Ethanol Fixation of Sputum Sediments for DNA-Based Detection of Mycobacterium tuberculosis

DIANA L. WILLIAMS, THOMAS P. GILLIS, AND WAYNE G. DUPREE
Laboratory Research Branch, G. W. Long Hansen’s Disease Center, Louisiana State University, Baton Rouge, and Central Laboratory, Louisiana State Health Department, New Orleans, Louisiana

Received 1 December 1994/Returned for modification 31 January 1995/Accepted 23 March 1995

The effect of ethanol fixation on PCR detection and viability of Mycobacterium tuberculosis in human sputum sediments was evaluated. M. tuberculosis seeded into sputum sediments was efficiently killed when treated for 1 h with 50, 70, or 95% ethanol. PCR amplification of a 123-bp fragment of the M. tuberculosis-specific IS6110 was not affected in ethanol-treated samples even when fixation was extended to 24 h. Ethanol fixation of sputum sediments did not affect the PCR detection of M. tuberculosis in clinical samples. PCR results from ethanol-treated clinical samples containing M. tuberculosis (smear positive and smear negative) or other respiratory pathogens correlated directly with the results by conventional detection methods for M. tuberculosis. Our results show that ethanol fixation of human sputum sediments containing M. tuberculosis significantly reduces the potential exposure of workers to viable M. tuberculosis without affecting DNA analysis by PCR. Also, ethanol fixation of sputum sediments provides a simple and inexpensive way to store and transport clinical specimens identified for DNA-based diagnostics without refrigeration.

Compounding the problem of resurgent tuberculosis in the United States is the emergence of strains of Mycobacterium tuberculosis resistant to many of the drugs used for therapy and prophylaxis (6). The time required for conventional laboratory identification, including drug susceptibility patterns for M. tuberculosis, remains an obstacle for rapid diagnosis and treatment. Newly developed, rapid methods for detecting M. tuberculosis and for determining drug susceptibility patterns from clinical specimens are being tested and may provide new approaches for shortening laboratory diagnostic procedures, thereby facilitating diagnosis and treatment. The successful integration of these tests into the clinical laboratory should help to reduce morbidity, mortality, and the spread of tuberculosis.

Many new tests designed to detect M. tuberculosis in clinical specimens (4, 7, 8, 11, 15) and for identifying drug resistance patterns (16, 17) are based on nucleic acid amplification procedures, exploiting their rapidity, sensitivity, and specificity. For most of these tests, the importance of rendering the clinical sample noninfectious at an early stage of processing to minimize the biohazard to laboratory workers and at the same time maintaining the integrity of the sample for subsequent storage, shipping, and analysis has not been considered. We report here that ethanol fixation of sputum sediments containing M. tuberculosis renders the bacterium nonviable, while preserving the integrity of M. tuberculosis genomic DNA in a state suitable for testing by PCR.

MATERIALS AND METHODS

Cultivation of M. tuberculosis. Log-phase cultures of M. tuberculosis H37Rv were obtained by inoculating M. tuberculosis into Middlebrook 7H9 medium (Difco, Detroit, Mich.) containing ADC supplement (Difco) and incubating cultures for 6 days at 37°C with 5% CO2. The bacteria were harvested and washed in phosphate-buffered saline (pH 7.6) containing 0.05% Tween 80 (PBST) by centrifugation. Briefly, preliminary quantitation by serial 10-fold dilutions and plating on 7H11 agar established that the number of M. tuberculosis organisms equivalent to a MacFarland nephelometric standard of 1 was equal to 2 × 106 acid-fast bacilli per ml. For seeding experiments, the bacteria were resuspended in PBST to produce a stock suspension at a final concentration of 109/ml. Stock suspensions were also quantitated further by plating serial dilutions onto 7H11 agar and determining the number of CFU. Stock M. tuberculosis suspensions were used to seed human sputum sediments to analyze the effect of ethanol fixation on the viability of M. tuberculosis and the ability of M. tuberculosis DNA to produce a 123-bp amplicon from IS6110 by PCR (4, 8).

