Journal of Clinical Laboratory Analysis 21:220–226 (2007)

Novel Method for Clearing Red Blood Cell Debris From BacT/ALERT® Blood Culture Medium for Improved Microscopic and Antimycobacterial Drug Susceptibility Test Results

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Even though automation in mycobacterial culture has immensely improved the detection of organisms, identification of species and antimycobacterial susceptibility testing from blood culture bottles remain cumbersome and error-prone due to the presence of intact red blood cells (RBCs). The removal or lysis of these RBCs and excessive protein from the blood components could theoretically help improve this process. The present study reports an effective method that uses ammonium chloride (NH_4Cl) and Triton X-100 to lyse the RBCs in blood culture medium. The method was optimized by preparing various concentrations of NH_4Cl and Triton X-100, and incubation conditions, leading to eight protocols. The lysis protocol with a concentration of 150 mM of NH_4Cl, 0.5% Triton X-100, and 1% potassium bicarbonate, pH 7.0, and incubation at 37°C for 15 min was found to be optimal. This method not only made the culture medium clear, the protein concentration decreased from 753.5 ± 39.4 to 53.2 ± 4.2 mg/mL in the *M. tuberculosis*-spiked culture medium and in the blood culture medium inoculated with the blood from tuberculosis patients. The method had no adverse effect on mycobacteria, and no depletion of *M. tuberculosis* colony-forming units was found. The lysate could be used for antimycobacterial susceptibility testing with no difficulty in setting the mycobacterial concentration of inoculum to 0.5 McFarland standards. Furthermore, this method had the added advantage in the microscopy and molecular methods for the speciation of *Mycobacterium* sp. J. Clin. Lab. Anal. 21:220–226, 2007.

Key words: MB/BacT blood culture; mycobacteremia; lysis of RBC; antimycobacterial drug susceptibility testing

INTRODUCTION

When choosing a better tuberculosis diagnostic modality, the sensitivity, specificity, and rapidity of the method are given top priority. The conventional methods for diagnosing mycobacteriosis, such as microscopy and culture on egg-based media, are either suboptimally sensitive or take an undesirably long time (1–3). The dual epidemic of HIV and TB has further limited the utility of conventional methods due to scanty excretion of mycobacteria in the body fluids and changing patterns of drug susceptibility (4–6). In recent times the importance of using blood for mycobacterial culture has increased because of the fact that the sputum positivity rate in suspected cases of HIV-TB coinfection is dismally poor (5). While the detection rate of mycobacteremia in HIV-positive patients by conventional methods was 15% in Brazil (7) and 10% in Thailand (8), the employment of an automated culture system resulted in a 30% detection rate in Brazil (9), 32% in South Africa (10), and 41% in Tanzania (11). To overcome the limitations of this poor detection rate and cumbersomeness, several automated culture detection

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Grant sponsor: bio-Mérieux (India) Pvt. Ltd; Grant sponsor: Indian Council of Medical Research, India.

Received 29 January 2007; Accepted 22 March 2007
DOI 10.1002/jcla.20169
Published online in Wiley InterScience (www.interscience.wiley.com).

systems have been developed that are based on principles of indicating growth of mycobacteria in the liquid medium radiometrically (BACTEC® 460; Becton Dickinson, Sparks, MD), fluorimetrically (BACTEC® MGIT 960, cat. no. L-000180JAA (G), 2005; Becton Dickinson, Sparks, MD) or colorimetrically (MB/BacT, bio-Mérieux, France). However, these systems were optimized only for clinical samples, such as sputum, cerebrospinal fluid, and pus (1,2,12). None of the present protocol is not recommended for antimycobacterial drug susceptibility testing due to the problem of red blood cells (RBCs) and excessive protein concentration in the culture medium. The process of setting up the inoculum to 0.5 McFarland turbidity standards is difficult and error-prone. Furthermore, the biochemical and gene amplification techniques (such as PCR and AccuProbe®) used for identification and speciation are inevitably delayed for 2–3 weeks until the subcultures are obtained on suitable solid media. Directly isolating DNA from the positive blood culture bottles also requires cumbersome and additional steps to lyse the RBCs and remove the excessive protein.

Therefore, this study was carried out to standardize and utilize a novel method for RBC lysis and removal of excessive protein concentration from the BacT/ALERT® MB blood culture medium.

