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Preparation of Mycobacterial DNA from Blood Culture Fluids by Simple Alkali Wash and Heat Lysis Method for PCR Detection

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A sodium iodide-isopropanol (NI) method was compared with an alkali wash and heat lysis (AH) procedure for the preparation and extraction of DNA from BACTEC 13A blood culture fluid samples from AIDS patients for use in a PCR for the detection and identification of mycobacteria. The sensitivity and efficiency of the DNA extraction methods were assessed by a multiplex PCR which detected the members of the genus *Mycobacterium* and differentiated between *M. intracellulare*, *M. tuberculosis*, and *M. avium* isolates with a limit of detection of between 0.28 pg (67 cells) and 120 pg (28,571 cells) of standard mycobacterial DNA. The PCR amplified mycobacterial DNA prepared by the AH procedure from 40 acid-fast bacillus-positive blood cultures with growth index values of >20 U but not from 48 blood cultures with growth index values of <21 U. The AH method was about 10 times more sensitive than the NI method for extracting DNA from 13 acid-fast bacillus-positive BACTEC fluid samples for PCR analysis. The study shows that the AH procedure in combination with the multiplex PCR is a simple, specific, and sensitive method which can be used in the routine diagnostic laboratory to detect and identify different members of the genus *Mycobacterium* in blood culture fluid samples from AIDS patients.

Mycobacterial infections are a major clinical problem in immunocompromised patients, particularly those with human immunodeficiency virus infection and AIDS (21). The most prevalent mycobacterial infections in AIDS patients are those caused by *Mycobacterium avium-M. intracellulare* complex (MAC), which occur at a frequency of about 50% during the late stage of disease or at autopsy (6, 24), and *M. tuberculosis* (1, 28). MAC infections can cause pulmonary diseases similar to tuberculosis, but the antibacterial treatment of *M. tuberculosis* infections is different from the triple or quadruple drug therapy of infections caused by MAC isolates resistant to antituberculosis drugs (21). The prognosis of pulmonary diseases may be worse when they are associated with *M. avium* than when they are associated with *M. intracellulare* (36). Consequently, a differential diagnosis of MAC infection or infections caused by other mycobacteria is important for patient management, antimicrobial treatment, and epidemiology.

Routine diagnosis of disseminated MAC infections is usually made by detecting bacterial growth radiometrically with a BACTEC blood culture system and identifying mycobacteria in positive blood culture fluids by Ziehl-Neelsen (ZN) staining and microscopy, biochemical methods, or thermophilicity (18, 31). However, these identification methods may have limited value because they lack sensitivity or specificity, are time-consuming, or cannot readily differentiate between *M. avium* and *M. intracellulare* isolates. Radioactive and nonradioactive nucleic acid probes offer a rapid and reproducible alternative method for identifying members of MAC causing an infection or differentiating between *M. avium* and *M. intracellulare* isolates, but generally, commercially available probes are expensive and some may have problems with specificity (7, 8).

Various investigators have described a PCR for the rapid and sensitive detection of mycobacterial DNA in clinical specimens and cultured isolates (5, 18, 25, 26). The routine application of PCR for the identification of mycobacteria in blood culture fluid specimens is still a problem because of the complicated methods used to extract genomic DNA for analysis. Extraction of mycobacterial DNA from clinical specimens and non-blood culture fluid specimens with phenol-chloroform or treatment with guanidinium salts and silica absorption is considered necessary to remove from the samples inhibitors that may interfere with the amplification of DNA (26). These multistep purification methods are time-consuming and may introduce contaminations resulting in the production of a significant number of false-positive results (26). Alkali treatment has been widely used as part of a digestion and decontamination procedure for the isolation of mycobacteria from urine and sputum specimens (16, 27) and has been effectively combined with heat lysis to detect mycobacterial DNA by PCR (9, 25). Heat lysis is a simple method for releasing DNA for PCR from mycobacterial cells that have been grown in solid and liquid cultures (5, 11), but it was found to be less sensitive than a sodium iodide-isopropanol (NI) method for preparing mycobacterial DNA from blood culture fluid specimens (18). The combination of an alkali wash with heat lysis (AH) has not been previously used to prepare mycobacterial DNA from blood culture fluid specimens, probably because of the presence of PCR inhibitors in blood.

In the present study, an NI method (18) was compared with an AH procedure to prepare and extract DNA from blood culture fluid specimens for the detection of mycobacterial DNA by PCR. The sensitivity and efficiency of the DNA extraction methods were assessed by a multiplex PCR method which detects the members of the genus *Mycobacterium* and differentiates between *M. intracellulare*, *M. tuberculosis*, and *M. avium* isolates (18, 34).

MATERIALS AND METHODS

Reference organisms. A collection of 143 organisms including reference strains of mycobacteria and other bacteria obtained from a routine diagnostic

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laboratory were used to assess and standardize the PCR methods as described previously (18).

