Alkaline Decontamination of Sputum Specimens Adversely Affects Stability of Mycobacterial mRNA

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Reverse transcriptase PCR (RT-PCR) is an important tool for *Mycobacterium tuberculosis* research and diagnostics. A standard procedure using *N*-acetyl-L-cysteine (NALC) and NaOH has been widely adopted for digestion and decontamination of sputum specimens for mycobacterial culture. The objective of this study was to determine the compatibility of this method with the recovery of RNA for RT-PCR assays. Nineteen sputum specimens were collected from smear-positive, pretreatment tuberculosis patients. After homogenization with NALC and glass beads, specimens were further processed by the addition of either NaOH, as per the standard decontamination protocol, or phosphate buffer. RNA was prepared by using a modified guanidine-phenol extraction method developed specifically for sputum sediments. DNA was isolated from the same specimens. Reverse transcriptions of alpha antigen (85B protein) mRNA and 16S rRNA were performed together, and aliquots were removed for separate PCRs. In all specimens, the 85B mRNA target was greatly diminished by treatment with NaOH; however, the 16S rRNA target remained unaffected. Storing sputum specimens for 48 h at 4°C before processing did not seem to affect the integrity or yield of RNA; however, some degradation occurred by 72 h. Data suggest that the NaOH-NALC method for processing sputum samples is not suitable for detecting mRNA targets in RT-PCR assays.

Tuberculosis continues to be the leading cause of morbidity and mortality by an infectious disease worldwide (12, 16). The need for rapid methods of diagnosis and determination of the response to therapy has led to the development of diagnostic tests for detecting *Mycobacterium tuberculosis* nucleic acid. Methods used to detect mycobacterium-specific DNA sequences include PCR and strand displacement amplification (1, 4–6, 8, 9, 21). Although these tests are useful for detection of mycobacterial DNA, they may be inappropriate for monitoring the response of patients to antituberculosis therapy. A recent study by Hellyer et al. (7) used PCR, strand displacement amplification, microscopy, and culture to study sputum specimens collected serially from patients throughout their course of treatment for pulmonary tuberculosis. This study showed that DNA amplification tests did not reflect response to treatment, since mycobacterial DNA persisted for long periods in sputum, even after the specimens were culture negative. Those authors suggested that an RNA-based assay would detect viable organisms and so appropriately reflect the efficacy of a particular therapy.

In cultured *M. smegmatis* cells, cell viability has been shown to correlate with rRNA levels, as measured by the nucleic acid-based sequence amplification (17). RNA-based tests specific for *M. tuberculosis* exist. For example, the Q-beta replicase system targets 23S rRNA, and the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test targets 16S rRNA. However, these methods are no more sensitive than routine culture for the detection of *M. tuberculosis* in sputum (15, 18, 20). This suggests that these methods may not have the necessary sensitivity to detect the small numbers of viable organisms likely to

be present in sputum specimens collected after the initiation of antituberculosis therapy.

In the present study, we developed a reverse transcriptase PCR (RT-PCR) assay for the mRNA of alpha antigen (85B protein) from *M. tuberculosis*. This target was selected because the 85B antigen constitutes up to 41% of the total mycobacterial protein in log-phase culture supernatants (22). It is reasonable to expect similarly high levels of 85B mRNA expression. For comparison, an RT-PCR assay directed towards the more stable and abundant 16S rRNA was also designed.

These RT-PCR assays were used to evaluate different sputum digestion and decontamination protocols. Methods of handling and storing specimens before processing were also addressed. Finally, a protocol for extraction of RNA from sputum specimens was developed by using a modification of the guanidine-acid phenol procedure with mechanical disruption. This method allowed isolation of RNA and DNA from the same specimen and was amenable to handling multiple samples.

MATERIALS AND METHODS

Sputum specimens. The 19 sputum samples used in this study were collected in a noninduced manner from 10 patients with symptomatic pulmonary tuberculosis at the tuberculosis clinic of the Hospital Universidade Cassiano Antonio de Moraes in Vitória, Brazil. All sputa contained at least one acid-fast bacillus per high-powered field ($\geq 3+$) on direct microscopic examination of Ziehl-Neelsen-stained smears. The presence of *M. tuberculosis* in these specimens was confirmed by growth in BACTEC 12B vials and by the presence of more than 100 colonies on Middlebrook 7H10 and 7H10S media. Species identification was made on the basis of *n*-acetyl-*para*-hydroxypropionophenone susceptibility and appropriate colony morphology. Sputum specimens were collected before the initiation of antituberculosis treatment, except in three cases in which the specimens were collected within 72 h of the start of therapy.