MATERIALS AND METHODS

To lyse the RBCs in culture-positive bottles, the following steps were used:

Preparation of Lysis Buffer

Four types of lysis buffer were prepared with different concentrations of ammonium chloride (Qualigens, India) and Triton X-100 (laboratory grade; Sigma, St. Louis, MO) while keeping the concentration of potassium bicarbonate (Qualigens) constant at 1 mM. The pH of all buffers was maintained at pH 7.0. For sterility, all buffers were filtered through a 0.45-μm membrane filter (Millipore, MA). The optimum buffer concentration and lysis treatment time were standardized at 37°C by using eight different protocols as follows:

- Protocol A: 150 mM of NH_4Cl, 0.5% Triton X-100, for 15 min.
- Protocol B: 150 mM of NH_4Cl, 0.5% Triton X-100, for 30 min.
- Protocol C: 150 mM of NH_4Cl, 1.0% Triton X-100, for 15 min.
- Protocol D: 150 mM of NH_4Cl, 1.0% Triton X-100, for 30 min.
- Protocol E: 300 mM of NH_4Cl, 0.5% Triton X-100, for 15 min.
- Protocol F: 300 mM of NH_4Cl, 0.5% Triton X-100, for 30 min.
- Protocol G: 300 mM of NH_4Cl, 1.0% Triton X-100, for 15 min.
- Protocol H: 300 mM of NH_4Cl, 1.0% Triton X-100, for 30 min.

Mycobacterial Strains

M. tuberculosis H37 Rv (a standard strain gifted by Dr. V.M. Katoch (Agra)) and *M. tuberculosis* (1900/04/SS, a clinical isolate from an HIV-positive patient) were identified and characterized as *M. tuberculosis* by both phenotypic and genotypic methods during our earlier studies and used in this study to prepare the inoculum. Both cultures were grown and maintained on LJ medium slants. A loopful of mycobacterial colony was suspended in sterile phosphate-buffered saline (PBS) and the turbidity was adjusted to 0.5 McFarland standards and used as an inoculum for spiking experiments.

Spiking of Blood Specimens With *M. tuberculosis*

Peripheral blood (10 mL) was collected aseptically from six laboratory volunteers in a sterile container containing 3.1% acid citrate dextrose. The blood from each donor was divided into two equal aliquots. One set of aliquotted samples, from each donor was were spiked with 0.5 mL of *M. tuberculosis* H37 Rv (sensitive to Streptomycin, Isoniazid, Rifampicin, and Ethambutol) and the other was spiked with 0.5 mL of a well-characterized clinical isolate of *M. tuberculosis* resistant to Isoniazid and Rifampicin. The spiked blood specimens were inoculated in BacT/ALERT® MB bottles and incubated in the system at 37°C.

Lysis of RBCs in Culture Medium

The growth of mycobacteria in the BacT/ALERT® MB culture bottle was read by the automated MB/BacT system using the algorithm of nonradioactive CO_2 production. About 3 mL of the culture medium from the positive flashed blood culture bottles were transferred to a ridge-capped, round-bottomed processing tube and centrifuged at 10,000 rpm for 5 min. The resulting pellet was added with 3 mL of lysis buffer, and the mixture was vortexed and incubated at 37°C for a specified time as indicated in the respective protocols described above. The nonlysed and lysed specimens were centrifuged again at 10,000 rpm for 5 min and the final pellet was washed and resuspended in 3 mL of phosphate buffer (pH 7.2). All of the above steps were

undertaken in sterile conditions in the biosafety cabinet (Kartos, India) following the biosafety guidelines and recommendations of the Centers for Disease Control (13).

The protein concentration in the nonlysed and lysed culture medium was measured spectrophotometrically (Biomate® 3; Thermo Electron Corp., Waltham, MA). The lysed and nonlysed positive culture medium was serially diluted and inoculated on Middlebrook 7H10-agar plates supplemented with OADC (Becton Dickinson) in triplicate to enumerate the colony-forming units (cfu).

The protocols were compared for the reduction in the concentration of protein concentration and its detrimental effect on the mycobacterial colony counts.

Lysis of RBCs in Culture Medium From Tuberculosis Patients

After standardization of the protocol on spiked samples, 5 mL of intravenous blood samples from 91 suspected cases of pulmonary tuberculosis was withdrawn under aseptic condition and directly inoculated in the BacT/ALERT® MB (bioméurieux, France) blood culture bottles. The bottles were incubated in the MB/BacT system (bioméurieux, France) at 37°C. Of these, 39 positive and five negative blood culture bottles were taken for further processing by the best protocol as determined earlier. The lysis steps were followed as mentioned above, and the efficiency of the method was demonstrated by estimating the protein concentration and enumerating the viable mycobacterial cells from the lysed and nonlysed culture medium.