Blood culture controls. *M. avium* ATCC 582, *M. intracellulare* ATCC 13590, and *M. tuberculosis* H37 RA type strains were grown by standard methods on egg-based medium (31). Colonies from each strain were resuspended in sterile distilled water by vigorous vortexing to obtain a suspension equivalent to that of a McFarland 0.5 opacity standard (Difco), or approximately 10^8 particles per ml. Positive controls were prepared by inoculating BACTEC 13A fluids with bacterial suspensions equivalent to 10^2 and 10^4 particles per ml in either the presence or the absence of 5 ml of normal uninfected human blood. The inoculated culture fluids were incubated at 37°C, and growth was examined with the BACTEC 460 radiometer (Becton Dickinson Diagnostic Instrument Systems) twice weekly for 41 days or until a growth index (GI) of >20 U was registered. Negative controls were BACTEC 13A fluids either with or without 5 ml of uninfected blood or sterile distilled water.

Collection, processing, and storage of blood culture specimens. Eighty-eight blood specimens were collected during 1994 from 29 AIDS patients and 9 immunocompromised patients for culture because of a suspected MAC infection. The culture medium in 13A BACTEC bottles (Middlebrook 7H12 medium; Becton Dickinson Diagnostic Instrument Systems) was inoculated with 5 to 7 ml of blood, and the bottles were incubated at 37°C for 6 to 7 weeks and examined for growth with the BACTEC 460 radiometer (Becton Dickinson Diagnostic Instrument Systems) twice weekly. A BACTEC GI of >20 U was considered positive, and a smear was prepared for ZN staining to detect acid-fast bacilli (AFB) by standard laboratory procedures. Following staining, a 4-ml aliquot was immediately removed from the ZN-positive bottles and was aseptically transferred into 5-ml polypropylene tubes, and the tubes were stored for 1 to 8 months at −70°C until they were required for DNA preparation and PCR analysis. The remaining blood culture fluid was sent to the PathCentre (Nedlands, Western Australia, Australia) for identification of mycobacteria by DNA hybridization with a commercially available probe (Gen-Probe Inc., San Diego, Calif.) and differential characterization of MAC infection by heat tolerance, assuming that the majority of *M. avium* isolates grow at 45°C, whereas *M. intracellulare* isolates do not (18, 30). Blood culture fluids that had GI values of <21 U after incubation for 6 to 7 weeks and that were negative by ZN staining were divided into aliquots (4 ml) and were stored at −70°C until they were required for DNA preparation and PCR analysis.

DNA standards and controls for PCR. Human placental DNA was purchased from Sigma Chemical Company (Sydney, New South Wales, Australia). Mycobacterial DNA standards were prepared from cultures of *M. avium* ATCC 582, *M. intracellulare* ATCC 13590, and *M. tuberculosis* H37 RA by phenol-chloroform extraction (18). Mycobacteria were harvested from Löwenstein-Jensen slants, washed in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and centrifuged (13,000 × *g* for 5 min). The pellet was resuspended in 10 mM Tris-HCl–10 mM EDTA–0.1% Tween 80–2 mg of lysozyme per ml, and the mixture was incubated for 2 h at 37°C with shaking and centrifuged as described before. The bacterial pellet was resuspended in TE containing 100 μg of proteinase K per ml, 100 μg of DNase-free RNase A per ml, and 1% sodium dodecyl sulfate, and the mixture was incubated for 1 h at 37°C and then at 60°C for 20 min. The lysate was extracted with an equal volume of TE-saturated phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol). The aqueous phase was removed and placed into a separate tube, 0.1 volume of cold 3 M sodium acetate (pH 5.2) was added, and the contents were mixed and placed on ice for 10 min before centrifugation in a microcentrifuge for 10 min. The supernatant was removed and placed into a separate tube, and nucleic acids were precipitated following the addition of 0.05 volume of cold 3 M sodium acetate and 2.5 volumes of ethanol. The nucleic acids were deposited by a brief centrifugation, and the pellet was washed twice with 70% ethanol, dried, and resuspended in TE. The DNA concentration and purity were determined by UV spectrophotometry at wavelengths of 260 and 280 nm. The integrity of the DNA was checked by electrophoresis of the DNA in 1% (wt/vol) agarose gels.

Preparation of DNA from blood cultures for analysis by PCR. All methods for the preparation or extraction of mycobacterial DNA from positive and negative blood cultures were performed in a class IIA biological safety cabinet, applying the biosafety guidelines recommended by the Centers for Disease Control and Prevention (Atlanta, Ga.) for laboratory work with *M. tuberculosis* isolates (14). Samples of DNA extracts were stored at −20°C in a sealed container until they were required for PCR, and the addition of sample extracts to PCR reagents was performed in a class IIA biological safety cabinet different from the one used for DNA extraction of specimens.

(i) AH method. For the standard AH method, a volume of 0.1 ml of blood culture fluid was added to 1.4 ml of alkali wash solution (0.5 M NaOH and 0.05 M sodium citrate) in a 1.5-ml Eppendorf tube, mixed by inversion by using a Hema-Tek aliquot mixer (model 4652; Miles Inc., Elkhart, Ind.) for 10 min at room temperature, and centrifuged at 13,000 × *g* for 5 min to deposit the bacterial cells. The cell pellet was resuspended in 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and was centrifuged as described before. This step was repeated at least once. After the final centrifugation, the cell pellet was resuspended in 0.1 ml of distilled water, heated at 95°C for 25 min in a heating block (Thermolyne Dri-Bath; type 17600; Lab Supply Australia Pty., Ltd., Perth, Western Australia, Australia) and stored in a sealed container at −20°C prior to PCR.

