, resuspended in 100 μl of distilled water, heated at 95°C for 15 min, and centrifuged at 10,000 × *g* for 1 min. An aliquot of 5 μl was used in a total reaction volume of 25 μl for PCR.

(iii) PC. A volume of 1.5 ml of blood culture fluid in an Eppendorf tube was centrifuged at 10,000 × *g* for 5 min to deposit the bacterial cells. The cell pellet was washed twice with distilled water, resuspended in 100 μl of 10 mM Tris-HCl (pH 8), and digested with lysozyme and proteinase K; the DNA was extracted twice with phenol-chloroform and once with chloroform and was then precipitated and washed with ethanol as described by Brisson-Noel et al. (3, 4). The pellet was resuspended in 100 μl of distilled water, and an aliquot of 5 μl was used for PCR in a total reaction volume of 25 μl.

PCR. All oligonucleotides used as either PCR primers or DNA hybridization probes were synthesized commercially by Biotech International (Perth, Australia). A multiplex PCR with primers which detect the 16S rRNA gene of members of the genus *Mycobacterium* and which distinguish between *M. avium* and *M. intracellulare* and the MPB70 gene of *M. tuberculosis* was prepared and used with Tth *plus* DNA polymerase (Biotech International) as described by Wilton and Cousins (31). The multiplex PCR primers (31) were MYCGEN-F (5′-AGA GTT TGA TCC TGG CTC AG-3′), MYCGEN-R (5′-TGC ACA CAG GCC ACA AGG GA-3′), MYCAV-R (5′-ACC AGA AGA CAT GCG TCT TG-3′), MY CINT-F (5′-CCT TTA GGC GCA TGT CTT TA-3′), TB1-F (5′-GAA CAA TCC GGA GTT GAC AA-3′), and TB1-R (5′-AGC ACG CTG TCA ATC ATG TA-3′). The MYCGEN-F and MYCGEN-R primers (31) are the same sequences as those described by Boddinghaus et al. (1) for primer 246 and reverse primer 264, respectively. An amplification product of 1,030 bp (MYCGEN-F and MYCGEN-R) was indicative of the genus *Mycobacterium*, and smaller fragments of 850 bp (MYCINT-F and MYCGEN-R), 372 bp (TB1-F and TB1-R), and 180 bp (MYCAV-R and MYCGEN-F) were positive signals for *M. intracellulare*, *M. tuberculosis*, and *M. avium*, respectively. Each PCR tube consisted of 25 μl of buffer (67 mM Tris-HCl [pH 8.8], 16.6 mM ammonium sulfate, 0.45% Triton X-100, and 200 μg of gelatin per ml), 2.0 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, primers (86 ng each of MYCGEN-F and MYC GEN-R, 80 ng of MYCINT-F, 40 ng of MYCAV-R, and 30 ng each of TB1-F and TB1-R), and 0.5 U of Tth *plus* DNA polymerase (Biotech International) overlaid with 50 μl of mineral oil (Sigma, Sydney, Australia). The cycling conditions with the Perkin-Elmer Thermal Cycler 480 were 1 cycle at 94°C for 10 min, 62°C for 1 min, and 72°C for 1 min; 35 cycles of 94°C for 30 s, 62°C for 1 min, and 72°C for 1 min; and 1 cycle of 94°C for 30 s, 62°C for 1 min, and 72°C for 10 min.

A human androgen receptor gene (HARE)-PCR and a β-globin gene (GLBN)-PCR were performed on DNA extracted from blood culture specimens to assess the presence of PCR inhibitors. An aliquot of human placental DNA (10 or 80 ng per tube) was added to a sample of the DNA extracts as an internal positive control. A 282-bp DNA fragment of the human androgen receptor gene was amplified from the human DNA template by the HARE-PCR with primers E1 (5′-CAA CCC GTC AGT ACC CAG ACT GAC C-3′) and E2 (5′-AGC TTC ACT GTC ACC CCA TCA CCA TC-3′) as described by Lubahn et al. (17). A 536-bp fragment of globin DNA was amplified from the human DNA template by GLBN-PCR with primers RS42 (5′-GCT CAC TCA GTG TGG CAA AG-3′) and KM29 (5′-GGT TGG CCA ATC TAC TCC CAG G-3′) as described by Kulski (16). The HARE-PCR and GLBN-PCR were performed with each primer at 25 ng per reaction and the same PCR reagents and cycling conditions described above for the multiplex PCR for the detection of mycobacterial DNA.