Ziehl Neelson Microscopy of the Lysed Culture Medium

Sample specimens were taken before and after the completion of lysis, and smears were made in duplicate from both the spiked medium and the patient's positive culture medium. The air-dried smears were heat-fixed and Ziehl Neelson (ZN)-stained (13). Acid-fast bacilli were counted under oil immersion objective (100 ×) and graded according to the World Health Organization (WHO) standards.

Culture Identification by AccuProbe® from the Lysed Culture Medium

Growth from the positive BacT/ALERT® MB culture bottles was confirmed by AccuProbe® MTB complex culture identification test (Gen-Probe Inc., San Diego, CA). The lysed culture medium was mixed well to a turbidity level of ≥ 1 McFarland nephelometer standard, and 100 µL of this were added into the Accuprobe® lysing reagent tubes. All of the remaining steps, including hybridization, incubation, and reading in an illuminometer, were done strictly according to the manufacturer's instructions (Gen-Probe cat. no. 2860; bio-Merieux).

Isolation of DNA and Species-Specific PCR From the Lysed Culture Medium

One milliliter of the lysed culture medium was kept in a water bath at 80°C for 20 min. The heat-killed mycobacteria-containing specimen was centrifuged at 10,000 rpm for 10 min and the pellet was resuspended in 0.5 mL of TE buffer (Sigma, St. Louis, MO). To this, 0.5% sodium dodecyl sulfate (SDS) and 1.2% Triton X-100 were added, followed by boiling for 1 hr (14). The extracted DNA was directly used for polymerase chain reaction (PCR) without further need of purification. For mycobacterium genus-specific PCR, the *hsp65* gene was targeted (15). For specific identification of *M. tuberculosis*, primers targeting the specific internal transcribed spacer (ITS) region designed by Park et al. (16) were used. The amplified products were electrophoresed through 2% agarose gel in Tris acetate buffer. Target bands of 441 bp for the genus-specific and 121 bp for the *M. tuberculosis* complex were visualized by staining with ethidium bromide.

Antimycobacterial Susceptibility Testing From the Lysed Culture Medium

One milliliter of lysed culture medium was set to 0.5 McFarland's standard using phosphate buffer (pH 7.2) and subcultured in fresh BacT/ALERT® MP bottles. After the instrument flashed positive, the antimycobacterial susceptibility to the first-line drug (Streptomycin (1 µg/mL), Isoniazid (1 µg/mL), Rifampin (1 µg/mL), and Ethambutol (2 µg/mL)) was done in MB/BacT by following the manufacturer's protocol. In brief, 0.5 mL of the mycobacterial inoculum (1:100 dilution in Middlebrook 7H9 broth) was added to the drug-containing and drug-free BacT/ALERT® MP proportional growth control bottles. The bottles were placed in the system and incubated at 37°C. An isolate was considered resistant if the bottle containing the drug flashed positive before or at the same time as the 1:100 growth control without drug, and susceptible if the drug-containing bottle flashed positive after growth control.

For quality-control assurance, antimycobacterial susceptibility testing of the first-line drugs (Streptomycin, Isoniazid, Rifampicin, and Ethambutol) was also done by means of the proportional agar method. The drug-containing plates were prepared by using the standard Middlebrook 7H-10 base enriched with oleic-albumin-

dextrose-catalase (OADC) supplement. The final concentrations of the drugs in the media were as follows: Streptomycin, 0.2 μg/mL; Isoniazid, 2.0 μg/mL; Rifampin, 1 μg/mL; and Ethambutol, 6 μg/mL (13).

Statistical Analysis

Statistical analysis of the results was done with the SPSS® statistics software (version 11.0) program for evaluation of the Student's *t*-test (paired) and chi-square test, and Microsoft™ Excel (version 7.0) was used for entering the data obtained from the study. *P*-values <0.05 were considered significant.

RESULTS

Optimization of the Lysis Method

Lysis of RBCs and Decolorization of Blood Culture Medium

In this study all of the 12 blood samples spiked with the standard strain and a clinical isolate of *M. tuberculosis* were detected as positive in 11.5 ± 3.1 and 10.2 ± 1.2 days, respectively, by the MB/BacT® automated culture detection system, indicating that our spiking methodology was correct and reproducible. As mentioned in the Materials and Methods section, the intact RBCs present in the culture medium bottles were lysed using eight protocols (Table 1). Our results showed that after the lysis steps, the red color of the culture medium disappeared completely and the medium became colorless. The lysis of RBCs and subsequent decolorization of the medium was accomplished by all of the eight protocols.