Clinical samples. All human sputum samples were obtained from the clinical microbiology laboratory at Charity Hospital (New Orleans, La.). Sputum samples were collected from individuals suspected of having tuberculosis and were processed for culture in BACTEC 12B medium. Species identification was performed by using AccuProbe (GenProbe, San Diego, Calif.) according to manufacturer’s instructions.

Briefly, sputum specimens were treated with N-acetyl-l-cysteine-NaOH and concentrated to approximately 1.0 ml in phosphate buffer (pH 7.0). BACTEC 12B vials were inoculated with 0.5 ml of sputum sediment and monitored for growth with a BACTEC 460 system for 6 weeks. A pool of M. tuberculosis-negative sputum samples was prepared from sputum sediments following confirmation of negative culture results for growth in BACTEC 12B medium. Sputum sediments for pooling were held at 4°C during the 6-week culture confirmation period.

Experiments designed to analyze the effect of ethanol on M. tuberculosis viability and PCR amplification of target DNA were performed using pooled sputum sediments seeded with M. tuberculosis and diluted with absolute ethanol to final ethanol concentrations of 50, 70, or 95%. Each individual patient’s sputum sediments (0.25 ml) for direct testing by PCR were diluted with 0.383 ml of 100% ethanol to bring the final ethanol concentration to 70%. Viability of M. tuberculosis in human sputum following ethanol treatment. Duplicate samples (250 μl) of M. tuberculosis-negative, pooled human sputum sediments were seeded with 2.5 × 108 M. tuberculosis organisms by adding 25 μl of M. tuberculosis stock suspension (109 organisms per ml). Absolute ethanol was added to duplicate samples to a final concentration of either 50, 70, or 95%, and samples were held for 1, 6, or 24 h at 25°C. At the completion of ethanol treatment, one of the two samples for each ethanol concentration was centrifuged at 12,000 × g for 5 min at 4°C, and the supernatant was discarded. Pellets were resuspended in 1.0 ml of spolysin (phosphate-buffered dithiothreitol solution; Calbiochem-Novabiochem, La Jolla, Calif.) to remove potential soluble inhibitors of PCR (personal observation) and mixed every 5 min for 15 min by vortexing. Samples were centrifuged as described above, and the pellets were resuspended in 1 ml of Hank’s balanced salt solution containing 0.05% Tween 80 (HBSST). Following a 15-s sonication of each sample, serial 10-fold dilutions were made in HBSST, and 0.1 ml was inoculated in duplicate into either Middlebrook 7H11 or BACTEC 12B medium for determinations of M. tuberculosis viability. BACTEC cultures were read every 3 days for 6 weeks, and 7H11 cultures were held at 37°C for 12 weeks. Control sputum specimens did not receive ethanol and were processed as described for ethanol-treated specimens.
TABLE 1. M. tuberculosis viability following ethanol treatment

<table>
<thead>
<tr>
<th>Sample treatment</th>
<th>Duration of treatment (h)</th>
<th>CFU/ml</th>
<th>BACTEC growth index</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1</td>
<td>$1.0 \times 10^7$</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>$0.9 \times 10^7$</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>$1.2 \times 10^7$</td>
<td>999</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* For Treatment with 50, 70, and 95% ethanol gave identical results.
* For controls, the growth index for BACTEC cultures was determined in duplicate after 17 days. For ethanol-treated cultures, the growth index was reached after 6 weeks of culture and never exceeded 2 for the entire experiment.

