

Detection and Identification of Mycobacteria by Amplification of RNA and DNA in Pretreated Blood and Bone Marrow Aspirates by a Simple Lysis Method

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Received 11 March 1997/Returned for modification 11 April 1997/Accepted 16 May 1997

A sodium dodecyl (lauryl) sulfate method was evaluated for the preparation of blood specimens and bone marrow aspirates for use in two amplification procedures (Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test [AMTDT] and Roche Amplicor *M. avium*/*M. intracellulare* [MAI] Test) for the detection and identification of *Mycobacterium tuberculosis* and *M. avium* and *M. intracellulare*, respectively. The AMTDT is based on amplification of rRNA, whereas the Amplicor MAI Test amplifies a specific DNA region of the 16S rRNA gene. The results of amplification techniques were compared with those of standard culture and culture in BACTEC 13A and BACTEC 12B liquid media. A total of 121 blood specimens and 15 bone marrow aspirates were collected from 136 AIDS patients. Mycobacterial growth was recovered for 103 specimens; 35 yielded *M. tuberculosis*, 62 yielded *M. avium*, 5 yielded *M. genavense*, and 1 yielded *M. kansasii*. The values of sensitivity and specificity in pretreated specimens for detection of *M. tuberculosis* by the AMTDT were 94.3 and 100%, respectively, and those for detection of *M. avium* by the Amplicor MAI Test were 91.9 and 100%, respectively. The simple lysis method described in the present work allows the recovery of mycobacteria from blood specimens and bone marrow aspirates and may be used in combination with the AMTDT and the Amplicor MAI Test to detect and identify different members of the genus *Mycobacterium*. This method might also be applicable for the identification of mycobacteria from blood culture fluids with acridinium-ester-labeled DNA probes.

Disseminated mycobacterial infections appear in patients with advanced human immunodeficiency virus (HIV) disease and are associated with increased mortality and morbidity (22).

Rapid identification of disseminated mycobacterial infections in AIDS patients is important for the early initiation of triple or quadruple drug therapy to improve patient outcome (23, 30). Blood cultures are an essential adjunct in the diagnosis of these infections caused principally by *Mycobacterium avium* complex (MAC) and *M. tuberculosis* complex (MTBC). Among the laboratory methods described for the recovery of mycobacteria from blood specimens is the initial processing of these specimens by lysis-centrifugation (in Isolator tubes; Wampole Laboratories, Cranbury, N.J.) with inoculation of concentrates into BACTEC 12B broth medium (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) for propagation and radiometric detection with a BACTEC 460 instrument, and inoculation onto solid media (1, 9, 16, 19, 36). Another technique utilized is the direct inoculation of blood specimens into BACTEC 13A radiometric bottles (Becton Dickinson Diagnostic Instrument Systems) (13, 26, 27). Once a positive BACTEC radiometric culture is obtained, a smear for Ziehl-Neelsen (ZN) staining is prepared and subcultures are inoculated onto solid media to identify the organisms. The identification of MTBC and MAC in blood culture-positive bottles by nucleic acid probes is expensive, and some problems

of sensitivity and specificity have been described previously (11, 12). In addition, because of the slow growth of mycobacteria and the variable numbers of organisms present in blood, a delay of a few days to 6 weeks may occur before growth is detected, and the delay may be longer before the organism is fully identified.

DNA amplification by PCR has the potential to reduce the time taken for the direct identification of MTBC and MAC by allowing the use of diverse genetic elements as target sequences (4, 8, 14, 18, 32, 40). However, there are still problems, including the presence of inhibitors of the PCR which may cause false-negative results (6). The isolation of small amounts of DNA from samples taken from different sites in the body needs improvement. Many samples, e.g., blood specimens, bone marrow aspirates, and pleural fluids, contain inhibitors, only some of which, such as hemoglobin and heparin, have been identified. These factors may persist even after DNA purification. The sodium dodecyl (lauryl) sulfate (SDS)-NaOH treatment has been widely used in the digestion and decontamination of respiratory specimens (33–35) and has been recently utilized on nonrespiratory specimens with great success (15, 34). SDS is a detergent which denatures proteins and enzymes and obviously eliminates most of the inhibitory compounds present in clinical specimens.