Additional experiments were performed to assess the use of NaOH at different concentrations on the efficiency of DNA extraction and PCR analysis. An aliquot of 0.1 ml of AFB-positive BACTEC 13A fluid was treated either with water or with alkali wash solution in concentrations ranging from 0.1 M NaOH and 0.01 M sodium citrate to 5 M NaOH and 0.5 M sodium citrate. The effects of increasing the heat lysis time to 35 min and using a 30-min proteinase K digestion step at 55°C prior to heat lysis were also investigated. Proteinase K was added to each tube to a final concentration of 0.3 μg/ml.

In other experiments, an erythrocyte lysis treatment step was used prior to the lysis step of the AH procedure. Essentially, a volume of 1.4 ml of erythrocyte lysis buffer (0.32 M sucrose, 0.01 M Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100) was added to 0.1 ml of blood culture fluid, and the contents were mixed for 1 min and centrifuged in an Eppendorf tube at 13,000 × *g* for 5 min to deposit the bacterial cells. The cell pellet was washed in 1.5 ml of erythrocyte lysis buffer, centrifuged (13,000 × *g* for 5 min), resuspended in alkali wash solution and treated by the standard AH method as described above.

(ii) NI method. For the NI method, blood culture fluid (0.1 ml) was added to 0.3 ml of NaI solution, the mixture was incubated at 60°C for 15 min, and the DNA was precipitated with 0.4 ml of isopropanol as described previously (18). Blue dextran (200 μg) instead of glycogen was added to the lysis solution to assist in the precipitation of the DNA pellet during two washes with 0.5 ml of 70% ethanol and in making the DNA pellet more visible. The DNA pellet was dried in a vacuum desiccator and was redissolved in 0.1 ml of distilled water. For direct comparison with the AH method, the NI method was used to extract DNA from 0.1 ml of blood culture fluid instead of from the 1.5 ml of blood culture fluid described previously (18).

PCR. DNA extracts that had been stored at −20°C were centrifuged at 13,000 × *g* for 2 min, and 0.01 ml of the supernatant was used in a total volume of 0.05 ml of PCR solution. A multiplex PCR (designated here as the GITA-PCR method) for the detection of members of the genus *Mycobacterium* (1,030-bp product) and differentiation between *M. intracellulare* (850-bp product), *M. tuberculosis* (372-bp product), and *M. avium* (180-bp product) isolates was used as described previously (18). Two additional PCR methods (the MAV- and MAIS-PCR methods) were used to confirm the results obtained by the multiplex GITA-PCR. The MAV-PCR was used with primers MAV22A and MAV22B to amplify a specific 90-bp DNA product of *M. avium* as described previously (10). The MAIS-PCR was used to amplify a 287-bp DNA product from the DNAs of *M. avium* and *M. intracellulare*. The primer and probe sequences for the MAIS-PCR were taken from the sequence of the Avi-3 gene of *M. avium* (35): MAV1 (5′-GACATGCCTCTGACCATCGG-3′) from positions −5 to 21, MAV4 (5′-TTGAAGTGGACGAACTCGCTGTCG-3′) from positions 284 to 260, and the hybridization probe MAV3 (5′-GACTACTTCACCACCATCACCAGC-3′) from positions 97 to 120. The MAIS-PCR contained primers at 50 ng each, 1.5 mM $MgCl_2$, and 1.5 U of *Tth* plus DNA polymerase (Biotech International, Perth, Western Australia, Australia) and was performed in a thermal cycler as described previously (18), with an initial denaturation step at 95°C for 5 min, annealing at 65°C for 0.5 min, and extension at 72°C for 2 min; 40 cycles of denaturation (95°C for 2 min), annealing (65°C for 0.5 min), and extension (72°C for 2 min), and a final cycle in which the extension step at 72°C was increased to 7 min. A human androgen receptor gene (HARE)-PCR and a β-globin gene (GLBN)-PCR were performed with DNA prepared from blood culture fluids to assess the presence of PCR inhibitors or carryover of human cellular DNA as described previously (18). The amplified PCR products (5 μl) were separated by electrophoresis in 3% agarose at 100 V for 45 min and were detected by ethidium bromide staining and Southern blot hybridization (18).

Semiquantitation of mycobacterial DNA by endpoint titration and PCR. Mycobacterial DNA was semiquantitated by determining the endpoint titer of 10-fold dilutions of samples of extracted DNA and mycobacterial DNA standards at which the amplified mycobacterial DNA bands were no longer detectable on the agarose gel (17). The endpoint titer was expressed as the negative $\log_{10}$ of the highest dilution that produced a specific DNA band that could be detected by ethidium bromide staining on an agarose gel. The concentration of mycobacterial DNA in culture fluid was calculated by multiplying the amount of standard mycobacterial DNA by the dilution factor needed to determine the limit of detection of mycobacterial DNA in positive samples. The concentration of mycobacteria was calculated either as the number of organisms ($\log_{10}$) per milliliter of culture fluid or the amount of mycobacterial DNA (in picograms) per milliliter of culture fluid. The number of organisms was estimated on the assumption that one mycobacterial cell is equivalent to 4.2 fg of mycobacterial genomic DNA.

