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Comparison of Amplified Qβ Replicase and PCR Assays for Detection of *Mycobacterium tuberculosis*

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Because of the long time required to isolate *Mycobacterium tuberculosis* in culture, there is an acute need for simple rapid methods for direct detection of *M. tuberculosis* from human sputum specimens. We have developed and characterized quantitative manual Qβ replicase and PCR assays for *M. tuberculosis*. The Qβ replicase assay was based on reversible target capture of *M. tuberculosis* 23S rRNA followed by amplification of a replicatable detector probe with Qβ replicase. For PCR assays, primers generating a 370-bp amplification product from the IS*6110* insertion element were used in combination with a control plasmid containing an internal deletion in the IS*6110* amplicon. Serial dilutions of *M. tuberculosis* were spiked into sputum and subjected to digestion and decontamination with *N*-acetyl-L-cysteine and NaOH. Assay conditions were optimized for hybridization and sample processing chemistries in order to maximize sample utilization. Following assay optimization, the sensitivities of the Qβ replicase and PCR assays of spiked sputum samples were 0.5 and 5.0 CFU per assay reaction, respectively. The effects of sputum matrix on each assay were examined by testing 20 patient sputum samples which had been cultured for *M. tuberculosis*. The culture-positive samples included smear-positive and smear-negative samples. The results of the Qβ replicase assay were not inhibited by sputum and were in 100% agreement with those of culture, including detection of 10 culture-positive specimens. However, using an internal control plasmid coamplified with each PCR as an indicator, we detected PCR inhibition in 9 of 20 samples tested. Decreasing the amount of sample assayed in the PCR 24-fold alleviated the inhibitory effects in all but two specimens, one of which was culture positive. The decreased sample utilization also resulted in a false-negative result with a third specimen which was culture positive for *M. tuberculosis*. Quantitative smear results and Qβ replicase assay estimates of the number of organisms present in these specimens were in close agreement. The Qβ replicase assay performed well in comparison with both culture and PCR and should offer a rapid means for detecting and controlling infection due to *M. tuberculosis*.

Disease caused by *Mycobacterium tuberculosis* has always been a serious world health problem. The World Health Organization estimated that in 1991 there were 8 million new cases of and 2.7 million deaths due to tuberculosis (12). The emergence of multi-drug-resistant strains and the increase in the number of new cases of tuberculosis have increased public health concerns in the United States.

Diagnosis of tuberculosis continues to depend on culture of *M. tuberculosis* from clinical specimens. Because of the long time required to isolate *M. tuberculosis* in culture, there is an acute need for simple rapid methods for direct detection of *M. tuberculosis* from patient specimens. The principal limitation of direct nonculture tests for *M. tuberculosis* has been a low level of sensitivity. Amplified nucleic acid hybridization assays such as PCR, the Gen-Probe amplified direct test, and the ligase chain reaction have shown promise in achieving the necessary sensitivity in in vitro systems (6, 7, 10, 23, 24). However, these assays have either shown poor sensitivity with smear-negative culture-positive specimens or have been hindered by the presence of inhibitors in patient sputum specimens, leading to a significant number of false-negative reactions (3, 7, 23, 26, 28).

Therefore, we have used an assay based on reversible target capture (RTC) of *M. tuberculosis* 23S rRNA followed by Qβ replicase amplification of a replicatable RNA detector molecule (19–22, 25) and compared it to a semiquantitative competitive PCR assay for the IS*6110* element of *M. tuberculosis*. The performances of the Qβ replicase and the PCR assays were compared in terms of the number of organisms detected, quantitative range, susceptibility to sample inhibition, and sample utilization. While both assays showed accurate performance in non-sputum-containing samples, sample inhibition significantly compromised the performance of the PCR assay. In contrast, the Qβ replicase assay was not affected by inhibitors in samples from patients and yielded consistent semiquantitative results.

MATERIALS AND METHODS

Clinical specimens and bacterial strains. Twenty human sputum sediment specimens previously characterized by culture and smear tests for the presence of *M. tuberculosis* were obtained from the Public Health Laboratories, Santa Ana, Calif. An avirulent *M. tuberculosis* strain (ATCC 25177) was obtained from the American Type Culture Collection (Rockville, Md.) and was used as a positive control in this study. Human sputum samples collected from patients with cystic fibrosis were obtained from Rainbow Babies and Childrens Hospital, Case Western Reserve University, Cleveland, Ohio.