IS6110 PCR following ethanol treatment. Samples were treated with 50, 70, or 95% ethanol as described above, except that following sputolysin treatment, bacterial pellets recovered by centrifugation were resuspended in 1 ml of 10 mM Tris–1 mM EDTA buffer (pH 7.6). The samples were centrifuged, and the pellets were resuspended in 50 μl of sterile deionized water, heated for 10 min at 100°C and centrifuged at 12,000 × g for 5 min. The supernatant containing crude cell lysate was removed and stored at −70°C prior to analysis by PCR. Two microliters of each crude cell lysate was added to PCR reagents including oligonucleotide primers which target a 123-bp region of IS6110 of M. tuberculosis (4). PCR was performed for 40 cycles following an initial denaturing step at 94°C for 7 min with a Perkin-Elmer 9600 DNA Thermal cycler by using the following program: denaturation, 94°C for 30 s; annealing, 60°C for 30 s; and extension 72°C for 90 s, with a final 10-min extension at 72°C. PCR products were analyzed by gel electrophoresis on either 3% NuSieve-SeaKem GTG (1:1) agarose (FMC Bio-products, Rockland, Maine) gels in 1× Tris-acetate-EDTA buffer (pH 8.3; Gibco/BRL, Grand Island, N.Y.) at 80 V for 60 min or on 6% nondenaturing polyacrylamide gels (NOVEX Corp., San Diego, Calif.) in 1× Tris-borate-EDTA buffer (Sigma Chemical Co., St. Louis, Mo.) at 150 V for 36 min. Gels were stained with ethidium bromide, and DNA was visualized by UV transillumination.

RESULTS AND DISCUSSION

M. tuberculosis viability following ethanol treatment. M. tuberculosis organisms seeded into human sputum sediments were effectively killed with all three concentrations of ethanol tested and as early as 1 h after treatment (Table 1). Recovery of viable M. tuberculosis in control samples following 1, 6, and 24 h of incubation at 25°C indicated that of the original 2.5 × 10^7 M. tuberculosis organisms seeded into control sputum sediments, only 40% (1 and 6 h) or 48% (24 h) of the organisms were recovered as viable. We repeated these experiments using M. tuberculosis grown to log phase as well as stationary phase and obtained similar levels of viability following incubation in pooled sputum sediments at room temperature. Whether the loss of CFU is due to death of M. tuberculosis during the incubation period or is the result of processing (e.g., centrifugation and/or sputolysin treatment) prior to plating was not determined. It is possible that either sputolysin or the centrifugation or both resulted in this decrease in viability.

PCR of M. tuberculosis following ethanol treatment. Electrophoretic analysis of PCR products following ethanol treatment indicated neither an enhanced nor a detrimental effect on the production of the M. tuberculosis-specific 123-bp amplicon (Fig. 1). On the basis of the original number of M. tuberculosis organisms seeded into sputum sediments and the final number analyzed after PCR amplification, the signal obtained represented 2 × 10^3 M. tuberculosis organisms. Similar results were obtained when the seeded M. tuberculosis was obtained from cultures grown to stationary phase (data not shown).

To obtain a more sensitive determination of possible effects of ethanol treatment on PCR, pooled human sputum sediments were seeded with decreasing amounts of viable M. tuberculosis and treated with 70% ethanol for 1 h. Following standard processing for and amplification by PCR, each sample was analyzed for production of the 123-bp amplicon on 6% polyacrylamide gels (Fig. 2). Compared with untreated M. tuberculosis-containing sputum sediments, no differences were observed in 123-bp amplicon production at concentrations of M. tuberculosis from 5 × 10^5 to 5 × 10^3 organisms. Enhanced resolution of PCR products by polyacrylamide gel electrophoresis indicated that in addition to the production of similar levels of M. tuberculosis-specific 123-bp amplicons, both control and ethanol-treated samples produced spurious bands of identical size, indicating similar behavior of the samples in PCR whether fixed or not fixed in ethanol.