Two newly developed, ready-to-use, direct-detection kits using amplification of genetic material are already available. The Amplified Mycobacterium Tuberculosis Direct Test (AMTDT; Gen-Probe Inc., San Diego, Calif.) uses the transcription-mediated amplification (TMA) method to amplify target rRNA via DNA intermediates, whereas the Amplicor *M. avium*/*M. intracellulare* Test (Amplicor MAI Test; Roche Diagnostic Sys-

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tems, Branchburg, N.J.) amplifies a specific DNA region, i.e., a 582-bp segment of the 16S rRNA gene (3).

The purpose of the present study was to evaluate the utility of a simple lysis method (10% SDS [pH 8.5]) in the recovery from blood specimens and bone marrow aspirates and direct detection of *M. tuberculosis* by the AMTDT and *M. avium* and *M. intracellulare* by the Amplicor MAI Test. The sensitivity and efficiency of this method were compared with those of conventional culture techniques for the isolation of mycobacteria from blood specimens and bone marrow aspirates.

MATERIALS AND METHODS

Patients and specimens. From April through December 1996, we investigated 121 blood specimens and 15 bone marrow aspirates from 136 AIDS patients who were suspected of having disseminated mycobacterial infections and were not receiving antituberculous therapy. Of the patients, 80% were men and 20% were women, with a median age of 31 years (range, 20 to 55 years). Of the HIV infected patients, 62% had <50 CD4 cells per μ l, 20% had 50 to 100 CD4 cells per μ l, and 18% had 100 to 200 CD4 per μ l.

Processing of clinical specimens. Five-milliliter samples of peripheral blood collected from 121 patients were inoculated into BACTEC 13A bottles and incubated at 37°C for 8 weeks and examined for growth with the BACTEC 460 radiometer twice weekly for the first 2 weeks and once weekly thereafter. A BACTEC growth index of >100 U was considered positive, and smears for ZN staining and cultures were prepared to detect acid-fast bacilli by using a small portion of the broth from the BACTEC bottles.

Further, from the 121 patients, 5-ml samples of peripheral blood were collected in EDTA-anticoagulated tubes. The 15 bone marrow aspirates were received in Isolator tubes (Wampole Laboratories). The processing of blood samples and bone marrow aspirates was performed in a class IIA biological safety cabinet, according to the biosafety guidelines recommended by the Centers for Disease Control and Prevention (Atlanta, Ga.) for laboratory work with *M. tuberculosis* isolates (24). Five-milliliter samples of blood and 1-ml samples of bone marrow aspirate were treated with 500 and 100 μ l, respectively, of 10% SDS. After being vortexed for 10 min at room temperature, the samples were washed with 30 ml of distilled water and centrifuged (3,300 \times g, 20 min) and the supernatant was removed. If the sediment still had hemorrhagic contents, the process was repeated. The cell pellets from all pretreated specimens were finally resuspended in 2.2-ml volumes of 0.067 M phosphate buffer (pH 6.8). Half of the sediment was stored at -80°C for the amplification techniques, and the other half was used for acid-fast staining and culture.

The smears were screened by staining with auramine-rhodamine fluorochrome. Positive slides were confirmed by the ZN technique (20) and were graded on a scale from 1 to 4+ (24).