Effect of Triton X-100 on Mycobacterial Viability

We also studied the detrimental effect of the lysis buffer on mycobacterial viability by enumerating the colony count of *M. tuberculosis* on Middlebrook-7H10 agar plates. The colony-forming units were enumerated from both the lysed and nonlysed culture media bottles. The protocol-A results showed that the colony count of *M. tuberculosis* H37Rv in the nonlysed culture medium was 25×10^7 cfu/mL. The lysis process had no inhibitory effect on the mycobacteria; rather, the colony count improved to 29×10^7 cfu/mL after lysis treatment. The increase in the colony counts after lysis treatment was more pronounced in the freshly isolated strain of *M. tuberculosis*. Using the same protocol A, the colony counts of this strain increased from 19×10^7 cfu/mL to 27×10^7 cfu/mL after lysis. However, the other lysis protocols resulted in reduced colony counts in both the standard and freshly isolated strains of *M. tuberculosis*. The reduction after lysis treatment was up to a log difference and statistically significant ($P = 0.001$), as shown in Table 1.

The statistical analysis of various protocols used in the study showed that mycobacterial colony counts were inversely proportional to the higher concentration of Triton X-100 and incubation time (Table 1).

Effect of the Lysis Method on the Protein Concentration of the Culture Medium

The total protein concentration in the blood culture medium without lysis treatment was found to be 753 ± 39.4 mg/mL. However, after the lysis protocols the protein concentration decreased to levels between

TABLE 1. The final protein concentration and mean mycobacterial colony count of the lysate from positive blood culture medium spiked with *M. tuberculosis* (H 37 Rv and a clinical isolate)*

	Lysis buffer concentration			Concentration of protein (mg/mL)		Mean colony count (cfu/mL)	
Lysis protocol	NH_4Cl (mM)	Triton X-100 (%)	Incubation time (min)	H 37 Rv	Clinical isolate	H 37 Rv	Clinical isolate
Lysed							
A	150	0.5	15	$53.2 \pm 4.2^{\dagger\dagger}$	$51.7 \pm 4.6^{\dagger\dagger}$	$27 \times 10^{7\dagger}$	$29 \times 10^{7\dagger}$
B	150	0.5	30	48.5 ± 3.6	45.3 ± 5.9	69×10^6	42×10^6
C	150	1.0	15	51.9 ± 2.1	51.9 ± 2.1	63×10^6	15×10^6
D	150	1.0	30	45.2 ± 1.2	47.5 ± 1.4	32×10^5	39×10^5
E	300	0.5	15	55.2 ± 3.5	53.2 ± 3.4	6×10^7	32×10^6
F	300	0.5	30	44.2 ± 2.5	43.5 ± 1.8	42×10^6	5×10^6
G	300	1.0	15	45.2 ± 3.1	52.5 ± 0.6	15×10^6	21×10^5
H	300	1.0	30	41.4 ± 2.4	40.5 ± 8.4	19×10^5	12×10^5
Nonlysed	—	—	—	$753.5 \pm 39.4^{\dagger\dagger}$	$712 \pm 12.6^{\dagger\dagger}$	$25 \times 10^{7\dagger}$	$19 \times 10^{7\dagger}$

*A–H are the arbitrary names of the protocols.
$^{\dagger}P$ value = 0.001.
$^{\dagger\dagger}P$ value = 0.0001.

41.4 ± 2.4 and 55.2 ± 3.5 mg/mL (Table 1, Fig. 1). This difference was statistically highly significant ($P = 0.0001$).

Based on these results, only protocol A was further used for application in the microscopy, PCR-based species identification, Accuprobe® culture detection test for *M. tuberculosis* complex and antimycobacterial susceptibility testing. Moreover, this protocol was further evaluated in lysing the RBCs tuberculosis patients.

Utility of the Method for Blood Samples Collected From Tuberculosis Patients

The efficiency of the lysis method was found to be unaltered in blood culture from tuberculosis patients. The medium became colorless after lysis with protocol A, and the protein concentration of the culture medium reduced from 623.5 ± 28.5 mg/mL (nonlysed) to 48 ± 3.9 mg/mL (lysed). The mycobacterial count was found to be slightly increased from 25×10^6 cfu/mL to 38×10^6 cfu/mL. The reduction in the protein concentration and the increase in the colony-forming units was found to be statistically significant ($P = 0.001$; Fig. 1).