Specimen processing. Sputum was refrigerated immediately upon collection and processed within 4 h, unless otherwise noted. Five milliliters of each sample was homogenized by vortexing with 4-mm glass beads and NALC (1 ml of a 2.5% *N*-acetyl-L-cysteine [NALC] solution in phosphate buffer [PB], 68 mM, pH 6.7, per specimen) and divided into halves for differential processing. The homoge-

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nate was processed by either standard alkaline decontamination (10) or sham decontamination with PB followed by storage in one of two different media.

The above protocol resulted in a total of four treatments per sputum specimen, as follows. (i) Samples were subjected to alkaline digestion by incubation for 15 min with an equal volume of 2% NaOH-sodium citrate followed by buffering with PB and then were pelleted by centrifugation at 10°C and 4,000 × g for 15 min. The supernatant was decanted, and the pellet was resuspended in PB and stored frozen at -70°C. (ii) Samples were treated as in treatment 1 except that they were stored frozen in GuSCN solution (4 M guanidine isothiocyanate, 50 mM Tris-HCl [pH 7.5], 25 mM EDTA, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). (iii) Samples were sham decontaminated without the addition of NaOH and stored frozen at -70°C in PB. (iv) Samples were treated as in treatment 3 except that they were stored frozen in GuSCN solution.

RNA and DNA isolation. RNA was isolated from broth cultures of *M. tuberculosis* and from processed sputum samples as follows. The sample was sedimented by centrifugation at 13,000 × g for 10 min, and all but 250 µl of supernatant was removed. The pellet was resuspended in 750 µl of TRIzol-LS (GIBCO-BRL, Grand Island, N.Y.), the mixture was transferred to a 2-ml tube containing glass beads (Blue matrix tube; Bio 101, La Jolla, Calif.), and 300 µl of phenol reagent was added (phenol-chloroform, 5:1 [Amresco, Inc., Solon, Ohio]; 50 mM sodium acetate, pH 4.5; 0.5% sarcosyl). The sample was processed in an FP 120 cell disrupter (Savant Instruments, Holbrook, N.Y.) at a setting of 6.5 for 45 s and allowed to stand for 5 min. Tubes were centrifuged at 500 × g for 5 min to pellet the glass beads, and the entire aqueous portion was transferred to a 1.5-ml polypropylene tube. Chloroform (200 µl) was added and the mixture was vortexed for 1 min, after which it was centrifuged at 13,000 × g for 15 min at room temperature. The aqueous portion was removed with caution to leave 10 to 20% of the interface, which was later used together with the organic portion for extraction of DNA. The aqueous portion was reextracted with 400 µl of acid phenol-chloroform (5:1) (Amresco, Inc.) and then with chloroform-isoamyl alcohol (24:1) and precipitated with an equal volume of isopropanol in the presence of 20 µg of glycogen (Boehringer-Mannheim, Indianapolis, Ind.). The pellet was allowed to air dry for 10 min, resuspended in 100 µl of distilled H₂O, and stored at -20°C.

DNA was isolated by a modification of the TRIzol (GIBCO-BRL) protocol for precipitation of DNA from the organic and interface layers remaining after isolation of RNA. Briefly, 1/2 volume of ethanol (EtOH) was added to the organic and interface layers and placed on ice for 15 min. Samples were centrifuged at 8,000 × g at 4°C for 10 min. Tubes were decanted, and the pellets were washed twice for 30 min each time in 0.1 M sodium citrate-10% EtOH and once in 75% EtOH, with centrifugation as described above separating the washes. The pellet was resuspended in 100 µl of 8 mM NaOH for 1 h at room temperature, neutralized with 10 µl of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; free acid form), and stored at -20°C.

RT-PCR assays. (i) **RT.** The reverse primers for both 85B and 16S rRNA PCRs were used for RT. The RT mixture consisted of 10 µl of RNA solution, 2 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris [pH 8.3], 0.1% gelatin [Perkin-Elmer, Norwalk, Conn.]), 10 pmol of 85B primer, 10 pmol of 16S rRNA primer, 1 µl of 40 mM deoxynucleoside triphosphates (dNTPs) (10 mM each dATP, dCTP, dGTP, and dTTP) (Pharmacia, Alameda, Calif.), 0.5 µg of RNase-free bovine serum albumin (BSA) (Boehringer-Mannheim), 1 µl of Prime RNase inhibitor (5 Prime 3 Prime, Inc., Boulder, Colo.) and 2.5 U of avian myeloblastosis virus RT (Boehringer-Mannheim) in a 20-µl total volume. The reaction mixture was incubated at 42°C for 1 h and placed on ice. Three microliters of this mixture was used in a 50-µl PCR volume.