For culture, equal aliquots (approximately 250 μ l) of the pretreated specimens were inoculated onto two solid slants, Löwenstein-Jensen and Coletsos. Coletsos medium is an egg-based medium containing pyruvate, 4% osein (gelatin), Berthelot's oligoelement solution, asparagine, glutamate, glycerin, and malachite green; this medium shows a short detection time for *M. bovis* and dysgonic strains of *M. tuberculosis* (7). The oligoelement solution contains salts of manganese, calcium, nickel, zinc, copper, and others (38). Slants were incubated at 37°C for 8 weeks in a humidified atmosphere. In addition, 500 μ l of the sediment was inoculated into BACTEC 12B medium (pH 6.8). Solid media were read weekly, and BACTEC cultures were read twice weekly for the first 2 weeks and once weekly thereafter. Moreover, 500 μ l of pretreated bone marrow aspirates was cultivated in BACTEC Pyrazinamide (PZA) Test Medium at pH 6.0 (Becton Dickinson Diagnostic Instrument Systems) (21). The blood specimens with negative results by BACTEC 12B and solid culture but with positive results by BACTEC 13A culture were subcultured by placing a small portion of the latter culture in BACTEC PZA Test Medium.

Quantitative cultures with 200 μ l of all pretreated specimens were performed by colony counting on solid culture medium. The pretreated specimens were spread onto 7H11 Middlebrook solid medium. The inoculated medium was incubated at 37°C in an atmosphere of 5% CO₂ for 4 to 8 weeks. Colony counts per milliliter of pretreated blood specimens were calculated. Routine biochemical methods (20), gas-liquid chromatography (28), and the Accuprobe culture confirmation tests (Gen-Probe Inc.) (17) were employed for the identification of isolates.

Amplification procedures. Amplification assays were run in three separate areas which had been set up in two rooms.

Gen-Probe AMTDT. The AMTDT is based on the isothermal amplification for detection of *M. tuberculosis*. This system uses the TMA method to amplify rRNA via DNA intermediates, followed by chemiluminiscent detection of amplicons with an acridinium-ester-labeled DNA probe. The AMTDT was performed according to the instructions supplied by the manufacturer. The AMTDT protocol consisted of the following steps. For lysis, 50 μ l of pretreated specimen was added to 200 μ l of specimen dilution buffer in a lysing tube, and the mixture was sonicated for 15 min in a model 1200 water bath sonicator (Branson Ultrasonics Corporation, Danbury, Conn.) at room temperature. For amplification, 25 μ l of

reconstituted amplification reagent was placed in a reaction tube and covered with 200 μ l of mineral oil. Fifty microliters of lysate was transferred to the amplification tube, incubated at 95°C for 15 min, and then cooled at 42°C for 5 min. An enzyme reagent mix (25 μ l) was added, and the mixture was incubated at 42°C for 2 h. To terminate amplification, 20 μ l of the termination reagent was added to each tube, and the mixtures were kept at 42°C for another 10 min. For detection, the reconstituted acridinium-labeled probe (100 μ l) was added to each tube, and the tubes were incubated at 60°C for 15 min; then, the selection reagent (300 μ l) was added and the mixtures were reincubated at 60°C for 10 min. All temperature-controlled incubation steps were carried out in heating blocks. All runs included AMTDT amplification-positive and -negative controls and hybridization-positive and -negative controls. Prior to being read in a luminometer (Leader 50; Gen-Probe Inc.), the tubes were cooled at room temperature for 5 to 10 min. The cutoff value was set at 30,000 relative light units (RLUs). Samples with values of >30,000 RLUs were considered positive; samples with values of <30,000 RLUs were considered negative. A ratio of sample/cutoff RLUs of \geq 1.0 was considered a positive result, as recommended by the manufacturer.