Sample processing of sputum specimens. All spiked specimens of sputum were digested and decontaminated by a modification of the *N*-acetyl-L-cysteine–NaOH (Nalc-NaOH) method (8, 11, 16, 17). An equal volume of Nalc-NaOH solution was mixed with 5 to 10 ml of sputum, the mixture was vortexed for 30 s, and the samples were kept at room temperature for 15 min. The samples were diluted to a volume of 50 ml with 0.067 M phosphate buffer (pH 6.8), and the samples were centrifuged at 3,000 × *g* for 15 min at room temperature. The fluid was decanted, and the pellet was washed once with 5 ml of 0.067 M phosphate buffer. The samples were centrifuged as before, the fluid was decanted, and the pellet was suspended in 2.0 ml of 0.2% bovine serum albumin (BSA).

A 100-μl portion of sediment, equal to 5% of the original sample, was inoculated on a Löwenstein-Jensen slant for culture, and 50-μl portions, equal to 2.5% of the original sample, were placed on duplicate slides for smear analysis. For both the Qβ replicase and PCR assays, 300-μl portions of the digested

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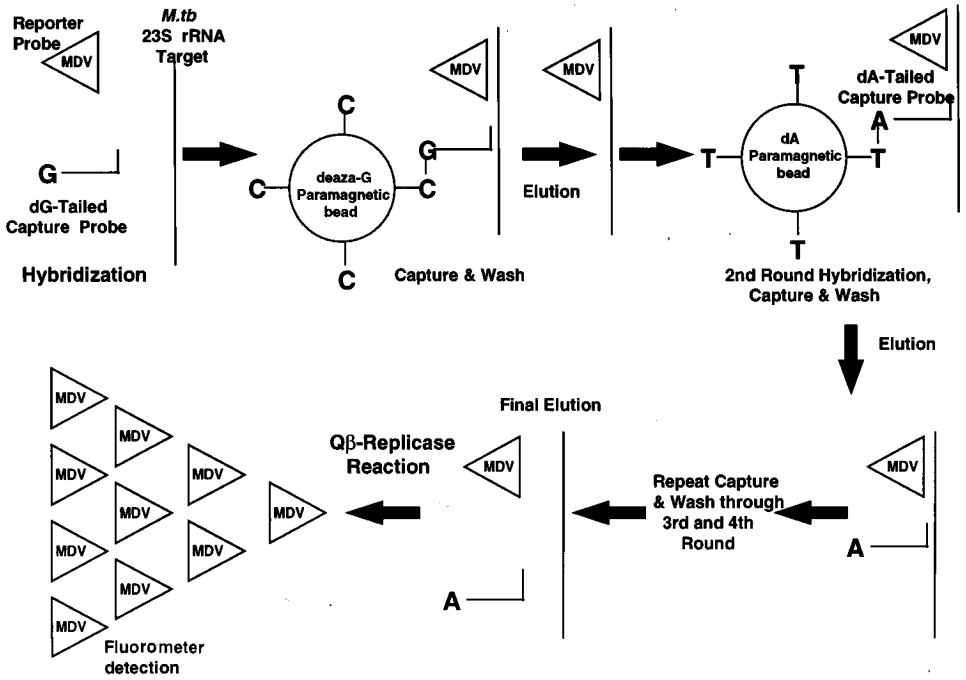

FIG. 1. Illustration of the Qβ replicase amplification RTC assay methodology. *M.tb*, *M. tuberculosis*; deaza-G, 7-deaza-deoxyguanosine.

sputum sediment were heated at 100°C for 15 min in a cryovial to inactivate mycobacteria. For the Qβ replicase assay, 250 μl of heat-inactivated sediment was added to a sample tube containing 400 μl of sample processing buffer (5.0 M guanidine thiocyanate, 400 mM Tris-HCl [pH 7.8], 100 mM EDTA, 1% Sarkosyl) and 0.5 g of zirconium oxide beads, and the mixture was shaken for 6 min at 5,000 rpm in a GENE-TRAK Sample Processor (27). Portions (250 μl) of the processed specimen, equivalent to 5.6% of the original sputum sample, were assayed by the Qβ replicase assay.

For the PCR assay, 125 μl of heat-inactivated sediment in 0.2% BSA was extracted with an equal volume of phenol before successive extractions with phenol-chloroform and chloroform. The samples were precipitated with 95% ethanol, dried, and suspended in 50 μl of H_2O. Either 48- or 2-μl portions, equivalent to 6 or 0.25% of the original sputum sample, respectively, were used for PCR assays.