(ii) **85B (alpha antigen) PCR.** A PCR assay for *M. tuberculosis* 85B was developed with the forward primer 5'-TCAGGGGATGGGGCCCTAG-3' and the reverse primer 5'-GCTTGGGGATCTGCTGCGTA-3'. The primers amplify a 130-bp region from nucleotide 588 to 718 of the 85B sequence from *M. tuberculosis* Erdman (GenBank accession number X62398). This primer pair is specific for the gene encoding the 85B protein in members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) (data not shown). The PCR mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 0.2 µM each primer, 200 µM dNTPs (dATP, dCTP, dGTP, and dUTP), 2 mM MgCl₂, 5 µg of BSA (Pharmacia), 1 U of uracil DNA glycosylase (GIBCO-BRL), and 1 U of *Taq* polymerase (Fisherbrand, Plano, Tex.) in a 50-µl reaction volume. To each reaction mixture 5 × 10⁵ cpm of ³²P-labeled oligonucleotides was added. The uracil DNA glycosylase decontamination system was used to prevent amplicon carryover contamination and consisted of a 10-min incubation of the reaction mixture at 50°C followed by 10 min at 94°C before PCR cycling. PCR was performed in a GeneAmp PCR system 9600 (Perkin-Elmer) for 32 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min followed by a 20-min extension reaction at 72°C.

(iii) **16S rRNA PCR.** PCR for 16S rRNA was performed as described above with the forward primer 5'-GCTTTAGCGGTGTGGGATGAGCC-3' and the reverse primer 5'-GCGACGCTCACAGTTAAGCCGTG-3'. This primer pair amplifies a 416-bp region from nucleotide 171 to 586 as per the 16S sequence of *M. tuberculosis* (GenBank accession number X52917) (14). All members of the genus *Mycobacterium* tested, except for *M. genavense*, were detected with this primer pair.

The products of the above PCRs were electrophoresed in 8% nondenaturing Tris-borate-EDTA (TBE) gels, dried, and analyzed by autoradiography. In ad-

FIG. 1. Effect of sputum processing methods on RT-PCR. Sputum was processed by one of four methods described in Materials and Methods. Lanes correspond to specimen treated with PB and stored frozen in PB (PP lanes), treated with PB and stored frozen in GuSCN solution (PG lanes), treated with NaOH and stored frozen in PB (NP lanes), and treated with NaOH and stored frozen in GuSCN solution (NG lanes). Control lanes contain no template (C lanes). Products of PCRs were electrophoresed in 8% nondenaturing TBE gels, dried, and exposed to film for 4 h. Arrows indicate the specific product of the reaction. The lower bands are the result of primer-dimers and free ³²P-end-labeled oligonucleotide primers.

dition, for each of the above primer pairs, nonradiolabeled PCR products were electrophoresed in both agarose and nondenaturing polyacrylamide TBE gels with DNA molecular weight markers to determine the sizes of the products generated. All products were of the expected sizes.

(iv) **IS6110 PCR.** PCR for the insertion element IS6110 was performed as previously described (5, 13).

RESULTS

Effect of sputum processing protocols on mycobacterial RNA. To assess the effect of specimen preparation on the stability of RNA, sputum specimens were collected and processed by four different methods (as described in Materials and Methods). An RNA extraction method, which we developed specifically for sputum specimens, was used to obtain RNA from the processed aliquots. RT-PCR assays for 85B mRNA and 16S rRNA targets were used to detect the presence of RNA in these samples.

The results for all specimens were similar, and an example is shown in Fig. 1. 85B mRNA was readily detected in the samples not exposed to NaOH (lanes PP and PG). Signal intensity for 85B message was greatly diminished in samples processed with NaOH (lanes NP and NG). In contrast, the signal for 16S rRNA was largely unaffected by the method of sputum treatment before RNA extraction. This pattern of diminished 85B signal and unaffected 16S rRNA signal was found for 16 of 19 specimens tested in this study. In three instances, the signal for 85B mRNA appeared to be unaffected by NaOH treatment of sputum when the specimen was subsequently stored in GuSCN solution (data not shown). This could be due to a stabilizing effect of GuSCN solution on RNA. However, since the effect was not consistent within the sample group and represented only a minor proportion of the samples tested, it was not considered a useful addition to the protocol.