The PCRs were performed with positive and negative controls, by sterile procedures, and by following contamination-free guidelines to prevent false-positive results. Sample and PCR reagents were prepared in separate biosafety hoods and laboratories that were separate from the laboratories used for DNA amplification and analysis.

A 5-μl aliquot of the DNA produced by the multiplex PCR was mixed with loading buffer and was separated on a 3% agarose gel by electrophoresis at 100 V for 45 min in 40 mM Tris-acetate–1 mM EDTA. The DNA was stained with ethidium bromide (0.5 μg/ml), and the resulting fluorescent DNA bands were visualized on a 254-nm UV transilluminator TS-15 (UVP, Inc., San Gabriel, Calif.) and photographed on Polaroid 667 instant film with a Polaroid Land camera.

For Southern blot hybridization, the PCR products were transferred from the agarose gels onto positively charged nylon membranes (Boehringer Mannheim, Sydney, Australia) by capillary blotting. The membranes were hybridized overnight at 10°C below the melting temperature of the oligonucleotide probe in hybridization buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 μg of yeast tRNA per ml, 0.5% sodium dodecyl sulfate, and oligonucleotide probe MAV-180Pr, Pr338, or MYCTBPr (Table 2) that had been 5′-end-labelled with T4 polynucleotide kinase (Bresatec, Adelaide, Australia) and [γ-^{32}P]ATP (10 mCi/ml, 3,000 Ci/mmol; Amersham, Sydney, Australia). After hybridization, the membranes were washed twice with 2.5× SSC at room temperature for 5 min each time and twice with 5× SSC at 10°C below the melting temperature of the oligonucleotide probe for 15 min each time and were then autoradiographed for 1 to 7 days.

The MAV-180Pr hybridization probe corresponding to nucleotide positions 98 to 126 (GenBank accession number M29572) of the *M. avium* 16S rRNA gene (1, 26) is complementary to a region of the 180-bp PCR product of *M. avium* internal to the sequence amplified by the PCR primers MYCGEN-F and MYCAV-R and to an internal region of the 1,030-bp PCR product amplified by the PCR primers MYCGEN-F and MYCAV-R. The hybridization probe Pr338 corresponds to nucleotide positions 326 to 343 (GenBank accession number M29572) of the *M. avium* 16S rRNA gene (1, 26) and is complementary to an internal region of the 850-bp PCR product of *M. intracellulare* amplified by PCR primers MYCINT-F and MYCGEN-R and to an internal region of the 1,030-bp PCR product amplified by PCR primers MYCGEN-F and MYCGEN-R. The MYCTBPr hybridization probe is complementary to an internal region of the MPB70 gene (22) and the 372-bp PCR product of *M. tuberculosis*. The sequences of the oligonucleotide probes MAV-180Pr, Pr338, and MYCTBPr are presented in Table 2.

