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Substances Interfering with Direct Detection of *Mycobacterium tuberculosis* in Clinical Specimens by PCR: Effects of Bovine Serum Albumin

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Interfering substances have been reported to inhibit PCR assays for the direct detection of *Mycobacterium tuberculosis* in clinical specimens. Using an internal control, we determined that 52% of respiratory specimens interfered with our PCR assay. On the basis of these findings, we tried to circumvent the problem by simply diluting prepared sediments. With sediment from a routinely processed sputum known to be inhibitory to PCR, one aliquot was prepared in a routine manner for PCR. Remaining sediment was diluted in phosphate-buffered saline, Middlebrook 7H10 broth, or BACTEC 12B broth; an internal control was added to all reaction mixtures and controls. Internal control was detected only in the sample diluted with BACTEC 12B medium. Components of the BACTEC 12B medium including PANTA reagent (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin), reconstituting fluid, 0.2% glycerol, 0.05% Tween 80, and 0.05% bovine serum albumin (BSA) were tested in a similar manner. Only 0.05% BSA resulted in amplification of the internal control DNA. Varying concentrations of BSA were added to 11 aliquots of a respiratory sediment known to be inhibitory to the PCR. Internal control was detected in all reaction mixtures containing 0.00038 to 0.1% BSA. To determine the ability of BSA to override inhibition, respiratory specimens were run in triplicate: undiluted, diluted 1:2 with BACTEC 12B medium, or diluted with 0.026% BSA. For 21 of 22 inhibitory specimens, BSA was able to override the presence of interfering substances. These data suggest that the presence of BSA in a PCR assay is critical for the direct detection of *M. tuberculosis* in respiratory specimens.

Tuberculosis remains the leading infectious cause of death in adults worldwide, accounting for nearly 3 million deaths annually. After years of declining case rates, the dramatic increase in tuberculosis in the United States underscored the immediate demand for development of more rapid and reliable laboratory diagnostic assays.

Although rapid, acceptance of amplification assays for the direct detection of *Mycobacterium tuberculosis* in clinical specimens has been hindered by multiple problems including inhibition of PCR assays (4, 7, 9, 10, 12); studies reporting rates of inhibition range from as low as 3% to greater than 15%. Although heme compounds present in blood specimens have been identified as inhibitors of PCR amplification, the nature of endogenous PCR inhibitors present in respiratory samples has not been delineated (1). Extraction procedures employing multiple steps to purify DNA from respiratory samples appear to have lower rates of inhibition (6, 9) compared with those with fewer steps (7). For example, Yuen et al. reported that inhibitors were found in 3.2% of sputum samples in which DNA was extracted by digestion with lysozyme and lysing by heat and a sodium dodecyl sulfate solution, followed by extraction with phenol-chloroform and ethanol precipitation (14).

Nolte et al. reported that 10% of specimens were inhibitory to PCR when samples were centrifuged and the pellets were lysed by detergent and heating and then centrifuged again (10). When DNA was extracted from inhibitory samples with phenol-chloroform, precipitated with ethanol, and retested, inhibitors were removed in all cases. Of interest, Kirschner and coworkers (8) reported that 21% of clinical specimens were inhibitory in their PCR assay using a commercially prepared kit (Amplicor *M. tuberculosis* Sputum Preparation Kit; Hoffman LaRoche, Grenzach-Wyhlen, Germany) to extract DNA for amplification. Using a simple extraction protocol of boiling and beating with glass beads routinely processed respiratory sediments, we initially estimated that 13% of specimens were inhibitory in a PCR assay developed in our laboratory (7). Subsequently, using an internal control (IC) in our reaction mixture (6), we prospectively analyzed 100 respiratory specimens with our PCR assay; 52% of these specimens showed evidence of interfering substances. Unfortunately, reextraction of DNA with phenol-chloroform and ethanol precipitation did not consistently remove substances in clinical specimens that were inhibitory to the PCR. On the basis of our findings, we attempted to find a more consistent means by which to reverse this inhibition. Inherent in our approach was to delineate a means of overriding this inhibition without engendering major changes since our PCR assay had been extensively evaluated by a simple and inexpensive specimen extraction protocol (7).

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MATERIALS AND METHODS

PCR assay. (i) Primers. Two sets of primers, one based on the IS*6110* repeated sequences of *M. tuberculosis* (5) and the other based on the 38-kDa protein antigen b (PAB) (11) of *M. tuberculosis*, were used in a multiplexed PCR assay to detect *M. tuberculosis* directly in respiratory sediments. The expected sizes of amplification products of the IS*6110* and PAB primers were 123 and 419 bp, respectively.