Colony counts. Quantitative cultures were performed with 13 blood culture fluid samples by colony counting on solid culture medium. Frozen samples of culture fluid were thawed and mixed by inversion for 1 h. An aliquot of 0.01 ml was diluted in sterile TE buffer in 10-fold increments to a final dilution of 1:100,000. The diluted samples were briefly vortexed and were spread onto 7H11 Middlebrook solid medium containing lincomycin (2 mg/liter), cycloheximide (400 mg/liter), and nalidixic acid (35 mg/liter) (PathCentre). The inoculated medium was incubated at 37°C in an atmosphere of 5% CO_2 for 4 to 8 weeks essentially as described by Havlir et al. (12). Colony counts per milliliter of blood culture fluid were calculated from the dilution containing 30 to 450 colonies.

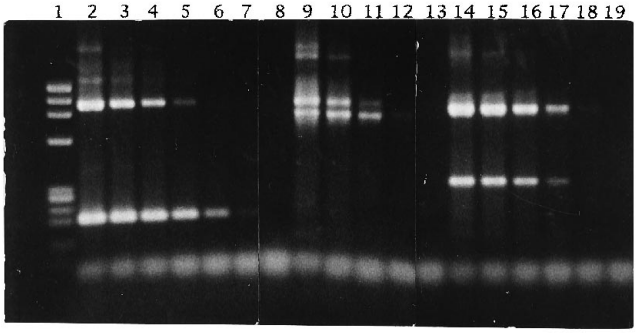

FIG. 1. Endpoint titration of 10-fold serial dilutions of standard mycobacterial DNA amplified by GITA-PCR. The preparation of mycobacterial standard DNA for endpoint titration and the conditions for GITA-PCR are described in Materials and Methods. The mycobacterial PCR products were separated by electrophoresis in 3% agarose and were detected by ethidium bromide staining. Lane 1, low-molecular-mass marker (ϕX174 DNA-*Hae*III; Promega, Sydney, New South Wales, Australia) representing (from top to bottom) DNA fragments of 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. The PCR products obtained by amplification of 10-fold serial dilutions of standard DNA of *M. avium* ATCC 582 are in lanes 2 (2.8 ng of DNA per PCR) to 8 (2.8 fg of DNA per PCR), *M. intracellulare* ATCC 13590 in lanes 9 (1.2 ng of DNA per PCR) to 13 (120 fg of DNA per PCR), and *M. tuberculosis* H37 RA in lanes 14 (5.0 ng of DNA per PCR) to 19 (50 fg of DNA per PCR).

RESULTS

Specificity of PCR. Previous studies have shown that the multiplex GITA-PCR is specific for the detection of members of the genus *Mycobacterium* and *M. intracellulare*, *M. tuberculosis*, and *M. avium* isolates (18, 34). The MAV-PCR was assessed for specificity by using 148 different isolates described previously (18), including cultured strains of *M. avium* and *M. intracellulare*, as positive and negative controls. All 23 *M. intracellulare* isolates (serotype 12, 14, 16, and 17 and nontypeable isolates) were negative, and 46 of 49 (94%) patient isolates of *M. avium* (serotype 1 to 4, 8, 10, 21 and nontypeable isolates) were positive by MAV-PCR. The three strains of *M. avium* (one serotype 8 isolate and two nontypeable isolates) that were negative by MAV-PCR were positive for *M. avium* by the multiplex GITA-PCR. In contrast, MAIS-PCR amplified a 282-bp DNA fragment from *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. ulcerans*, *M. marinum*, *M. asiaticum*, and *M. neoaurum* but not from *M. aurum*, *M. bovis*, *M. chelonae*, *M. chitae*, *M. duvalii*, *M. fortuitum*, *M. gadium*, *M. gilvum*, *M. haemophilum*, *M. kansasii*, *M. parafortuitum*, *M. phlei*, *M. smegmatis*, and *M. vaccae.*

Sensitivity of PCR. The sensitivity of PCR by ethidium bromide staining of the amplification products was estimated with 10-fold dilutions of standard amounts of DNA of *M. avium* ATCC 582, *M. intracellulare* ATCC 13590, and *M. tuberculosis* H37 RA. The results for the stained PCR products in agarose electrophoretic gels after endpoint titration by multiplex GITA-PCR are provided in Fig. 1. The limit of detection by GITA-PCR was approximately 0.28 pg of *M. avium* DNA (equivalent to 67 cells), 12 pg of *M. intracellulare* DNA (2,857 cells), and 5 pg of *M. tuberculosis* DNA (1,190 cells) with the species-specific primers and 5 to 120 pg of mycobacterial DNA (1,190 to 28,571 cells) with the genus-specific primers. Increasing the number of GITA-PCR cycles from 35 to 40 did not increase the sensitivity of detection of standard DNA by another 10-fold. The limit of detection by MAV-PCR was 1.4 pg of *M. avium* DNA (330 cells), and the limit of detection by MAIS-PCR was 1.2 pg of *M. avium* DNA or *M. intracellulare* DNA (290 cells).