RESULTS

Assessment of PCR with reference strains and cultured organisms. The multiplex PCR for the detection and identification of members of the genus *Mycobacterium* and differentiation between *M. tuberculosis*, *M. avium*, and *M. intracellulare* was assessed and standardized for sensitivity and specificity by using 143 different cultured reference strains of mycobacteria and typed cultures originally isolated from patients (Table 1). HL, PC, and NI of a standardized suspension of mycobacteria released sufficient DNA from the cells for the analysis, detection, and identification by multiplex PCR. However, HL was used to release the DNA from the 143 isolates for PCR because it was simpler and more convenient to use than PC or NI. Figures 1 and 2 show the electrophoretic separation of the PCR products obtained by multiplex PCR of genomic DNAs of some of the different mycobacterial isolates that were tested and that are listed in Table 1. There was a strong correlation between the PCR results and the mycobacteria identified by routine biochemical procedures (Table 1). All of the 138 mycobacteria that were tested (Table 1) were positive by PCR for the 1,030-bp fragment of members of the genus *Mycobacterium* by using primers MYCGEN-F and MYCGEN-R, whereas all of the 5 nonmycobacterial isolates were negative. From only *M. tuberculosis* (24 samples), *M. bovis*, and *M. bovis* BCG (7 samples) was a 372-bp fragment amplified by the primers TB1-F and TB1-R. Fifty of the 55 *M. avium* isolates produced a 180-bp fragment that was amplified by the primers MYCGEN-F and MYCAV-R, and 30 of the 32 isolates of *M. intracellulare* produced an 850-bp fragment with the primers MYCINT-F and MYCGEN-R. However, 5 of the 55 isolates of *M. avium* were identified as *M. intracellulare* by PCR instead of as *M. avium* by thermophilic differentiation of growth at 45°C. In addition, 2 of the 32 isolates of *M. intracellulare* were identified as *M. avium* by PCR instead of as *M. intracellulare* by thermophilic differentiation of growth at 45°C.

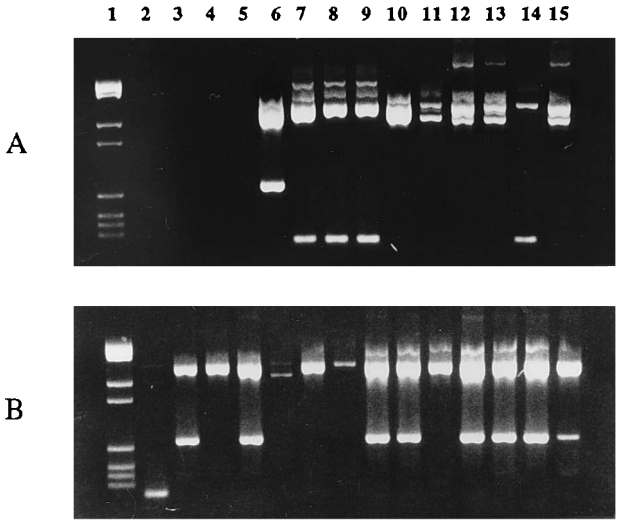

FIG. 1. Electrophoretic separation of PCR products obtained by multiplex PCR of genomic DNAs of the bacterial isolates listed in Table 1. (A) The DNA for each lane is as follows: lane 1, low-molecular-mass markers representing DNA fragments of 1,746, 1,434, 800, 634, 303, 279, 249, and 222 bp (Bio-Rad); lane 2, *A. pyogenes*; lane 3, *N. asteroides*; lane 4, *N. brasiliensis*; lane 5, *R. equi*; lane 6, *M. tuberculosis*; lanes 7 to 9, *M. avium*; lane 10, *M. chelonae*; lanes 11 to 13, *M. intracellulare*; lane 14, *M. avium*; and lane 15, *M. intracellulare*. (B) The DNA for each lane is as follows: lane 1, low-molecular-mass markers (Bio-Rad); lane 2, *M. avium*; lane 3, *M. tuberculosis*; lane 4, *M. intracellulare*; lane 5, *M. tuberculosis*; lanes 6 and 7, *M. intracellulare*; lane 8, *M. gordonae*; lanes 9 and 10, *M. tuberculosis*; lane 11, *M. chelonae*, subsp. *chelonae*; and lanes 12 to 15, *M. tuberculosis*. Genomic DNA was prepared from bacterial isolates by HL, 35 amplification cycles were performed by PCR, and 20% of the reaction volume was analyzed by electrophoresis in 3% agarose gels as described in the text.