Stability of RNA in sputum specimens over time. We next wanted to determine how rapidly a sample must be processed to maintain the integrity of the RNA. This has practical applications in the field, where it is not always possible to process specimens as soon as they are collected. To measure RNA stability, a specimen was collected, homogenized with NALC and glass beads, divided into either two or three aliquots, and held at 4°C for 24 to 72 h. This experiment was performed on samples from a total of three patients, and a representative result is shown in Fig. 2. The RT-PCR results were similar to those shown in Fig. 1, with the 85B mRNA target being detected in greater amounts in samples processed without the

FIG. 2. Effect of storage of sputum specimens before processing. 85B RT-PCRs were performed on aliquots of sputum that had been held at 4°C for 24, 48, and 72 h. Lanes are as in Fig. 1. Reactions were performed under the same conditions either with (+RT lanes) or without (–RT lanes) RT.

addition of NaOH. Furthermore, the intensity of the signal does not appear to be affected by additional storage time at 4°C. As with all control reactions, no signal was detected in the absence of RT (Fig. 2, –RT lanes), indicating that the signal was dependent on an RNA origin and not a result of DNA contamination.

Quantitation of relative amounts of mRNA and rRNA. To estimate the amount of RNA present for each of the target types (mRNA and rRNA), RT reactions were performed on dilutions of RNA. RNA isolated from sputum was diluted before the RT reaction to determine the endpoint at which the product could no longer be detected by amplification. Results for two specimens are shown in Fig. 3. The top panel shows an RT-PCR analysis of RNA isolated from the sputum from patient A, in which 85B signal was detected at a 1/250 dilution and 16S rRNA remained detectable at a 1/12,500 dilution (Fig. 3A). Patient B's sample had a reduced 85B signal (faint band at a 1/10 dilution). The 16S rRNA band was detectable at the greatest dilution; however, the signal loses much intensity after a 1/500 dilution (Fig. 3B).

Figure 3C shows the DNA controls for these reactions. For both 85B and 16S targets, 10, 100, and 1,000 genomic equivalents of *M. bovis* 410 (ATCC 19210) DNA were tested in the PCRs (i.e., 40, 400, and 4,000 fg of *M. bovis* DNA per reaction) (2).

Less product was detected for the 16S rRNA gene, since this assay demonstrates 10-fold less sensitivity than the 85B gene. For that reason, control reactions to test for contamination of RNA with DNA (without RT) were done by using the 85B mRNA assay in this example.

The different dilution patterns for samples from patients A and B suggest that different amounts of RNA are present in these two specimens. The results also indicate that a dilution of

FIG. 3. Quantitation of relative amounts of specific RNA by limiting dilution. 85B mRNA and 16S rRNA RT-PCRs were done with serial dilutions of RNA from two sputum specimens. The numbers above the lanes correspond to the inverse dilution of specimen RNA before the RT reaction. Lanes –RT, reactions performed without RT. Control reactions contained 10, 100, and 1,000 genomes of *M. bovis* 410 DNA. All gels were exposed to film for 4 h.

the specimen RNA before the RT reaction is likely needed to appropriately estimate the amount of specific target present in a sample. The lowest dilutions of both 85B mRNA and 16S rRNA appear to have similar intensities of bands for a given probe, suggesting that the RT reaction is saturated (Fig. 3A, 85B lanes 10 and 50 and 16S lanes 10, 100, and 500).

Parallel isolation of DNA from specimens used for RNA extraction. For some studies it would be beneficial to isolate DNA, in addition to RNA, from sputum specimens. For example, in the experiment described above a quantitation of DNA could be used to standardize the number of organisms present in a particular sample. Since TRIzol was a component of the RNA isolation system, it seemed logical to isolate DNA from the same samples. This was readily accomplished by precipitation of DNA from the interface and organic layers with ethanol. The results of IS6110 PCR assays for DNA isolated from two patient specimens are shown in Fig. 4. DNA was easily detectable under all conditions of specimen treatment. The amount of DNA detected was comparable to that obtained by using standard DNA isolation protocols (5).