GITA-PCR of DNA prepared by the AH method from blood culture controls. The optimal concentration of NaOH and the time required to lyse mycobacterial cells for the AH method were determined with one AFB-positive specimen. The intensities of the fluorescent PCR products increased slightly with increasing concentrations of NaOH from 0.1 to 5 M, whereas the endpoint titers for the GITA-PCR of mycobacterial DNA extracted by the AH method remained the same. An increase in the time of heat lysis from 15 to 35 min or the addition of a proteinase K digestion step prior to heat lysis resulted in no observable increase in sensitivity over that of the standard AH procedure.

The efficiency of the standard AH method for preparing mycobacterial DNA for GITA-PCR was further tested with simulated growth fluids and blood culture fluids that were either inoculated with *M. avium*, *M. intracellulare*, and *M. tuberculosis* or left uninfected as negative controls. A summary of the results is presented in Table 1. The time for inoculated mycobacterial reference strains to register a detectable GI (GI

TABLE 1. GITA-PCR of blood culture controls positive and negative for mycobacteria

Control culture	Organism[a]	Growth fluid BACTEC 13A[b]	Time of growth (days)	GI	GITA-PCR result[c]	Endpoint dilution for positive GITA-PCR result
PCMA-1	*M. avium*	Blood	41	117	+	1 in 100
PCMA-2	*M. avium*	Blood	16	35	+	1 in 100
PCMA-3	*M. avium*	No blood	6	42	+	1 in 100
PCMA-4	*M. avium*	No blood	6	531	+	1 in 100
PCMI-1	*M. intracellulare*	Blood	13	27	+	1 in 100
PCMI-2	*M. intracellulare*	Blood	13	548	+	1 in 10
PCMI-3	*M. intracellulare*	No blood	6	21	+	1 in 10
PCMI-4	*M. intracellulare*	No blood	6	231	+	1 in 10
PCTB-1	*M. tuberculosis*	Blood	35	35	+	Neat
PCTB-2	*M. tuberculosis*	No blood	19	22	+	Neat
NC-1	None	Blood	1	<21	−	
NC-2	None	No blood	1	<21	−	

[a] *M. avium* ATCC 582 was used to inoculate PCMA-1 (10^2 organisms), PCMA-2 (10^4 organisms), PCMA-3 (10^2 organisms), and PCMA-4 (10^4 organisms). *M. intracellulare* ATCC 13950 was used to inoculate PCMI-1 (10^2 organisms), PCMI-2 (10^4 organisms), PCMI-3 (10^2 organisms), and PCMI-4 (10^4 organisms). *M. tuberculosis* (H37 RA) was used to inoculate PCTB-1 and PCTB-2, each with 10^4 organisms.

[b] The volume of uninfected blood added to PCMA-1, PCMA-2, PCMI-1, PCMI-2, and NC-1 was 5 ml. An aliquot of 0.5 ml of growth fluid was used to extract cellular DNA for GITA-PCR by the AH method as described in Materials and Methods.

[c] The GITA-PCR result was positive (+) or negative (−).

TABLE 2. Colony counts and endpoint titers by GITA-PCR of mycobacterial DNA prepared from 13 AFB-positive blood culture specimens

Specimen no.	Patient no.	GITA-PCR endpoint titers ($-\log_{10}$) by the following DNA preparation method[b]		Colony counts ($\log_{10}$)	Species identification[c]
		AH method	NI method		
1	1	2.0	1.0	6.0	*M.avium*
2	2	3.7	2.7	7.0	*M. avium-M. intracellulare*
3	3	2.0	1.0	6.0	*M. avium*
4	4	3.7	2.0	8.0	*M. intracellulare*
5	5	3.7	1.7	8.0	*M. avium*
6	6	2.7	2.0	7.0	*M. avium*
7	6	3.0	2.7	7.0	*M. avium*
8	6	3.0	2.7	7.0	*M. avium*
9	6	3.0	1.7	7.0	*M. avium*
10	7	2.0	2.0	7.0	*M. avium*
11	7	2.7	1.0	6.0	*M. avium*
12	7	3.7	2.0	7.0	*M. avium*
13	7	2.7	1.7	6.0	*M. avium*

[a] Endpoint titers represent the average result of PCR analysis for duplicate samples.
[b] The DNA preparation methods are described in Materials and Methods. For the NI method, NI lysis (without blue dextran) of 0.1 ml of blood culture fluid was used.
[c] Identification was based on PCR results.

of >20 U) in the BACTEC fluids was at least twice as long in the presence of blood than in the absence of blood. The time for the detection of growth ranged between 6 and 41 days and was not dependent on the strength of the inoculum, which was equivalent to 10^2 or 10^4 organisms per ml. The GITA-PCR detected and identified the members of the mycobacteria in the positive controls, with the endpoint titers of the PCR ranging between 1 in 10 and 1 in 100. No PCR products were detected in the negative BACTEC fluids with or without blood (Table 1). The sensitivities of PCR determined by endpoint titration of DNA preparations by the AH procedure with or without prior treatment with erythrocyte lysis buffer were similar, suggesting that the initial washing step with erythrocyte lysis buffer was unnecessary. Therefore, the basic AH method (without an erythrocyte lysis treatment) was chosen for the comparative analysis with the NI method.