Roche Amplicor MAI Test. The Amplicor MAI Test utilizes PCR nucleic acid amplification and DNA hybridization for the detection of *M. avium* and *M. intracellulare* organisms in blood. The amplification target for both *M. avium* and *M. intracellulare* is a portion of the mycobacterial 16S rRNA gene. The Amplicor MAI Test was done according to the manufacturer's instructions. It consists of three steps: specimen preparation, amplification, and detection. In brief, a 200- μ l aliquot of the pretreated specimen was mixed with wash solution and centrifuged (13,000 \times g) for 10 min. After centrifugation, the supernatant was removed and lysis reagent was added to the pellet. After being vortexed, the suspension was incubated at 60°C for 45 min to complete lysis of the mycobacteria. The lysed material was then neutralized by the addition of neutralization reagent. For amplification, 50 μ l of the neutralized specimen was added to 50 μ l of the master mix reagent. The latter was prepared by the addition of 100 μ l of uracyl *N*-glycosylase enzyme (Amperase; Roche Molecular Systems, Inc.) to an amplification mixture containing nucleotides, biotinylated primers, and thermostable *Taq* polymerase just prior to the amplification process. The primers used (KY75 and KY18) amplify a 582-bp sequence located in a highly conserved region of the 16S rRNA gene of *Mycobacterium* spp. The PCR procedure was carried out by using a 37-cycle program in a Thermocycler TC 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). Amplification of *M. avium* DNA produces a 582-bp amplicon that is detected by an *M. avium*-specific detection probe (KY167) coated on the *M. avium* microwell plate (MWP). Amplification of *M. intracellulare* DNA also produces a 582-bp amplicon that is detected by an *M. intracellulare*-specific detection probe (KY169) coated on the *M. intracellulare* MWP. Hybrid detection was then accomplished with an avidin-horseradish peroxidase conjugate-tetramethylbenzidine substrate system. The reaction was stopped by the addition of hydrosulfuric acid, and the absorbances were read at a wavelength of 450 nm. Specimens giving an absorbance value (AV) of >0.350 were considered positive. Positive and negative amplification controls were included in each run.

The evaluation of inhibition was realized in all pretreated specimens by utilizing the Mycobacterium Internal Control (Myc IC; Roche Diagnostic Systems, Inc.), which has been added to the Amplicor MAI Test. The Myco IC is a DNA plasmid with primer binding regions identical to those of mycobacterium target sequences, a randomized internal sequence of similar length and base composition as the target sequences, and a unique probe binding region that differentiates the Myco IC from the target amplification reaction. The Myco IC is introduced into each amplification reaction and is coamplified with target DNA from the clinical specimens and is detected by a specific detection probe coated on the internal control MWP.

Patient clinical evaluation. In the cases where results from the blood cultures and amplification techniques were discrepant, clinical data and other microbiological results of the patient were analyzed. Clinical assessment included the patient's history, symptoms, chest X-ray, laboratory results, cytological and histological results of specimens, result of the tuberculin skin test, and history of drugs administered.

Retested samples with discrepant results. The sensitivity and specificity of the AMTDT and the Amplicor MAI Test were calculated in comparison with culture results and, separately, in comparison with culture results plus the patient's clinical data. For all specimens with discrepant results, new aliquots of the same pretreated specimens were retested.

RESULTS

Analytical performance of AMTDT and Amplicor MAI Test. Positive and negative results were clearly distinguished by the magnitude of both RLUs and AVs. The majority of samples with positive results had >500,000 RLUs by the AMTDT and >1.2 AVs by the Amplicor MAI Test. Samples with negative results had values below the cutoffs of 30,000 RLUs for the AMTDT and 0.350 AV for the Amplicor MAI Test.

Clinical results. Mycobacterial growth was recovered for 103 specimens. Thirty-five specimens yielded *M. tuberculosis*,