Direct PCR of ethanol-treated sputum specimens. Sputum sediments from one smear-negative (culture-positive) and five smear-positive (culture-positive) tuberculosis patients and from seven patients not infected with M. tuberculosis were tested for PCR amplification of target DNA following ethanol treatment. Sputum sediments (250 μl) were collected at Charity Hospital, treated in 70% ethanol, and shipped to G. W. Long Hansen’s Disease Center, where they were tested by
PCR within 1 week of acquisition. All smear-positive sputum samples produced a strong 123-bp band following PCR (Fig. 3, lanes 2 to 5). The smear-negative, culture-positive sputum samples also produced a visible 123-bp band, albeit weak, on the 6% polyacrylamide gel (Fig. 3, lane 8) compared with the smear-positive sputum sediments.

Of the remaining sputum samples collected for M. tuberculosis testing, two contained Mycobacterium avium-M. intracellulare complex organisms as determined by AccuProbe (Gen-Probe), one contained Mycobacterium chelonii, and one contained Streptococcus pneumonia; two sputum samples were devoid of culturable organisms. These sputum samples tested negative for the M. tuberculosis-specific 123-bp PCR product (Fig. 3). These results confirm earlier reports on the specificity of the IS6110 PCR assay for M. tuberculosis and demonstrate the utility of ethanol fixation for processing infectious sputum samples for subsequent M. tuberculosis DNA testing.

Our incentive for developing a simple procedure capable of rendering M. tuberculosis-containing sputum specimens noninfectious and at the same time suitable for PCR analysis comes from the practical need to minimize the biohazard associated with such specimens and to preserve samples for safe shipping and long-term storage. Most DNA-based amplification tests for detecting M. tuberculosis in sputum sediments and other biological specimens have not been evaluated for potential biohazards associated with the procedural aspects of each test. A notable exception is the work by Zwydik et al. (18) in which heating M. tuberculosis suspensions at 100°C for 30 min was found to be effective in killing M. tuberculosis as well as extracting DNA from the bacterium for both PCR and strand displacement amplification analysis.

We chose to evaluate ethanol as a decontaminant for sputum sediments on the basis of our previous experience in detecting Mycobacterium leprae by PCR in skin biopsies fixed in ethanol (5) and because of its general disinfectant properties. For example, 70% ethanol is generally considered an effective tuberculocidal agent (3, 12–14). However, effective killing of M. tuberculosis by ethanol can be compromised by the length of contact time and the organic milieu in which M. tuberculosis is found. For example, Best et al. (1) found 70% ethanol to be effective against M. tuberculosis in suspension in the absence of sputum only when exposures were held to 1 min. Lind et al. (9) found that a 15-min exposure of M. tuberculosis to 70% ethanol reduced the titer of M. tuberculosis more than 1,000-fold. Our studies extended exposure times to 1, 6, and 24 h, with the rationale that sputum sample disinfection and processing for M. tuberculosis detection by PCR should be completed within 24 to 48 h. All three exposure times tested for 50, 70, and 95% ethanol met this criterion, offering flexibility in scheduling sample processing for DNA-based detection of M. tuberculosis.

Ethanol fixation also allows for long-term storage and safe shipping of potentially dangerous strains of M. tuberculosis without refrigeration. Sputum samples have been processed after 6 months of storage in ethanol at room temperature in our laboratory without detrimental effects on the PCR detection of M. tuberculosis (data not shown). M. tuberculosis isolates have been scraped from solid medium (Lowenstein/Jensen) or pelleted from broth cultures (Middlebrook 7H11 and 7H12), fixed in 70% ethanol, and shipped to our laboratory for PCR amplification and direct sequencing of the PCR product (17). Viavable organisms were not detected in these samples by using both BACTEC and 7H11 media for culture. Other DNA sequences have been amplified and sequenced from ethanol-fixed samples including fragments from 16S rRNA, rplS, inhA, katG, and rpoB genes (2, 10, 17), indicating the utility of this type of processing for many DNA-based analyses and suggesting that ethanol fixation may be used for universal decontamination of biological specimens containing M. tuberculosis identified for PCR evaluation.

ACKNOWLEDGMENTS

We thank Don Greer and Alan Sanders for providing patients’ samples and Penne Cason for preparing the manuscript.

This work was supported in part by NIAID grant AI35274.

REFERENCES