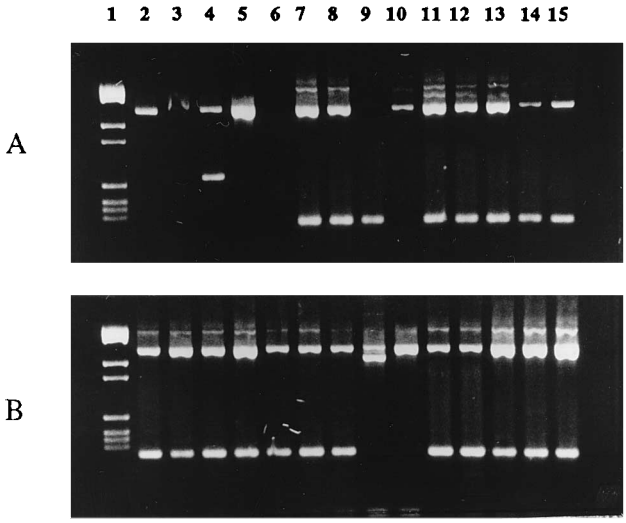

FIG. 2. Electrophoretic separation of PCR products obtained by multiplex PCR of genomic DNAs of *M. avium*, *M. intracellulare*, and the other mycobacterial isolates listed in Table 1. (A) The DNA for each lane is as follows: lane 1, low-molecular-mass markers representing DNA fragments of 1,746, 1,434, 800, 634, 303, 279, 249, and 222 bp (Bio-Rad); lane 2, *M. scrofulaceum*; lane 3, *M. intracellulare*; lane 4, *M. bovis*; lane 5, *M. terrae*; lane 6, *M. intracellulare*; lanes 7 to 9, *M. avium*; lane 10, *M. intracellulare*; and lanes 11 to 15, *M. avium*. The DNAs in lanes 3, 6, and 10 were determined to be that of *M. intracellulare* by PCR in repeated experiments. (B) The DNA for each lane is as follows: lane 1, low-molecular-mass markers (Bio-Rad), lanes 2 to 8, *M. avium* serotypes 2, not typed, 21, 9, 4, not typed, and 8, respectively; lanes 9 and 10, *M. intracellulare*; lanes 11 to 15, *M. avium* serotypes not typed, not typed, 2, not typed, and 4, respectively. The genomic DNA was prepared from mycobacterial isolates by HL, 35 amplification cycles were performed by PCR, and 20% of the reaction volume was analyzed by electrophoresis in 3% agarose gels as described in the text.

The specificities of the PCR amplicons were confirmed by Southern blot DNA hybridization with mycobacterial genus-specific oligonucleotide probes MAV-180Pr, Pr338, and MY-CTBPr for the detection of the PCR products of *M. avium*, *M. intracellulare*, *M. tuberculosis*, and members of the genus *Mycobacterium* by using a sample of the organisms that had tested positive and negative by PCR. The hybridization results between the oligonucleotide probes and PCR products are summarized in Table 2. The present study of 143 different cultured reference strains of mycobacteria and typed cultures confirmed the findings of Wilton and Cousins (31) that the multiplex PCR is specific for identifying members of the genus *Mycobacterium* and differentiating between *M. tuberculosis*, *M. avium*, and *M. intracellulare* and that the limit of sensitivity of the multiplex PCR ranged from 10 to 100 organisms (data not shown).