DISCUSSION

Although several amplification protocols for the rapid detection of *M. tuberculosis* exist (1, 4–6, 8, 9, 21), they detect

FIG. 4. IS6110 PCRs with DNA isolated from two different specimens. Lanes are as in Fig. 1.

DNA and therefore do not distinguish between living and dead bacilli. An RNA-based test is likely to reflect only nucleic acid from living organisms since the turnover rate of RNA, particularly mRNA, is high in prokaryotic cells, with an estimated half-life of 3 min (19). Additionally, the analysis of gene expression in patient specimens would be facilitated by RT-PCR since the numbers of organisms present is usually inadequate for conventional methods.

Sputum is the most common specimen collected for confirming the diagnosis of tuberculosis. Here we describe a method for isolation and analysis of RNA from sputum samples. We found that the traditional method of decontamination and digestion of sputum with NALC and NaOH results in a dramatic decrease in the amount of 85B mRNA obtained. It is probable that the results obtained for 85B mRNA could be extrapolated to other mRNAs. The effect on 85B mRNA was most likely due to damage or death of the cells during treatment with NaOH rather than simple loss of organisms during processing. Processing steps were the same for both nondecontaminated and NaOH-decontaminated aliquots, thus subjecting them to the same potential loss of organisms during centrifugation. In addition, Yajko et al. (23) demonstrated a 10 to 20% loss in colony counts during decontamination procedures but no corresponding loss in the amount of amplifiable nucleic acid present, suggesting the bacilli were still present but nonviable. Finally, the consistent level of 16S rRNA obtained, regardless of the processing method used, supports the idea that observed differences in RT-PCR for mRNA were due to reagents used for processing samples and not loss of organisms.

Maintenance of cell viability is also an important factor in developing methods for isolation of mRNA. Procedures which reduce cell viability are likely to decrease the amount of message detected. We have found that the best method for processing sputum seems to involve minimum specimen handling. Specimens should be homogenized in the presence of NALC and glass beads and stored at -70°C until RNA extraction can be performed. Although freezing can be detrimental to cell viability, it does not necessarily damage RNA. For cultured *M. tuberculosis* cells from which RNA was prepared before or after cells were frozen at -70°C , no difference in the yield or integrity of either 85B mRNA or 16S rRNA as measured by RT-PCR, dot blot analysis, and gel electrophoresis was detected (data not shown). However, specimens should be processed in a timely manner. Although RNA remains after 3 days of storage at 4°C , the concentration in two of three sample pairs was reduced by approximately 5- to 10-fold by day 3, as determined by endpoint dilutions of the RNA before and after storage (data not shown). It appears that 24 to 48 h at 4°C may be acceptable, allowing some flexibility in the timing of specimen processing.

Dilutions of RNA isolated from patient specimens were also

made prior to the RT reaction in order to estimate the amount of specific target present in different RT-PCR assays. In all cases, a signal for 16S rRNA was easily visible at a higher sample dilution than that for 85B mRNA, usually by approximately 100-fold. This is in agreement with rRNA being found in a 100-fold-greater abundance than mRNA in prokaryotes (11). The greater stability of 16S rRNA could also play a role in the relative levels of mRNA and rRNA detected.

RNA could be diluted more than 10,000-fold with persistence of the signal for 16S rRNA. If quantitative culture information is available, these dilutions can be used to approximate the amount of 16S rRNA present. For example, the specimen in Fig. 2A had a colony count of 10^4 CFU/ml. On the basis of calculation of the total volume of specimen and correction for the amount of specimen used in the PCR after extraction, a 1/12,500 dilution contains the equivalent of 1/140 CFU. Experiments are under way to modify these RT-PCR assays to include a quantitative format with an internal control to measure the relative differences in these gene products more precisely. However, even without exact quantitation, it is clear that the ribosomal transcript is more abundant than that for 85B mRNA; this suggests that this method may be very sensitive in the detection of mycobacterial transcripts.

We conclude that these methods will be useful for the isolation of mycobacterial RNA from patient sputum. This work demonstrates that both 85B mRNA and 16S rRNA are expressed in patients' sputum, and their amplification products can easily be detected. Furthermore, the RT-PCR assays developed here for 85B mRNA and 16S rRNA might be good candidates for detection of viable bacilli in sputum. With the resurgence of tuberculosis and in particular the increase in drug-resistant strains of *M. tuberculosis* (3, 16), a sensitive assay, such as RT-PCR, would be beneficial in the evaluation of different treatment regimens.

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