Comparison between AH and NI methods for preparation of DNA from 13 AFB-positive blood culture specimens. The number of organisms per milliliter of blood culture fluid was determined by colony counting with 13 AFB-positive blood culture specimens from 7 AIDS patients and was found to range between 10^6 and 10^8 CFU/ml. Eight of the 13 specimens contained approximately 10^7 CFU/ml. The results of the detection and identification of *M. avium* and *M. intracellulare* in the 13 blood culture fluid specimens by GITA-PCR after DNA preparation by the AH or the NI method are presented in Table 2. Specific PCR products were detected in all 13 AFB-positive cultures by using DNA prepared by the AH or the NI method. However, the sensitivity of PCR determined by endpoint titration was approximately 10 times higher for DNA prepared by the AH method than for DNA prepared by the NI method, showing that the AH method is more efficient than the NI method.

Figure 2 shows an example of the PCR products detected by GITA-, MAV-, or MAIS-PCR of mycobacterial DNA extracted by the AH method from some of the AFB-positive (Table 2) and negative blood culture specimens. It is noteworthy that two of the MAV-PCR products were the 200-bp product instead of the expected 90-bp product (Fig. 2C, lanes 3 and 6), suggesting that these two *M. avium* strains from patients 2 and 5 (Table 2) may have an additional 110-bp insert in the 90-bp sequence amplified by MAV22A and MAV22B primers

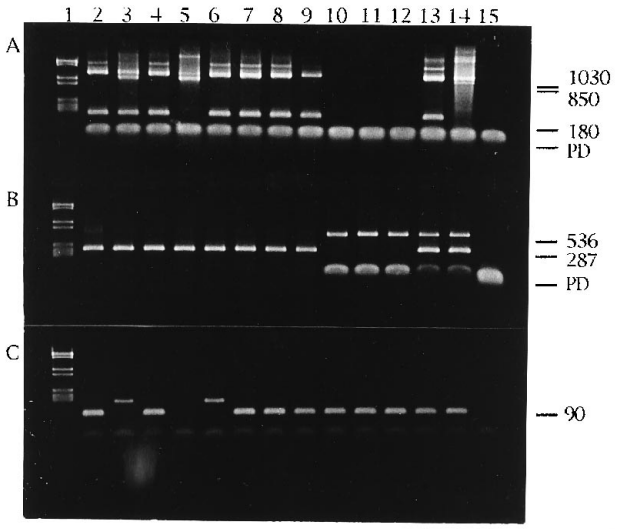

FIG. 2. Electrophoretic separation of DNA products obtained by GITA-PCR (A), MAV-PCR (B), and MAIS-PCR (C) of mycobacterial DNA extracted by the AH method from various AFB-positive and -negative blood culture fluid specimens. The preparation of mycobacterial DNA from blood culture fluids by the standard AH method and PCR protocols are described in Materials and Methods. The mycobacterial PCR products were separated by electrophoresis in 3% agarose and were detected by ethidium bromide staining. Lane 1, low-molecular-mass marker representing DNA fragments of 1,746, 1,434, 800, 634, 303, 279, 249, and 222 bp (Bio-Rad). (A and B) Lanes 2 to 6, AFB-positive blood culture fluid specimens from patients 1 to 5; lanes 7 to 9, three consecutive AFB-positive blood culture fluid specimens from patient 6 (Table 2); lanes 10 to 12, three different AFB-negative blood culture fluid specimens; lane 13, *M. avium* DNA standard; lane 14, *M. intracellulare* DNA standard; lane 15, distilled water as a reagent-negative control. (B) Human placental DNA (10 ng) was added to the AFB-negative blood cultures and mycobacterial DNA standards, and a β-globin DNA fragment (536 bp) was amplified by GLBN-PCR as described in Materials and Methods. (C) Lanes 2 to 6, AFB-positive blood culture fluid specimens from patients 1 to 5; lanes 7 to 10, four consecutive AFB-positive blood culture fluid specimens from patient 6; lanes 11 to 14, four consecutive AFB-positive blood cultures from patient 7 (Table 2); lane 15, distilled water. The molecular sizes of the PCR products are presented on the right, where the 1,030-bp band represents the genus *Mycobacterium*, the 850-bp band represents *M. intracellulare*, and the 180-bp band represents *M. avium* by GITA-PCR (A), the 287-bp band represents mycobacterial DNA by MAIS-PCR, the 536-bp band represents β-globin DNA by GLBN-PCR (B), and the 90-bp band represents *M. avium* DNA by MAV-PCR (C). PD, primer-dimer or nonspecific amplification product.