Assessment of PCR with DNA extracted from blood cultures. The results of the detection and identification of *M. avium* and *M. intracellulare* in blood culture fluid of 16 AIDS patients by routine identification procedures and multiplex PCR are presented in Table 3. Specific PCR products were detected by multiplex PCR in all 41 (100%) AFB-positive cultures after NI, in 18 (44%) after HL, and in 5 (12%) after PC (Fig. 3). The six AFB-negative cultures obtained from 4 of the 16 AIDS patients (Table 3, patients 1, 2, 4, and 12) were PCR negative after extraction of DNA by HL, NI, or PC. Figure 4 shows an example of amplified mycobacterial DNA detected by ethidium bromide staining of agarose electrophoretic gels after multiplex PCR of DNA extracted by HL, NI, or PC from five AFB-positive cultures of blood and from two AFB-negative cultures of blood obtained from 4 of the 16 AIDS patients studied (Table 3). In some of these samples, the specific products of *M. avium* (180 bp) were amplified in the absence of the genus-specific signal (1,030 bp), as seen in Fig. 4 (lanes 4, 10, and 15).

Of the 41 AFB-positive cultures, 38 were identified as *M. avium* and 2 were identified as *M. intracellulare* by routine methods and confirmed by multiplex PCR. However, the mycobacteria in a sample obtained from patient 16 (Table 3) that was identified as *M. intracellulare* by the heat tolerance method was identified as *M. avium* by multiplex PCR (Fig. 5, lane 6).

Six blood culture specimens that were assessed as negative for mycobacteria by ZN staining, gene probing, and PCR were used as negative control specimens in the multiplex PCR assays after HL, PC, and NI (Table 3, patients 1, 2, 4, and 12). The PCR results for the negative control specimens, uninoculated BACTEC 13A fluid and BACTEC 13A fluid that had been inoculated with uninfected normal human blood, were negative after HL, NI, or PC when they were assayed by the multiplex PCR. By contrast, blood-free BACTEC 13A fluids inoculated with reference strains of *M. avium* or *M. intracellulare* that had registered a GI of >20 U were positive after HL, NI, or PC when they were assayed by the multiplex PCR. The addition of 5 ml of human blood to the BACTEC 13A fluids that had been inoculated with reference strains of mycobacteria and that had registered a GI of >20 U reduced the sensitivity of the PCR after HL and PC but not after NI. The addition of internal control DNA (human placental DNA) to samples of DNA extracted from blood cultures and the amplification of a DNA fragment from either the androgen receptor gene of the control DNA by either HARE-PCR or the β-globin gene by GLBN-PCR confirmed the absence of PCR inhibitors in most (>95%) of the extracts obtained by HL and NI and the presence of PCR inhibitors in most of the extracts obtained by PC.

TABLE 3. Detection and identification of *M. avium* and *M. intracellulare* by routine methods and multiplex PCR in 47 blood cultures of 16 patients with HIV and AIDS[a]

Patient no.	Sex[a]	Age (yr)	Identification method		No. of cultures with positive GI (>20 U)	
			Routine[b]	PCR	Mycobacteria-positive cultures[c]	Mycobacteria-negative cultures[c]
1	M	66	Negative	Negative	0	2
2	M	43	*M. avium*	*M. avium*	4	2
3	M	34	*M. avium*	*M. avium*	2	0
4	M	36	*M. avium*	*M. avium*	1	1
5	M	36	*M. intracellulare*	*M. intracellulare*	3	0
6	M	45	*M. avium*	*M. avium*	4	0
7	M	40	*M. avium*	*M. avium*	6	0
8	M	48	*M. avium*	*M. avium*	1	0
9	M	43	*M. intracellulare*	*M. intracellulare*	3	0
10	M	30	*M. avium*	*M. avium*	9	0
11	M	36	*M. avium*	*M. avium*	4	0
12	M	34	Negative	Negative	0	1
13	M	33	*M. avium*	*M. avium*	1	0
14	M	47	*M. avium*	*M. avium*	1	0
15	M	40	*M. avium*	*M. avium*	1	0
16	F	37	*M. intracellulare*	*M. avium*	1	0
Total					41	6

[a] M, male; F, female.
[b] Routine identification methods included ZN staining, DNA hybridization with MAC probe, and differentiation by growth at 45°C.
[c] The result of a mycobacterial-negative or -positive culture was determined both by PCR and routine methods.