Utility of the Lysis Method For ZN Microscopy

The efficiency of the lysis buffer in the microscopic examination of ZN-stained smears was evident. For this 12 BacT/ALERT® MB blood culture bottles spiked with *M. tuberculosis* and 39 culture positive bottles from the patient's blood specimen were taken and their medium was divided into two portions from each. One portion was lysed and the other was used as a control without lysis treatment. The grading of ZN-stained smears for acid-fast bacilli was done according to the WHO standards. On microscopy of the unlysed direct specimens, from 12 spiked bottles, 80% were smear positive and, only one smear (8.33%) showed 2+ for Acid Fast Bacilli (AFB), nine showed 1+ for AFB (75%), and two (16.66%) were negative. After lysis treatment 10 smears (83.33%) showed 2+ AFB and two (16.67%) were graded as 1+ Acid Fast Bacilli (AFB) positive. No smear was negative. The difference was highly significant ($P = 0.0001$). The same enhanced sensitivity was also achieved in the patient's blood culture medium and the difference was found to be statistically significant ($P = 0.0001$; Table 2).

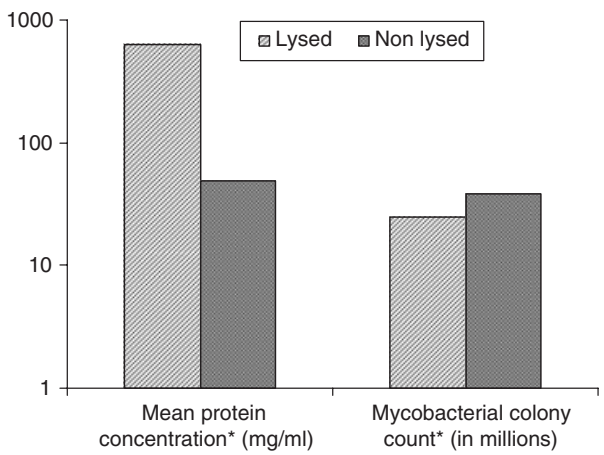

Fig. 1. The final protein concentration and mycobacterial colony-forming units of the lysate from a patient's blood culture. Values are in logarithmic scale.

Utility of the Lysis Method for Isolation of DNA and Species-Specific PCR

All three methods (the genus and species-specific PCR and AccuProbe®) correctly identified isolates from the spiked culture medium as *M. tuberculosis*. Thirty-seven of 39 clinical isolates from tuberculosis patients were identified as *M. tuberculosis* by species-specific PCR and the AccuProbe® culture detection system. The remaining two were identified as *M. avium* and *M. kansasii* based on their phenotypic and biochemical reactions and hsp65 RFLP patterns.

Utility of the Lysis Method for Performing Anti Mycobacterial Drug Susceptibility Testing

Protocol A was used for the first time for antimycobacterial susceptibility testing directly from the positive BacT/ALERT® MB blood cultures. Drug susceptibility testing of two *M. tuberculosis* strains (six bottles each) was done by the MB/BacT system and the conventional proportional method. After lysis treatment the turbidity could easily be set to 0.5 McFarland standards, but this was impossible with the unlysed samples. The sensitivity pattern was reproducible in all of the spikes by MB/BacT, *M. tuberculosis* H37Rv showed a similar sensitivity pattern in all, and the resistant clinical isolate

TABLE 2. Improved sensitivity of the Ziehl Neelson microscopic observation of the positive culture medium of tuberculosis patients

AFB grading[a]	Spiked culture medium (n = 12)		Patient's blood culture medium (n = 39)	
	Nonlysed	Lysed	Nonlysed	Lysed
Negative	2	–	18	3
Scantly positive	–	–	3	1
1+	9	2	12	18
2+	1	8	5	11
3+	–	2	1	6

[a]Per WHO guidelines.

showed a similar resistance pattern to Isoniazid and Rifampicin except in one strain.

Antimycobacterial susceptibility testing was done for all of the 39 *Mycobacterial* isolates by both MB/BacT and the conventional proportional method. Of 39 *Mycobacterial* strains, 21 (53.84%) were found to be sensitive to all four first-line drugs, eight (20.51%) were mono-resistant, seven (17.94%) were resistant to two drugs, while three (7.7%) was found to be resistant to all Streptomycin, Isoniazid, and Rifampicin. Of 39 antimycobacterial susceptibility results by MB/BacT, 34 (87.2%) were concordant with the conventional proportional method.