(10). One of the specimens also contained a mixture of *M. avium* and *M. intracellulare* (Fig. 2A, lane 3), whereas the other specimen appeared to contain only *M. avium* (Fig. 2A, lane 6), as determined by the multiplex GITA-PCR. The results of the GLBN-PCR of human placental DNA (10 ng) that was added as internal positive control to three AFB-negative blood cultures and mycobacterial standards are shown in Fig. 2B (lanes 10 to 14), which shows a 536-bp PCR product.

PCR of DNA prepared by the AH method from 88 blood culture fluid specimens. DNA was prepared by the AH method from 88 specimens of blood culture fluid (including the 13 specimens described in Table 2) to determine the presence of mycobacterial DNA by GITA-, MAV-, and MAIS-PCRs. Of 40 blood cultures that had a GI of >20 U and that were positive for AFB by ZN staining, 28 (70%) were positive for *M. avium* and 12 (30%) were positive for *M. intracellulare* by multiplex GITA-PCR and Southern blot hybridization of the PCR products. All of the GITA-PCR-positive blood cultures were also positive for mycobacterial DNA by MAIS-PCR. The 28 blood cultures that were positive for *M. avium* by multiplex GITA-PCR were also positive by MAV-PCR, whereas the 12 blood cultures that were positive for *M. intracellulare* by GITA-PCR were negative by MAV-PCR. In addition, all of the mycobacterial species identified by PCR were confirmed by DNA hybridization with the commercially available Gen-Probe, and *M. avium* isolates were differentiated from *M. intracellulare* isolates by growth at 45°C. All of the 48 blood cultures that had a GI of <21 U were negative by GITA-, MAV-, and MAIS-PCRs and Southern blot hybridization of the PCR products. The absence of PCR inhibitors in the DNA prepared by the AH method from the 88 blood culture specimens was confirmed by HARE-PCR of human placental DNA (10 ng) that was added as internal positive control.

DISCUSSION

The sensitivity of PCR for the detection and identification of mycobacteria in blood culture fluids can be markedly affected by the method of preparing mycobacterial DNA. The preparation of crude mycobacterial lysates by heat lysis in sterile water is a simple method which has been used successfully to extract mycobacterial DNA for PCR from pure mycobacterial isolates (5, 11, 34) and sputum samples (15). However, heat lysis of mycobacteria in water is often inefficient, produces variable results, and requires additional treatments such as boiling in nonionic detergents, digestion with proteinase K, freezing and thawing, sonication (4, 20, 25, 29), or mechanical disruption with glass beads (9). For example, Liedtke et al. (20) found that processing mycobacterial samples by heating at 70°C for 12 h and sonication for 12 min was 10-fold more sensitive than boiling the samples for 10 min. Sritharan and Barker (29) found that boiling for 30 min in the presence of 1% Triton X-100 lysed 95% of the mycobacteria in a sample and detected fewer than 10 mycobacteria by PCR, whereas boiling in distilled water was ineffective. On the other hand, Buck et al. (4), in comparing several simple methods for preparing *M. tuberculosis* isolates for PCR, concluded that boiling with Triton X-100 was relatively ineffective and recommended sonication. In our study, the preparation of mycobacterial DNA from sediments harvested from 0.1 ml of blood culture fluid with water washes and heat lysis in water for 25 min was only 46% efficient (19), and the result by that method was similar to our previous result with 1.5 ml of blood culture fluid and heat lysis in water for 10 min (18). By contrast, mycobacterial DNA prepared from 0.1 ml of blood culture fluid with alkali washes and heat lysis in water for 25 min was 100% efficient and permitted the detection of mycobacterial DNA by GITA-PCR in samples diluted to 1 in 100,000. No PCR inhibitors were detected by GLBN- or HARE-PCR in the DNA samples prepared by either water or alkali wash and heat lysis methods. The preparation of mycobacteria in NaOH prior to heat lysis in water may increase the susceptibility of the mycobacterial cell wall to heat and, consequently, improve the efficiency of release of DNA from the heat-lysed cells.

The preparation of mycobacterial DNA for PCR from blood culture fluid by NI extraction was significantly more effective than either water wash and heat lysis or phenol-chloroform extraction (18) but was less sensitive than the AH method (Table 2). A disadvantage of the NI method is the inhibitory action of residual NaI from the DNA pellet on PCR (18) and the occasional loss of the DNA pellet during the alcohol washing step to remove the NaI. During the course of the present study, we observed that blue dextran coprecipitates with DNA in the presence of isopropanol and allows the DNA pellet to be more easily visualized during the washing step. The presence of residual blue dextran in the PCR solution had no obvious inhibitory effect on amplification of mycobacterial or human DNA by PCR. The inclusion of blue dextran in the NI solution has helped to reduce the loss of DNA during the wash step and to improve the reproducibility and sensitivity of PCR results, in some cases by 10- to 100-fold (19).