Growth of *M. tuberculosis*. *M. tuberculosis* ATCC 25177 was grown on Middlebrook 7H10 medium (Remel, Lenexa, Kans.) and was incubated at 37°C in 10% CO_2 for 2 to 3 weeks. Isolated colonies were subcultured in small tissue culture flasks containing 10 ml of Middlebrook 7H9 medium supplemented with albumin, dextrose, and catalase (ADC) and were incubated at 37°C in 10% CO_2. After 3 to 4 weeks, 0.5 ml of this culture was added to 50 ml of Middlebrook 7H9 medium supplemented with ADC and was incubated at 37°C in 10% CO_2 for 3 to 4 weeks. The cultures were quantitated by a standard plate count method (11). Typically, the cultures contained between 10^7 and 10^8 CFU/ml.

Preparation and quantitation of *M. tuberculosis* nucleic acid. Mycobacterial RNA was purified as previously described (1, 2). *M. tuberculosis* rRNA was extracted by a combination of phenol extraction and mechanical lysis in a GENE-TRAK Sample Processor. RNA was purified by centrifugation in CsTFA (Pharmacia Inc., Piscataway, N.J.). The purified RNA was quantitated by spectrophotometry, and the concentration of RNA was confirmed by densitometric analysis of ethidium bromide-stained agarose gels (NIH Image; National Institutes of Health, Bethesda, Md.).

Oligonucleotides and nucleic acid probes. Two primers, F6 (5′-TGGCTAAC CCTGAACCGTGAG-3′) and R6 (5′-TTCAGGTCGAGTACGCCTTCT-3′), were used for PCR amplification of the *M. tuberculosis* IS*6110* insertion sequence. PCR amplification was confirmed by hybridization with one of two ^{32}P-labeled oligonucleotide probes. The Cp probe (5′-CCTGCGAGCGTAGGC GTCGGT-3′) detected both the 370-bp *M. tuberculosis* amplicon and a 247-bp amplicon generated by amplification of a control plasmid, IS*247*. The Rp probe (5′-GGCGGTTAGGTGCTGGTGGT-3′) detected only the 370-bp *M. tuberculosis* amplicon.

The RTC and Qβ replicase amplification procedures were carried out with two capture probes, a 7-deaza-deoxyguanosine-tailed capture probe referred to as capture probe A (5′-ACACGCCACTATTCACACGCGCGTAT-3′) and a deoxyadenosine-tailed capture probe referred to as capture probe B (5′-GAAC ACGCCACTATTCACACGCGCGTAT-3′). Both capture probes were complementary to the same target sequence located near the 5′ end of the 23S rRNA of each of the members of the *M. tuberculosis* complex. Tailing was performed as previously described (21, 22, 25).

The replicatable detector probe was a recombinant midivariant (MDV) RNA (19) containing a sequence complementary to the *M. tuberculosis* 23S rRNA target (5′-TGTGGGTCGCCCTATTCAGACTCGCTTTCGCTGCG-3′). A vector plasmid containing a cDNA copy of the MDV detector probe was linearized by digestion with *Sma*I, and the detector probe was prepared by transcription with T7 RNA polymerase (Megascript kit; Ambion Inc., Austin, Tex.). The MDV detector probe was designated MZ28.

Paramagnetic particles. Paramagnetic particles were obtained from Advanced Magnetics, Inc. (Cambridge, Mass.). The particles were derivatized with either oligo$(dT)_{14}$ or oligo$(dC)_{25}$ as previously described (9, 21, 22, 25).

RTC. The Qβ replicase assay was performed as outlined in Fig. 1. The assays were run in a 96-well format using sample racks and magnetic separators as previously described (19, 20, 25). A 250-μl portion of either purified 23S RNA, lysed organisms or a processed sputum sample was combined with 400 μl of sample processing buffer and shaken at 5,000 rpm for 6 min in a GENE-TRAK Sample Processor. Samples consisting of spiked RNA or lysed organisms were assayed in quadruplicate, while clinical specimens were assayed in duplicate. A 250-μl portion of each specimen was removed and analyzed for the presence of *M. tuberculosis* 23S rRNA in the RTC Qβ replicase-amplified assay. Capture probe A and the *M. tuberculosis*-specific MDV detector probe, MZ28, were diluted in probe dilution buffer (100 mM Tris-HCl [pH 7.8], 20 mM EDTA) to a final concentration of 600 ng/ml each. Hybridization reactions (final concentration of 2.5 M guanidine thiocyanate in a total volume of 300 μl) were prepared by combining 250 μl of the sample with 50 μl of the probe mixture, yielding a final concentration of 100 ng/ml for both the capture and detector probes. The probes were hybridized with the target nucleic acid for 30 min at 37°C. Following hybridization, 50 μl of a suspension of 0.03% oligo$(dC)_{25}$ paramagnetic particles in blocking buffer (100 mM Tris [pH 8.0], 20 mM EDTA, 4% BSA, 0.5% Sarkosyl, 0.01% antifoam) was added, and the reaction mixture was incubated further at 37°C for 4 min. The particles were collected on the sides of the reaction tubes by exposure to a magnet for 2 min. The particle-bound ternary capture probe A–target-detector probe complexes were washed with 200 μl of low-salt wash buffer containing 25 mM NaCl, 25 mM Tris-HCl (pH 8.1), 5 mM EDTA, 0.2% Sarkosyl, 0.05% BSA, and 0.05% bronopol. The samples were vortexed, the particles were collected as before, and the wash cycle was repeated three times. The binary target-detector probe complex was released from the particles by the addition of 100 μl of release buffer containing 3.5 M guanidine thiocyanate, 300 mM Tris-HCl (pH 7.8), 50 mM EDTA, 0.5% Sarkosyl, and 0.5% BSA. Under these conditions, capture probe A remains bound to the first set of particles. After a 5-min incubation in release buffer, the particles were again collected and the supernatant containing the binary target-detector probe complex was transferred to a fresh tube.