(ii) IC. Recombinant DNA serving as an IC was kindly provided by K. D. Eisenach and M. D. Cave (University of Arkansas). IC DNA was constructed so as to produce a 600-bp amplified product with the same primers as those for the

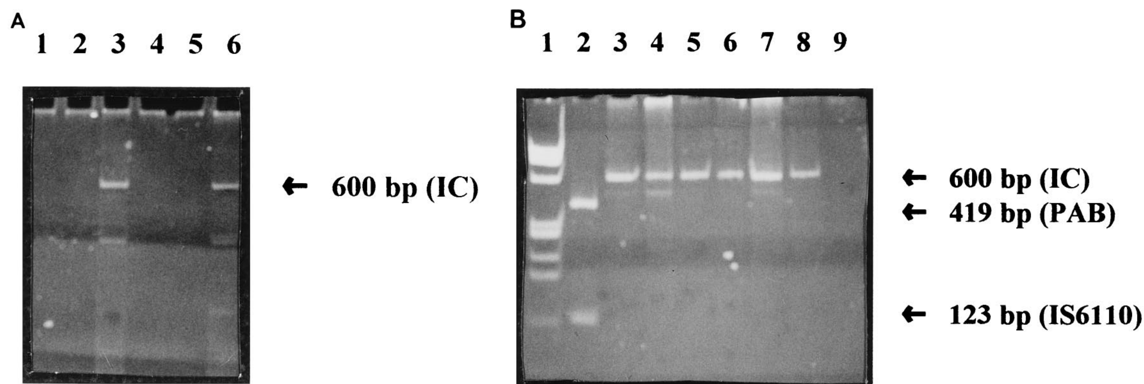

FIG. 1. DNA from a sputum specimen known to be inhibitory to PCR was amplified with components of BACTEC 12B medium to determine which might be responsible for overriding PCR inhibition. (The inhibitory specimen was culture negative for *M. tuberculosis*; therefore, only the IC product was expected. (A) Results with BACTEC 12B components: lane 1, 1:2 dilution with PANTA; lane 2, 1:2 dilution with reconstituting fluid; lane 3, 1:2 dilution with BACTEC 12B broth without PANTA; lane 4, 0.2% glycerol; lane 5, 0.05% Tween 80; lane 6, 1:2 dilution with BACTEC 12B broth and PANTA. (B) Results with BSA added to reaction mixture in varying concentrations: lane 1, ΦX174 digested with *Hae*III; lane 2, positive control; lane 3, 0.1% BSA; lane 4, 0.05% BSA; lane 5, 0.026% BSA; lane 6, 0.013% BSA; lane 7, 0.006% BSA; lane 8, 1:2 dilution with BACTEC 12B broth; lane 9, undiluted.

IS*6110* target sequence (6). Prior to use, the amount of IC added to each reaction mixture was optimized; approximately 200 fg of IC was subsequently added to each reaction mixture for use in all PCR assays. Importantly, our assay was reevaluated to determine whether inclusion of the IC affected the PCR assay's sensitivity. Sensitivity was determined as previously described (7) by preparing serial 10-fold dilutions in pooled, *M. tuberculosis* culture-negative sputa, extracting the DNA, and then amplifying. The sensitivity of our PCR assay with the inclusion of the IC was equivalent to that without the IC present, fewer than one *M. tuberculosis* organism was detected by the IS*6110* primers while the PAB primers detected about five organisms. Results following amplification of a preparation containing *M. tuberculosis* and IC DNA using the IS*6110* and PAB primers are shown in Fig. 1B for illustration.

(iii) Sample processing for PCR and reaction conditions. Following routine processing for mycobacterial smear and culture, aliquots of respiratory sediments were boiled for 10 min. A 200-μl portion was suspended in glass beads to which 16 μl of Tween 20 had been added. The suspension was beaten for 3 min, and 10 μl was added to a tube containing 85 μl of amplification master mixture containing 12 pmol of IS*6110* primers, 20 pmol of PAB primers, 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and 200 fg of IC in PCR buffer II (Perkin-Elmer) (7). The reaction mixture was overlaid with mineral oil, boiled for 8 min, and chilled on ice, and 2.5 U of *Taq* polymerase was added. Reaction mixtures were subjected to 40 cycles of amplification. Amplified products were visualized with UV light following polyacrylamide gel (8%) electrophoresis and ethidium bromide staining.