The AH method appears to be better suited for the routine laboratory than the NI method because it is simpler, more sensitive, and more economical than the NI method and the alkali solutions are easier to prepare and are more stable during long-term storage. Alkali also is widely used in routine laboratories to harvest mycobacteria from nonblood culture fluids and sputa for identification by culture or PCR (16, 25). For blood culture fluids, NaOH was used principally to lyse and wash out the human erythrocyte and leukocyte components from the remaining mycobacterial cell pellet prior to heat treatment. The most convenient volumes for recovering bacteria from blood culture fluids for heat lysis were between 0.1 and 0.5 ml in 1.5-ml Eppendorf centrifuge tubes. Therefore, the AH method was optimized for use with 0.5 M NaOH and 0.1 to 0.5 ml of blood culture fluid, but larger volumes and concentrations of NaOH may be required to remove potential inhibitors of PCR if more than 0.5 ml of blood culture fluid is used. The AH method could also be applied for PCR detection of fungi that are grown in blood culture fluid or other organisms that are resistant to lysis by alkali alone (22, 23).

The titers of mycobacterial DNA in 13 blood culture specimens (Table 2) were determined by endpoint dilution by GITA-PCR after preparing DNA by the AH method, and the results correlated with those obtained by colony counting. Dilutions for the 13 blood culture specimens ranged between 2 and 4 log units (equivalent to a concentration range of 10^6 to 10^8 organisms per ml) and correlated with the colony counts within 1 order of magnitude (Table 2). In this regard, the number of mycobacteria in 0.1 ml of blood culture fluid was sufficient for the sensitive detection of mycobacterial DNA by PCR after the DNA was prepared by the AH method. When DNA was prepared from 0.1 ml of blood culture fluid, all 48 AFB-positive blood cultures with GIs of >20 U were positive by PCR, whereas all of the negative blood cultures (0.1 or 0.5 ml of sample) with GIs of <21 U were negative by PCR even after DNA hybridization of the PCR products. Consequently, we do not see the need to increase the sample volume for added sensitivity in exchange for the convenience and economy of processing small volumes (0.1 to 0.5 ml) of blood culture fluid. However, the present method could be scaled up with 1

to 10 ml of blood culture fluid to potentially improve the sensitivity of PCR an additional 10 to 100 times if required.

The relative efficiencies of the DNA preparation methods were assessed with different PCR systems for amplification of mycobacterial and human DNA by using the multiplex GITA-PCR as the "gold standard" to detect members of the genus *Mycobacterium* and identify *M. avium*, *M. intracellulare*, and *M. tuberculosis* isolates in a single-tube assay (18, 34). The MAIS-PCR that amplifies the DNA of members of the *M. avium-M. intracellulare-M. scrofulaceum* complex is described here for the first time. The MAIS-PCR can also be used as a multiplex PCR in combination with other primers including the HARE- and GLBN-PCR primers for the detection of human DNA products (18), the TB1 primers for the detection of *M. tuberculosis* and the MYCOGEN primers for the detection of members of the genus *Mycobacterium* (19). Both the MAV-PCR (10) and the MAIS-PCR, in addition to DNA hybridization with specific probes, helped to confirm the reliability and the specificity of the multiplex GITA-PCR for use in the detection and identification of members of the genus *Mycobacterium* and *M. avium* and *M. intracellulare* isolates, which are the most commonly detected mycobacteria in cultures of blood from patients at our hospital (18). Other PCR assays could be developed with primers that detect mycobacterial species that have been identified in clinical specimens or blood culture fluids from AIDS patients, including *M. genavense* (3) and *M. simiae* (32). Alternatively, amplified mycobacterial 16S rRNA genes or genes which are resistant to chemical therapy, such as rifampin therapy, may be detected by PCR and identified by direct sequencing or DNA restriction enzyme analysis (13, 33). The detection of amplified PCR products is also amenable to various forms of automation, such as capillary electrophoresis and laser gel scanner analysis, for greater accuracy and throughput of samples (2).

The cultivation of mycobacteria from blood by using BACTEC blood culture systems remains central to the routine diagnosis of disseminated mycobacterial infection in AIDS patients. The detection and identification of members of the genus *Mycobacterium* and *M. avium*, *M. intracellulare*, and *M. tuberculosis* isolates harvested from blood culture fluids are relatively simpler and cheaper by a single multiplex PCR assay than with expensive commercial DNA probes or by subcloning procedures or biochemical tests. The relative advantage of the use of a multiplex PCR over the use of a single diagnostic DNA probe is that a number of different mycobacterial species can be identified by a single assay, which conserves costs, time, and effort. In addition, AccuProbe tests for the detection of mycobacteria (Gen-Probe) cannot be performed directly on BACTEC 13A blood cultures because false-positive results are obtained because of the presence of interfering substances in the blood, a problem which has been circumvented by subculturing to BACTEC 12B medium in the absence of blood (7) or by repeated washing of the sediment from the BACTEC broth (8). In this regard, preparation of mycobacterial sediments from blood culture fluids by the alkali wash method described in the present study might also be applicable for the identification of mycobacteria with acridinium ester-labeled DNA probes.

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