Fifty microliters of a 300-ng/ml solution of capture probe B in probe dilution buffer was added, and the tubes were incubated for 30 min at 37°C. Following

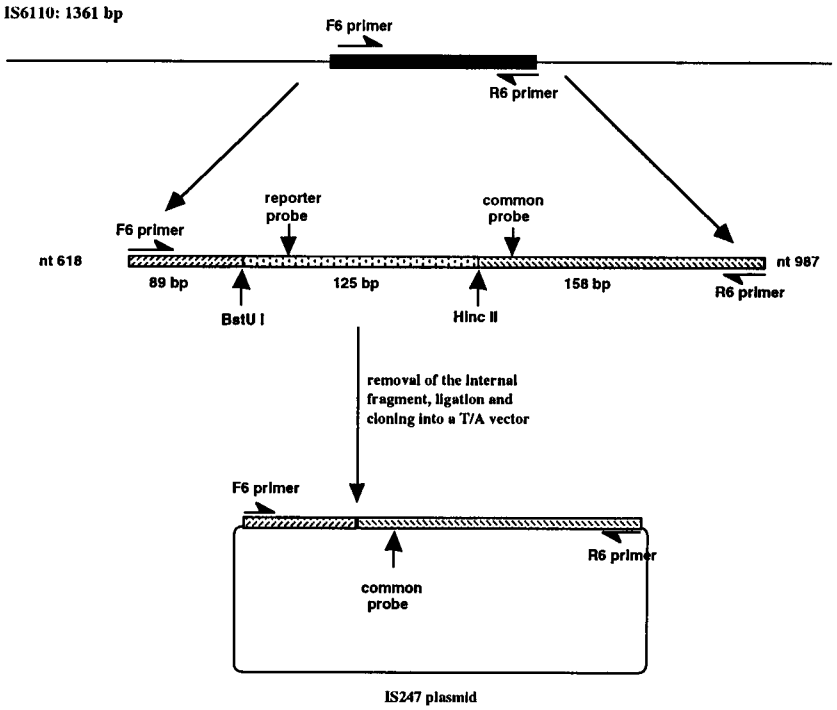

FIG. 2. Strategy for construction of the IS*247* PCR internal control plasmid. The PCR amplicon obtained by amplification of the IS*6110* sequence from *M. tuberculosis* (ATCC 25177) was digested with *Bst*UI and *Hinc*II, and the 158- and 89-bp external fragments were isolated, ligated, and cloned into a T/A vector. Amplification of the IS*247* plasmid with primers F6 and R6 yielded a 247-bp amplicon. Amplification of IS*6110* with F6 and R6 yielded a 370-bp amplicon.

hybridization of capture probe B to the binary target-detector probe complex, 150 μl of 0.03% oligo$(dT)_{14}$ particles in blocking buffer were added and the ternary capture probe B–target-detector probe complexes were captured on the particles for 4 min at 37°C. The particles were separated as before and were washed three times with 200 μl of high-salt wash buffer (300 mM NaCl, 100 mM Tris-HCl [pH 8.1], 0.5% Sarkosyl, 0.5% BSA, 0.05% bronopol). The ternary complexes were eluted from the particles by the addition of 100 μl of low-salt wash buffer. The particles were collected as before, and the supernatant containing the ternary complexes was transferred to a fresh tube.