DISCUSSION

The rapid detection and identification of mycobacterial pathogens in blood culture are necessary for the effective treatment of disseminated infection in AIDS patients. The mycobacteria identified in AIDS patients at our hospital and elsewhere (18) are predominantly *M. avium* and *M. intracellulare*, but *M. tuberculosis* has also been isolated. We use the BACTEC system for cultivation of mycobacteria from the blood of AIDS patients, and once sufficient growth has occurred the BACTEC fluid is sent to a mycobacteria reference laboratory for identification of the organism by using nucleic acid Gen-Probes and/or conventional mycobacteriology methods. Recently, a multiplex PCR was described for the identification of members of the genus *Mycobacterium*, *M. avium*, *M. intracellulare*, and *M. tuberculosis* in a single-tube assay with mycobacterial reference strains and clinical isolates grown on solid media (31). Our study shows that this multiplex PCR can be applied successfully in conjunction with blood cultures by using the BACTEC system to rapidly identify *M. avium* and *M. intracellulare* directly from the culture fluid in a way similar to that in which DNA probes are used. With the exception of one case, there was good agreement between the results obtained by multiplex PCR and routine methods for the detection and identification of *M. avium* and *M. intracellulare* in the 47 blood culture samples that were analyzed (Table 3). Differentiation between *M. avium* and *M. intracellulare* by multiplex PCR is quicker and more reliable than by growth at 45°C (27) for diagnosis or epidemiological studies of mycobacteria. In addition, if the mycobacteria in the blood cultures are detected only by amplification of the genus-specific 1,030-bp fragment, then the species can be identified either by direct sequencing of the 1,030-bp PCR product (24) or by DNA restriction enzyme analysis (28).

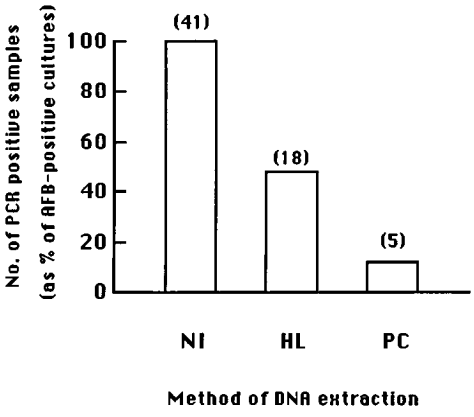

FIG. 3. Effect of different DNA extraction methods on the frequency of detection of mycobacteria by PCR in AFB-positive blood cultures of AIDS patients. DNA was extracted by NI, HL, or PC. The numbers of PCR-positive samples are given in parentheses above the bars. All 41 samples were radiometrically positive for bacterial growth by using the BACTEC system and were confirmed to be AFB positive by ZN staining.

Although the multiplex PCR is easy to use, the method chosen to extract the mycobacterial DNA from a small volume of blood culture fluid is an added complicating step which can affect the outcome of the PCR results. Because the mycobacterial cell wall is a complex structure resistant to simple lysis in strong alkali or acid, several enzymatic steps (lysozyme and proteinase K) and protein denaturants (phenol and chloroform) have been used to achieve cell lysis (1, 3, 4, 11). The preparation of crude mycobacterial lysates by heating in sterile water is a simpler method which has been used successfully for extracting mycobacterial DNA for PCR from pure mycobacterial isolates or infected nonhematological tissues from which mycobacteria had been cultured and isolated in solid or liquid medium (5, 10). Neither of these two DNA extraction methods, however, was efficient in our study with a sample of 1.5 ml of blood culture fluid. This was unexpected since at least 10^4 AFB per ml were detected by ZN staining (33) in positive blood cultures, which is within the detection limit of the multiplex PCR. The poor sensitivity of detection of mycobacteria