Sample dilution. One aliquot of a processed sample (200 μl) known to be inhibitory to PCR amplification was prepared for PCR in a routine manner by beating for 3 min; a processed sample was considered inhibitory if no 600-bp product was detected following amplification. The remaining boiled sediment was diluted 1:2 in one of the following prior to beating: phosphate-buffered saline, Middlebrook 7H10 broth (Difco Laboratories, Detroit, Mich.), and BACTEC 12B medium (Becton Dickinson, Sparks, Md.). IC was added to all reaction mixtures and controls prior to amplification. Amplification was carried out as previously described. Inhibition was considered to be overridden if the presence of the 600-bp IC was observed.

Addition of BACTEC 12B components. With a routinely processed sputum known to be inhibitory to PCR, one boiled aliquot was again prepared for PCR in a routine manner by beating for 3 min. The remaining boiled sediment was first diluted 1:2 in one of the following prior to beating: PANTA reagent (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) (Becton Dickinson), reconstituting fluid (polyoxyethylene stearate; Becton Dickinson), 0.2% glycerol, 0.05% Tween 80, and BACTEC 12B broth with and without PANTA reagent. Remaining boiled sediment was also added to tubes containing bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Mo.). Since 0.05% BSA is present in BACTEC 12B broth, BSA was added in varying concentrations prior to beating: 0.1, 0.05, 0.025, 0.013, and 0.006%. Amplification was then carried out as previously described.

PCR optimization with BSA. Again, a routinely processed sputum known to be inhibitory to PCR was boiled for 10 min. One aliquot (200 μl) was prepared for PCR in a routine manner by beating for 3 min. The remaining boiled sediment was added to tubes containing twofold serially diluted BSA from 0.026 to 0.000001% prior to beating. All samples were then amplified as previously described.

To determine whether BSA dependably reversed the inhibitory effect of clinical samples, 28 routinely processed respiratory specimens were amplified with and without BSA; 26 of these 28 specimens were known to interfere with our PCR assay. All samples were boiled as previously described and then processed by three different methods in preparation for PCR. First, aliquots (200 μl) were beaten and 10 μl of the undiluted preparation was directly added to the standard reaction mixture and amplified. A second aliquot was diluted 1:2 in BACTEC 12B broth, beaten, and amplified as previously described. Finally, a third aliquot was beaten with the determined optimum concentration of BSA (0.026%) and amplified as previously described.

RESULTS

Dilution with BACTEC 12B broth components. In order to override inhibition yet maintain a simple extraction protocol that would be feasible in a routine clinical laboratory setting, we first attempted to dilute out possible interfering substances. For all experiments, a routinely processed sputum, known to be inhibitory to PCR, was employed. Only BACTEC 12B broth was able to override the interfering substances present in a clinical specimen. Therefore, DNA from a sputum known to be inhibitory to PCR was amplified with various components of BACTEC 12B medium to determine which component might be responsible for overriding PCR inhibition. These results are shown in Fig. 1. Only BSA alone and BACTEC 12B broth with or without PANTA were able to nullify the inhibitory effects of the clinical sample on the PCR.

BSA. Once it was determined that BSA was responsible for overriding the inhibition, the optimum concentration of BSA was determined for use in the PCR assay. On the basis of the presence of the IC and intensity of the product band following gel electrophoresis, we determined that a concentration of 0.026% BSA was optimum.

Finally, 28 routinely processed sediments were amplified in triplicate, either undiluted, diluted 1:2 in BACTEC 12B broth, or diluted with 0.026% BSA. Twenty-two of these specimens were inhibitory to PCR. In only one instance was BACTEC 12B broth or BSA unable to override the inhibition. Results of these 21 specimens are summarized in Table 1. Figure 2 illustrates the overriding effects of BACTEC 12B broth or 0.026% BSA on interfering substances present in clinical specimens from three patients. Six specimens, considered noninhibitory (for example, Fig. 2, patient 2 and patient 5), were amplified under all three reaction conditions. While only the IS*6110* target was detected in the undiluted specimen for two of these six patients, addition of BACTEC 12B broth and BSA to the reaction mixture resulted in the amplification of both the IS*6110* and PAB targets; results for one of these patients are

TABLE 1. Summary of culture and amplification results following addition of BSA to the reaction mixture for the 21 inhibitory specimens

Culture result	No. of specimens	Amplification product(s) detected
No growth	14	IC only
No growth	1	None
Mycobacterium avium complex	2	IC only
M. tuberculosis	1	IC, IS*6110*, and PAB
	2	IS*6110* only
	2	IC and IS*6110*

shown in Fig. 2 (patient 2). These findings suggest that these specimens were only partially, rather than completely, inhibitory to PCR. Of note, PCR results on all *M. tuberculosis* culture-positive specimens showed the appropriate *M. tuberculosis* product(s) and either a reduction or disappearance of the 600-bp IC product, regardless of whether BSA was required or not for detection (Fig. 2), thereby indicating that the IS*6110* primers preferentially amplify *M. tuberculosis*-specific target sequences compared with the IC.