A 100-μl portion of a fresh 0.03% suspension of oligo$(dT)_{14}$ particles and 200 μl of dilution buffer (100 mM Tris-HCl [pH 7.8], 20 mM EDTA, 8 M GuHCl) were added, and the ternary complexes were captured and washed as described above for the second round. The elution and capture process was repeated through a fourth round, and the bead-bound complexes were washed with buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 300 mM KCl, 0.5% Nonidet P-40, and 0.5% succinylated BSA. Following completion of the washing steps, the target-detector probe complex was released by the addition of 200 μl of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% Nonidet P-40 and the eluted material was transferred to a fresh tube after 4 min at 37°C. Typically, the overall efficiency of the RTC reaction was between 1 and 10%.

Qβ replicase assay. A 100-μl portion of the released hybrid complexes from the RTC procedure was combined with an equal volume of Qβ replicase buffer (50 mM Tris-HCl [pH 8.0]; 1 mM EDTA; 0.5% Nonidet P-40; 1.2 mM [each] GTP, ATP, CTP, and UTP; 2.0 μg of propidium iodide per ml; and approximately 110 μg of Qβ replicase per ml in 25% glycerol) in a closed tube. The reaction components were mixed in sealed reaction tubes which were placed in a custom-built recording fluorometer at 37°C (4). The reaction mixtures were illuminated at 510 nm, and the fluorescence was measured at 615 nm through the bottom of the tube by using a split fiber optic bundle. Fluorescence was monitored in all 96 reactions by simultaneously reading each tube at 40-s intervals for 45 min. Fluorescence increased in those reaction mixtures containing the replicatable detector probe as a result of the binding of propidium iodide by the RNA product of the amplification. The time interval required to produce sufficient RNA such that its fluorescence was detectable above the basal level was defined as the response time and corresponds to the presence of approximately 3×10^{11} molecules of the amplified detector probe (4). The response time is inversely proportional to the log of the number of detector molecules which initiate the amplification reaction (19, 20, 25). Thus, a short response time would indicate the presence of a large number of detector molecules and a correspondingly large number of target molecules in the initial sample. Positive and negative controls for each assay consisted of samples containing known numbers of either 23S rRNA molecules, lysed organisms, or TE (Tris-EDTA) buffer.

PCR assay. The IS*6110* element was chosen as the target for PCR assay (6). For PCR, sputum samples or control samples processed as described above were amplified in the presence of 1× amplification buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 2.5 mM $MgCl_2$, and 2.5 U of Amplitaq polymerase (Perkin-Elmer Cetus) in a total volume of 100 μl. Amplifications were performed in a thermocycler (Perkin-Elmer Cetus) and consisted of 40 cycles of denaturation at 94°C for 1 min, renaturation at 55°C for 45 s, and polymerization at 72°C for 45 s. All PCR assays were carried out under the recommended containment conditions (18) and were performed multiple times with each sample. The 370-bp amplicon generated with primers F6 and R6 was detected on ethidium bromide-stained agarose gels after electrophoresis and was confirmed by Southern blot hybridization using one of the radiolabeled oligonucleotide probes specific for the amplicons.

Construction of an internal control plasmid for PCR assays. An internal control plasmid for the PCR assay was constructed as shown in Fig. 2. The 370-bp amplicon derived from amplification of IS*6110* was isolated and digested with *Bst*UI and *Hinc*II, and the two end fragments (89 and 158 bp) containing the primer sites were purified, ligated, and cloned into a T/A vector (Invitrogen, San Diego, Calif.). A plasmid (designated IS*247*) containing an internally deleted 247-bp fragment of the IS*6110* amplicon was isolated and was used as an internal standard in all PCR assays.

Analysis of the effects of human clinical sputum specimens on assay performance. Samples to be analyzed by the Qβ replicase assay were prepared and tested as described above. Quantitated serially diluted 23S rRNA was amplified in each experiment as a control. Six replicates of each control serial dilution were assayed. Amplification of the serial dilution series was used to establish the lower limit of sensitivity. Clinical samples were assayed in duplicate.

For PCR analysis of clinical samples, the processed sputum specimens were combined with 10^2 molecules of the IS*247* plasmid and were subjected to 40 cycles of amplification. In order to minimize differences in PCR conditions, all reagents were added from a single master mixture. Samples and controls were also amplified and analyzed simultaneously by using a single thermocycler and agarose gel. The optical density of the 247-bp amplicon resulting from amplification of the IS*247* plasmid was determined by densitometry and was used to detect the presence or absence of PCR-inhibitory substances. For samples in which amplification occurred, 10 μl of PCR product was analyzed by agarose gel electrophoresis, stained with ethidium bromide, photographed, and quantitated by densitometric scanning (NIH Image Software; National Institutes of Health). The samples were analyzed in duplicate.