DISCUSSION

Automated culture systems have added new dimensions in mycobacteriology laboratories (7,9). However, most of the automated culture systems use clinical samples other than blood. Direct blood samples are used only in the MB/BacT® (bio-Merieux, France) system, which has been on the market for several years. Even though bio-Merieux has a protocol for mycobacterial isolation, no protocol is recommended by the manufacturer for performing antimycobacterial drug susceptibility testing from blood culture-positive BacT/ALERT® MB bottles. Furthermore, we found no published protocol in the English literature. Indeed, there are no recommendations or protocols at all regarding how to carry out antimycobacterial susceptibility testing. In earlier studies we used subcultures on solid media for antimycobacterial susceptibility testing, which is very time-consuming, tedious, and error-prone. We also tried to do antimycobacterial susceptibility testing directly from these bottles, but heavy concentrations in the patient's blood made the process impossible, particularly in adjusting the growth to a turbidity of 0.5 McFarland standards. Hence, the system's utility remained limited to isolation of the mycobacteria.

The red coloration of the culture medium becomes colorless after lysis. The lysing buffer prepared in this study has ammonium chloride as one of the most important constituents, which causes an osmotic imbalance and rupture of the cell membrane and leads to lysis of the RBCs (17). Ammonium chloride has been used to lyse RBCs during the isolation of peripheral blood mononuclear cells in various immunological studies (18,19).

The lysis buffer also contained Triton X-100, a nonionic detergent that denatures the cell membranes of bacteria by binding with their phospholipids (20), thereby may reduce the viability of the cell (21). Recently, Blackwood et al. (22) demonstrated that higher concentrations of nonionic detergents could be detrimental for the viability of mycobacterial cells. But the present study shows that the concentration (0.5%) of Triton X-100 used in protocol A was just optimal. Concentrations lower than this could not lyse the RBCs while the higher concentrations lysed the RBCs, they also lysed some mycobacterial cells. The incubation time also plays an important role in the viability of mycobacteria in the presence of strong detergents like Triton X-100. Triton X-100 had a desirable effect not only on contaminating RBCs, but also on the excessive proteins in the blood culture medium. These contaminating proteins are present in a significant amount in blood specimens.

After lysis treatment, 10 out of 12 smears (83.33%) showed 2+ AFB, and two (16.67%) were graded as 1+ AFB positive. No smear was negative. While in unlysed spike specimens 2 were missed the difference was highly significant ($P = 0.0001$). This was an important finding and can be used for processing of hemoptotic samples under the Directly Observed Treatment, Short Course (DOTS) program with high efficacy. Detection of AFB in blood-stained sputa, particularly in peripheral laboratories, is extremely difficult since most such laboratories perform direct microscopy without a decontamination process. To overcome this problem, most investigators (23–25) use fluorescence staining to confirm the AFB from culture bottles. With lysis treatment, the need for fluorescence microscopy could be obviated effectively.

This method was also found to be very handy and useful for isolating mycobacterial DNA. The DNA could successfully be isolated from the lysis buffer-treated medium using a single boiling step, without the need to add proteinase K, lysozyme, or SDS. The isolated DNA was successfully used for genus- and *M. tuberculosis*-specific PCR and the AccuProbe® culture identification test for *M. tuberculosis* complex. Inhibitors of PCR are a major concern, and special steps must be taken during the isolation of DNA from the blood for accurate PCR results (26). The specificity of the PCR protocol standardized here was evaluated as an additional step using blood culture bottles that were positive for *M. avium* and *M. kansasii*. The PCR and AccuProbe® results were negative for both of these isolates (details not presented here).

In conclusion, the lysis protocol reported here was found to be extremely useful for direct microscopy, subculturing the isolate, DNA isolation, AccuProbe® analysis, and antimycobacterial susceptibility testing. The buffer preparation is simple and extremely cost-effective, and could be utilized in peripheral district-level health centers responsible for DOTS programme. The lysis treatment significantly improved the smear and culture positivity rate, and obviated the need for

cumbersome sample processing steps and costly equipment. A patent has been filed for this innovation (Singh S, Gopinath K, 943/DEL/2007).

ACKNOWLEDGMENTS

We thank Dr. V.M. Katoch (Agra) for his kind gift of the *M. tuberculosis* H37Rv standard strain.

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