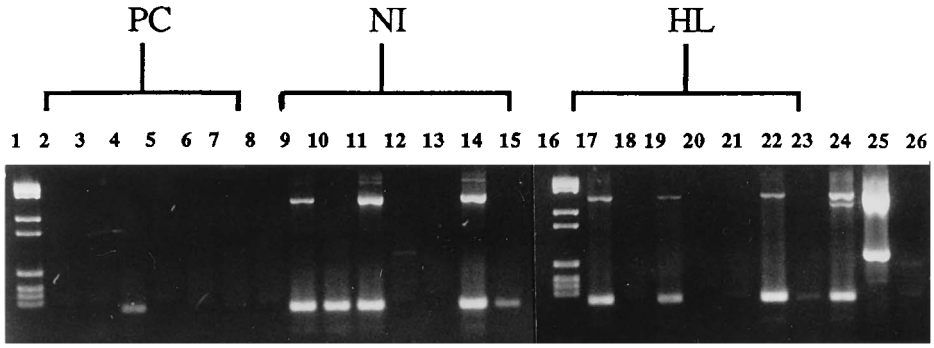

FIG. 4. Electrophoretic separation of PCR products obtained by multiplex PCR of genomic DNA extracted from five AFB-positive and two AFB-negative blood cultures from four AIDS patients. The DNA was extracted from blood culture fluid by PC (lanes 2 to 8), NI (lanes 9 to 15), and HL (lanes 17 to 23). The samples used for PCR were two AFB-negative cultures of blood from patient 1 (lanes 5, 6, 12, 13, 20, and 21), three AFB-positive cultures of blood from patient 2 (lanes 2 to 4, 9 to 11, and 17 to 19), and one AFB-positive cultures of blood from patient 3 (lanes 7, 14, and 22) and patient 4 (lanes 8, 15, and 23). Bio-Rad low-molecular-mass markers are in lanes 1 and 16, *M. avium* DNA (10^3 copies) and *M. intracellulare* DNA (10^4 copies) are in lane 24, *M. tuberculosis* DNA (10^5 copies) is in lane 25, and distilled water is in lane 26. Bio-Rad low-molecular-mass markers represent DNA fragments of 1,746, 1,434, 800, 634, 303, 279, 249, and 222 bp. The extracted DNA was amplified by multiplex PCR for 35 cycles, and 20% of the reaction volume was analyzed by electrophoresis in 3% agarose gel as described in the text.

by PCR after PC or HL may be due to inefficient lysis of cells, loss of DNA during purification, or in some cases, the presence of PCR inhibitors. The loss of significant amounts of DNA during PC is likely to occur by entrapment of DNA between the aqueous and solvent phases and loss during transfer of the aqueous phase after each solvent extraction step, during precipitation of DNA with ethanol, and during the final washing steps. In addition, a few clinical specimens may contain PCR inhibitors which are not fully removed by phenol extraction of DNA (3), and this may be an added problem when using blood culture sediments for extraction (21). However, the poor sensitivity of the detection of mycobacterial DNA and an internal control DNA by PCR in at least 88% of the samples after PC indicates that PCR inhibition was probably due mainly to the presence of residual solvents (phenol, chloroform, or ethanol) remaining in the resuspended DNA solution. By comparison, the inefficiency (44%) of the HL method appears to have been due mainly to the inefficient lysis of cells and only occasionally to the blood components and PCR inhibitors remaining in the crude cell lysate. Since HL is a simple method which is well suited to a routine laboratory, further efforts to improve its efficiency are warranted. In this regard, the HL method recently has been improved to 100% efficiency in comparison with the NI method by including a blood cell lysis and wash step with NaOH (23) to remove PCR inhibitors and by optimizing the time required for heat treatment to release the cellular DNA (15a).