TABLE 1. Comparison of results obtained for blood specimens and bone marrow aspirates by various methods^a

| Type of specimen (n) | No. of specimens | Culture result in: | | | | AMTDT result | Amplicor MAI test result | |
|----------------------------|---------------------|----------------------|----------------------|--------------|------------------------|-----------------|-----------------------------|--------------------------|
| | | BACTEC 13A medium | BACTEC 12B medium | Solid medium | | | <i>M. avium</i> | <i>M. intracellulare</i> |
| | | | | CFU/ml | Species identified | | | |
| Blood (121) | 28 | Pos | Pos | ≤10->100 | <i>M. tuberculosis</i> | Pos | Neg | Neg |
| | 2 | Pos | Pos | ≤10 | <i>M. tuberculosis</i> | Neg | Neg | Neg |
| | 56 | Pos | Pos | ≤10->100 | <i>M. avium</i> | Neg | Pos | Neg |
| | 5 | Pos | Pos | ≤10-60 | <i>M. avium</i> | Neg | Neg | Neg |
| | 4 | Pos | Pos ^b | | <i>M. genavense</i> | Neg | Neg | Neg |
| | 1 | Pos | Pos | 80 | <i>M. kansasii</i> | Neg | Neg | Neg |
| | 25 | Neg | Neg | | | Neg | Neg | Neg |
| | | | | | | | | |
| Bone marrow aspirates (15) | 5 | | Pos | ≤10->100 | <i>M. tuberculosis</i> | Pos | Neg | Neg |
| | 1 | | Pos | >100 | <i>M. avium</i> | Neg | Pos | Neg |
| | 1 | | Pos ^b | | <i>M. genavense</i> | Neg | Neg | Neg |
| | 8 | | Neg | | | Neg | Neg | Neg |

^a Neg, negative; Pos, positive.^b Growth in BACTEC PZA Test medium.

62 yielded *M. avium*, 5 yielded *M. genavense*, and 1 yielded *M. kansasii*. All 103 specimens were recovered in BACTEC 12B and solid media after pretreatment lysis of blood specimens and bone marrow aspirates (Table 1). The results of acid-fast staining were negative for all pretreated samples.

Blood specimens. Of 121 blood specimens examined, 28 were AMTDT positive, Amplicor MAI negative, and culture positive for *M. tuberculosis*. Two specimens were AMTDT negative, Amplicor MAI negative, and culture positive for *M. tuberculosis* (considered AMTDT false-negative results). Fifty-six specimens were AMTDT negative, Amplicor *M. avium* positive and *M. intracellulare* negative, and culture positive for *M. avium*. Five specimens were AMTDT negative, Amplicor MAI negative, and culture positive for *M. avium* (considered Amplicor *M. avium* false-negative results). Four specimens were AMTDT and Amplicor MAI negative and culture positive for *M. genavense*. One specimen was AMTDT and Amplicor MAI negative and culture positive for *M. kansasii*. Twenty-five blood specimens were AMTDT and Amplicor MAI negative and culture negative (Table 1).

Bone marrow aspirates. Of the 15 bone marrow aspirates examined, 5 were AMTDT positive, Amplicor MAI negative, and culture positive for *M. tuberculosis*. One specimen was AMTDT negative, Amplicor *M. avium* positive and *M. intracellulare* negative, and culture positive for *M. avium*. One specimen was AMTDT and Amplicor MAI negative and culture positive for *M. genavense*. Eight specimens were AMTDT and Amplicor MAI negative and culture negative (Table 1).

Retested samples with discrepant results. The seven specimens (two AMTDT and five Amplicor MAI false-negative results) with discrepant results were retested with new aliquots of the same pretreated specimens. These seven specimens were confirmed as false negatives after repetition of the amplification assays.

Inhibition. We also examined the 136 pretreated specimens for the presence of inhibitors, utilizing the Myco IC which has been added to the Amplicor MAI Test. None of the 136 specimens presented inhibitors.

On the basis of these data, the values of sensitivity and specificity in pretreated specimens for detection of *M. tuberculosis* by the AMTDT were 94.3 and 100%, respectively, and those for detection of *M. avium* by the Amplicor MAI Test were 91.9 and 100%, respectively.