DISCUSSION

We developed a PCR assay that proved to be a useful rapid diagnostic test for the detection of *M. tuberculosis* directly in clinical specimens. Although we estimated that interfering substances were present in about 13% of specimens, we found that about half of the specimens were in fact inhibitory to PCR by using an IC in each PCR. Therefore, we attempted to circumvent the problem of these substances in clinical specimens without significantly changing the extraction protocol by simply diluting prepared sediments. This approach led to the finding that BSA was able to override endogenous PCR inhibitors. We confirmed that BSA consistently reversed the effects of these inhibitors in clinical samples.

Although the presence of PCR inhibitors in various clinical samples is well recognized, few studies have been performed to specifically address this issue. In an attempt to delineate a simple, yet reliable, method for treating clinical specimens containing mycobacteria for PCR analysis, Victor et al. (13) and Buck and colleagues (3) investigated different extraction methods. Using a sucrose purification step prior to amplification, Victor et al. (13) were able to theoretically remove inhibitory substances and provide more reproducible and accurate results; however, 11 of the 169 samples still required reamplification. Buck et al. reported the successful use of sonication to obtain nucleic acid for amplification (3). Nevertheless, PCR failed to detect *M. tuberculosis* in 2 of 26 culture-positive samples. One false-negative PCR result was determined to be due to sampling error while the other was caused by inhibition; an overall rate of inhibition was not reported.

More recently, Amicosante et al. (2) compared the ability of an anion-binding resin, GeneReleaser (Bio Ventures), with that of phenol-chloroform extraction to remove interfering substances from 35 culture-positive specimens. Inhibition rates for PCR of phenol-chloroform extraction alone and GeneReleaser were 22.8 and 8.6%, respectively; of interest, use of extraction in combination with GeneReleaser removed all inhibition.

The incorporation of BSA had a significant impact upon the inhibition rate of our PCR assay in all but one instance. The mechanism as to how BSA decreases the rate of inhibition remains to be determined. On the basis of these data, BSA could override the effects of endogenous inhibitors present in respiratory samples inhibitory to PCR and/or increase the efficiency of the PCR assay. Of significance, this finding has allowed us to retain our simplified extraction procedure in which routine processed sediments are boiled, beaten, and then amplified (7). In addition, costs to implement the routine use of BSA for the direct detection of *M. tuberculosis* in clinical samples are relatively low, particularly compared with commercially available alternatives to DNA extraction such as GeneReleaser. On the basis of these data, the incorporation of BSA into PCR assays might be a very useful potential tool to override endogenous inhibitors present in clinical samples.

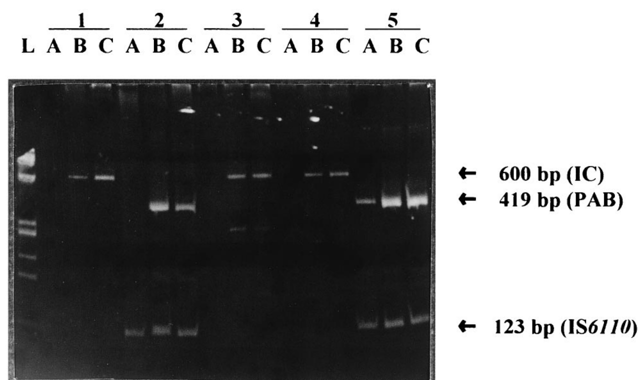

FIG. 2. Clinical evaluation of PCR assay employing 0.026% BSA. Routinely processed sediments were amplified in triplicate, either undiluted, diluted 1:1 in BACTEC 12B broth, or diluted with 0.026% BSA. Specimens were obtained from five patients (labeled 1 through 5 above the lane markers): lane L, ΦX174 digested with *Hae*III; lanes A, undiluted sediment; lanes B, sediment diluted 1:1 in BACTEC 12B broth; lanes C, sediment amplified in the presence of 0.026% BSA. Cultures of specimens from patients 1, 3, and 4 were culture negative and PCR negative for *M. tuberculosis*; therefore, only an IC amplification product was observed. Specimens from patients 2 and 5 were culture positive and PCR positive for *M. tuberculosis*.

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