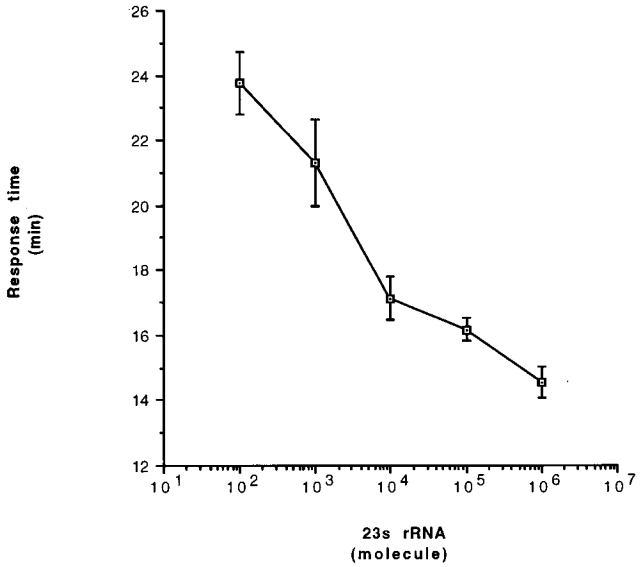

FIG. 3. Quantitative range of the Qβ replicase assay. Quantities of serially diluted *M. tuberculosis* 23S rRNA ranging from 10^6 to 10^2 molecules were examined by the Qβ replicase assay, and the response times were correlated with the number of molecules of rRNA used in the initial reactions. The error bars indicate standard deviations.

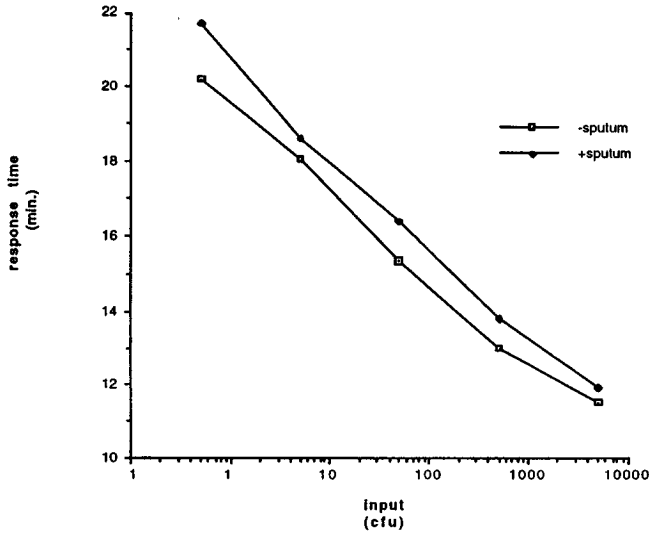

FIG. 4. Effects of digestion and decontamination by Nalc-NaOH on the recovery of *M. tuberculosis* 23S rRNA from spiked sputum samples. Various concentrations of organisms were combined with sputum and subjected to digestion and decontamination. Samples containing equal numbers of organisms in buffer were prepared as controls and were not digested and decontaminated. All samples were combined with sample processing buffer and treated in a GENE-TRAK Sample Processor. Processed samples were assessed for their content of *M. tuberculosis* 23S rRNA by Qβ replicase assay.

RESULTS

Characterization of the Qβ replicase assay for *M. tuberculosis*. Serial dilutions of known amounts of 23S rRNA as well as *M. tuberculosis* organisms were used to establish the characteristics of the Qβ replicase assay. Figure 3 illustrates the relationship between the number of molecules of 23S rRNA used in the assay to the response time. An inverse semilogarithmic relationship between target concentration and response time was maintained over the 10,000-fold range of target concentration tested. All samples containing 10^2 or more molecules of 23S rRNA produced positive results in the assay. Below this level, however, the detection rate was less than 100%.

The Qβ replicase assay was characterized for its performance in detecting *M. tuberculosis* in human sputum. *M. tuberculosis*-negative sputum collected from cystic fibrosis patients was spiked with known amounts of *M. tuberculosis* before digestion and decontamination by the Nalc-NaOH procedure (8, 11, 16, 17). Specimens containing the same levels of *M. tuberculosis* prepared with 0.2% BSA but not subjected to digestion and decontamination were used as controls. When *M. tuberculosis* organisms were extracted directly without digestion and decontamination, the assay had a lower limit of detection of approximately 0.5 CFU (Fig. 4). The response times generated by the samples spiked into sputum and subjected to digestion and decontamination were slightly slower than those for undigested samples in 0.2% BSA (Fig. 4). However, the overall assay sensitivity was unaffected. When *M. tuberculosis* organisms were spiked directly into sputum sediments, there was no difference between the response times of the spiked sputum specimens and those of organisms in buffer, indicating that this effect was not due to inhibition of the Qβ replicase reaction (data not shown). Instead, the delay in response times reflects the loss of organisms which occurs during the digestion and decontamination procedure. At initial inputs of 10^4, 10^3, 10^2, and 10 CFU per 5 ml of sputum, the efficiencies of nucleic acid recovery following digestion and decontamination were 43.58, 64.44, 45.64, and 48.32%, respectively. The average recovery efficiency of intact target nucleic acid after Nalc-NaOH digestion and decontamination was 50.5%.