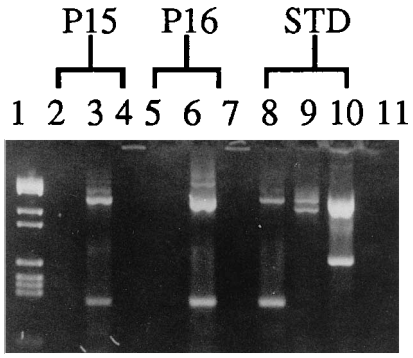

FIG. 5. Electrophoretic separation of amplified *M. avium* DNA extracted from AFB-positive cultures of blood from patient 15 (P15; lanes 2 to 4) and patient 16 (P16; lanes 5 to 7). The DNA was extracted from the blood cultures by PC (lanes 2 and 5), NI (lanes 3 and 6), and HL (lanes 4 and 7). Bio-Rad low-molecular-mass markers are in lane 1. The mycobacterial DNA standards (STD) are *M. avium* DNA (10^3 copies) in lane 8, *M. intracellulare* DNA (10^4 copies) in lane 9, and *M. tuberculosis* DNA (10^5 copies) in lane 10, and distilled water is in lane 11. The extracted DNA was amplified by multiplex PCR for 35 cycles, and 20% of the reaction volume was analyzed by electrophoresis in 3% agarose gel as described in the text.

The NI method was 100% efficient for cell lysis and DNA extraction of mycobacteria from blood cultures and is easier and cheaper to use than the PC method, which includes relatively expensive enzymatic steps. NI was also slightly more efficient than HL or PC for extracting DNA from pure mycobacterial isolates (data not shown). After cell lysis to release the cellular DNA with NaI, DNA can be recovered in high yields (>90%) by precipitation with isopropanol even from small amounts (<1 μg) of starting DNA (15). However, because residual contamination of the PCR solution with NaI or isopropanol can suppress DNA amplification, it is essential to wash all of the NaI from the DNA pellet with 40% isopropanol and redissolve the DNA pellet in water or a buffer free from contaminating isopropanol. Analysis of the effect of NaI-isopropanol solution on the DNA amplification of human cellular DNA by PCR showed that the presence of individual components of the NaI solution at concentrations greater than 1 mM NaI, 0.04% sodium *N*-lauroylsarcosine, 0.4 mM EDTA, or 2% isopropanol in the PCR completely inhibited DNA amplification with the Tth *plus* DNA polymerase purchased from Biotech International (15b). The successful amplification of DNA by PCR in the 41 AFB-positive blood cultures after NI extraction confirmed that sufficient numbers of organisms were present in 1.5 ml of growth fluid and that potential inhibitors of PCR had been washed from the isolated DNA. However, the use of an internal DNA control for the PCR assay, such as human placental DNA that is described in this report or a modified fragment of mycobacterial DNA described by Nolte et al. (20), is needed to assess the presence of potential inhibitors of the PCR. Alternatively, routine ZN staining of a portion of BACTEC fluid in conjunction with PCR is recommended to confirm that sufficient mycobacteria are present in the growth medium for DNA extraction and PCR. In this regard, ZN staining can be used as an indirect check for false-negative results which may occur because of the presence of PCR inhibitors or the loss of DNA during the extraction procedure.

Currently, the cultivation of mycobacteria remains central to the routine diagnosis of *M. avium* complex in the blood of AIDS patients. The multiplex PCR is sufficiently simple, rapid, specific, and sensitive for routine use without the need to perform a hybridization step to identify members of the genus *Mycobacterium*, *M. avium*, *M. intracellulare*, and *M. tuberculosis* in blood culture fluid. The multiplex PCR is estimated to cost at least half that of a commercial DNA probe which is used to identify only one of the three pathogens that can be identified by the mycobacterial multiplex PCR assay. In this regard, the use of the multiplex PCR could provide large savings in time, costs, and laboratory resources compared with the use of the expensive commercial DNA probes, subcloning procedures, and biochemical tests. Recently, Irula et al. (14) reported the detection of *M. avium* in extracts of blood leukocytes by using PCR and radioactive dot blot DNA hybridization. Further studies will help to establish whether the multiplex PCR can also be used to detect and identify mycobacteria directly in blood specimens and blood cultures at an earlier stage of growth (GI, <20 U) to reduce the time to the diagnosis of disseminated mycobacterial infection in immunocompromised patients.

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