DISCUSSION

Mycobacterial infections are a major clinical problem in immunocompromised patients, particularly those with HIV infection and AIDS (2). The most prevalent disseminated mycobacterial infections in AIDS patients are those caused by MAC, which occur at a high frequency during the late stage of disease or are detected at autopsy (10, 31), and *M. tuberculosis*. A rapid differential diagnosis of MAC infections or infections caused by *M. tuberculosis* or other mycobacteria is important for patient management, antimicrobial treatment, and epidemiology (2, 37).

The routine diagnosis of disseminated mycobacterial infection is usually made by detecting bacterial growth in blood specimens and identifying mycobacteria by ZN staining and microscopy, by biochemical methods, and with commercial DNA probes. These identification methods are time-consuming, and DNA probes cannot be performed directly on BACTEC 13A blood cultures because false-positive results are obtained due of the presence of interfering substances in the blood, a problem which has been circumvented by subculturing in BACTEC 12B medium in the absence of blood (11) or by repeated washing of the sediment from the BACTEC broth (12).

Several investigators have described PCR procedures for the rapid and sensitive detection of mycobacterial DNA in blood specimens (13, 26, 27). The sensitivity of amplification assays for the identification of mycobacteria in blood specimens can be markedly affected by the method of specimen preparation for recovery of mycobacteria (13, 26, 27). 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 (26). Another problem associated with the use of nucleic acid amplification techniques with blood specimens is the frequent failure of amplification due to the presence of inhibitors, which may interfere with the activity of the reaction.

Treatment with SDS has been widely used as part of a digestion and decontamination procedure for the isolation of mycobacteria from sputum specimens (33, 35). Several authors have reported success in pretreating different types of clinical specimens with SDS, a detergent which denatures proteins and enzymes, and obviously eliminates most of the inhibitory compounds present in body fluids (15, 34).

In the present study, the AMTDT and Amplicor MAI techniques were employed for the direct amplification and detection of RNA of MTBC and DNA of MAC, respectively, from blood specimens and bone marrow aspirates pretreated by a simple and rapid SDS lysis method. The proposed pretreatment protocol with 10% SDS uses extensive washing to remove any traces of the detergent that might interact with the enzymes and interfere with the amplification techniques (15, 29).

The values of sensitivity and specificity in pretreated specimens for detection of *M. tuberculosis* by the AMTDT were 94.3 and 100%, respectively, and those for detection of *M. avium* by the Amplicor MAI Test were 91.9 and 100%, respectively.

We obtained only a total of seven false-negative results, two with the AMTDT and five with the Amplicor MAI Test (for *M. avium*). These specimens exhibited positive results by radiometric culture between 25 and 50 days. As none of the 136 specimens tested showed evidence of inhibitory compounds of amplification, we believe that the results clearly illustrate the sampling problem commonly encountered with small loads of mycobacteria as a result of their tendency to clump together.

Among the laboratory methods described for the recovery of mycobacteria from blood specimens, the use of lysis-centrifugation and inoculation of the sediment into solid or liquid medium have been recommended (1, 9, 16, 19). Previous reports have investigated the efficacy of blood culture for mycobacteria following lysis-centrifugation (1, 9, 16). Recently, it has been reported that blood cultures collected by the Isolator system with lysing anticoagulant solution have an inhibitory effect on the growth of *M. avium* in BACTEC 12B medium compared with growth in Löwenstein-Jensen medium (9, 39). The proposed pretreatment protocol with 10% SDS has been adequate for the recovery of mycobacteria from blood and bone marrow aspirates. All 103 mycobacterial isolates were recovered in BACTEC 12B and solid media after pretreatment of the samples. Therefore, we believe that the lysis method described in this work allows the recovery of mycobacteria under optimal conditions in liquid and solid media without any inhibitory effect on growth.

The present study shows clearly that the proposed SDS procedure in combination with the AMTDT and the Amplicor MAI 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 samples and bone marrow aspirates from AIDS patients. Additionally, preparation of mycobacterial sediments from blood culture fluids by the 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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