Using the Qβ replicase assay, we were able to estimate that the number of 23S rRNA molecules per CFU was approximately 6.5×10^3. This number may show some culture-to-culture variation.

Optimization and characterization of a competitive PCR assay for *M. tuberculosis*. Competitive PCR was optimized initially by determining the concentration of the IS*247* plasmid which resulted in the greatest molecular sensitivity. We determined that the use of 50 to 100 molecules of the IS*247* plasmid per assay yielded optimal sensitivity. As shown in Fig. 5, when 100 molecules of IS*247* were coamplified with decreasing amounts of a plasmid containing the IS*6110* sequence in PCR buffer, one of four samples at the single-molecule level were amplified whereas all of the samples at or above the five-molecule level were amplified. Thus, the assay exhibited Poisson failure at the single-molecule level when PCR buffer was used. Hybridization of Southern blots with either radiolabeled Rp (Fig. 5B) or Cp (Fig. 5C) probe confirmed the sensitivity. Since there are 10 to 20 copies of the IS*6110* gene per CFU of *M. tuberculosis* (6), this result represents less than 1 CFU of *M. tuberculosis*.

On the basis of densitometric analysis of the target amplicon after gel electrophoresis, the PCR assay was linear over a range of 1 to 500 molecules of IS*6110* plasmid DNA under the conditions used in the study (Fig. 6).

Human sputum was examined for inhibitory effects on PCR. Pooled *M. tuberculosis*-negative sputum obtained from patients with cystic fibrosis was digested, decontaminated, and processed by a series of phenol extractions as described above. After ethanol precipitation, samples of the processed sputum were spiked with 100 molecules of the IS*247* control plasmid and serial dilutions of a plasmid containing the complete IS*6110* sequence prior to amplification by PCR. As shown in Fig. 7, the assay sensitivity was significantly decreased in the

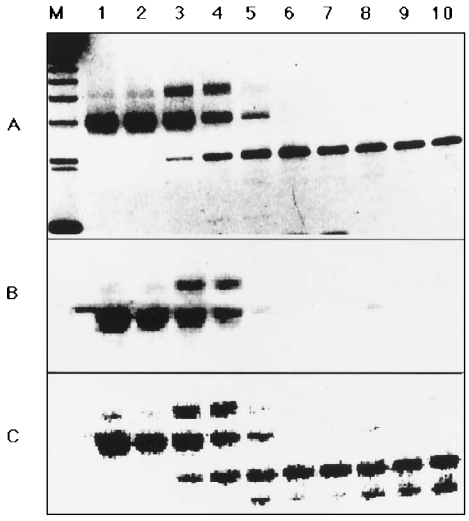

FIG. 5. Coamplification of IS*6110* DNA with IS*247* DNA. (A) Ethidium bromide-stained gel containing the reaction products of coamplification of 10^2 molecules of IS*247* DNA with various amounts of IS*6110* DNA. Lane 1, 5×10^4 molecules; lane 2, 5×10^3 molecules; lane 3, 5×10^2 molecules; lane 4, 5×10^1 molecules; lane 5, 5 molecules; lanes 6 to 9, 0.5 molecules; lane 10, IS*247* only. (B) Southern blot of the gel in panel A hybridized with the Rp probe. (C) Southern blot of the gel in panel A hybridized with the Cp probe. Lane M, marker DNA.

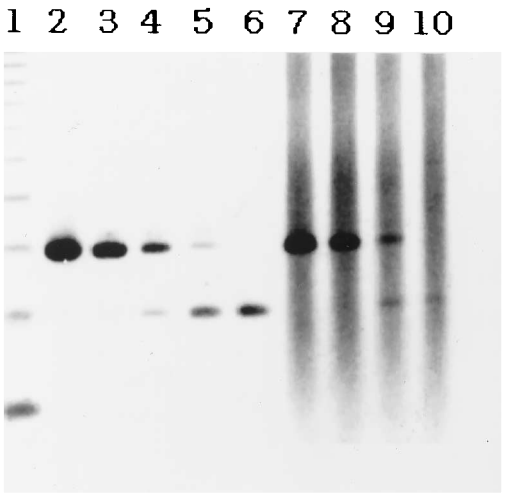

FIG. 7. Effect of sputum on PCR amplification of DNA added to sputum pellets. Various concentrations of DNA were combined with either TE buffer or sputum pellets which had been subjected to Nalc-NaOH digestion and decontamination followed by suspension in TE buffer alone. Samples were combined with 50 molecules of IS*247* DNA before being amplified. Lanes 2 to 5 show amplifications in the absence of processed sputum. Lanes 7 to 10 show amplifications in the presence of processed sputum. Lane 1, 123-bp ladder; lanes 2 and 7, 5×10^3 molecules of IS*6110* DNA; lanes 3 and 8, 5×10^2 molecules of IS*6110* DNA; lanes 4 and 9, 5×10^1 molecules of IS*6110* DNA; lanes 5 and 10, 5 molecules of IS*6110* DNA; lane 6: IS*247* DNA only.

presence of sputum, indicating the presence of PCR inhibitors. The sensitivity limit of the PCR assay in sputum was 500 molecules of 23S rRNA, equivalent to approximately 2 to 5 CFU of *M. tuberculosis.*

We determined the combined effects of PCR inhibition and losses in recovery of DNA targets due to the digestion and decontamination procedure. Mock sputum samples were prepared by spiking various quantities of *M. tuberculosis* organisms into sputum, digesting and decontaminating the samples, extracting the material from the sputum pellets with phenol as described above, and assessing recovery by PCR. For comparison, samples containing the same number of organisms in buffer were prepared and were subjected to phenol extraction without digestion and decontamination. As shown in Fig. 8, the combined effect of sample loss due to digestion and decontamination as well as PCR inhibition was a significant loss of the signal. The loss of the signal was due to the combined effects of digestion, decontamination, and PCR inhibition. However, judging from the average recovery of 50.5% of the viable organisms after digestion and decontamination (as discussed above), the majority of the loss in signal is attributable to matrix effects on the PCR amplification efficiency. The lower limit of detection of the PCR assay for organisms in the absence of sputum was 0.5 CFU. However, the lower limit of detection of the PCR assay on organisms spiked into sputum and processed by digestion-decontamination and phenol extraction was 5 CFU of *M. tuberculosis.*

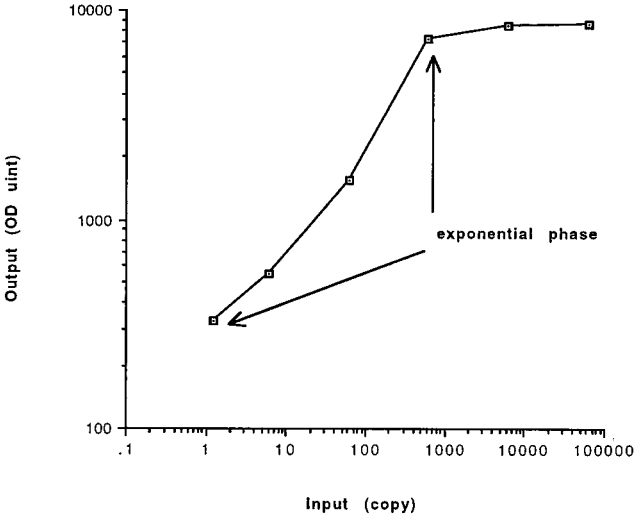

FIG. 6. Quantitative range of the PCR assay. The products of the PCR assay were separated on an agarose gel, stained with ethidium bromide, and analyzed by densitometry. The optical densities (OD) of the gel bands from the IS*6110* amplicons were plotted against the number of molecules of IS*6110* DNA assayed.

Analysis of the effects of human clinical sputum samples on assay performance. In order to examine the effects of individual patient sputum samples on each assay, 20 patient sputum samples previously characterized by culture and stained smears were tested. For Qβ replicase assay analysis of clinical samples, *M. tuberculosis* culture-positive or -negative sputum specimens were processed as described above and assayed. For PCR analysis, a 2-μl sample and a 48-μl sample of each of the processed sputum specimens were combined with 100 molecules of the IS*247* plasmid and subjected to 40 cycles of amplification.

The results of the analysis are shown in Table 1. The Qβ replicase assay was in 100% agreement with the culture results. Based on an estimate of 6.5×10^3 molecules of 23S rRNA per CFU derived from Qβ replicase assay quantitative calculations, the number of *M. tuberculosis* CFU ranged from 2 to 9.8 $\times 10^3$ per assay point.

In contrast, significant inhibition was observed when 48-μl samples of sputum were examined by PCR assay. Complete inhibition was observed in 9 (45%) of 20 samples. Reducing

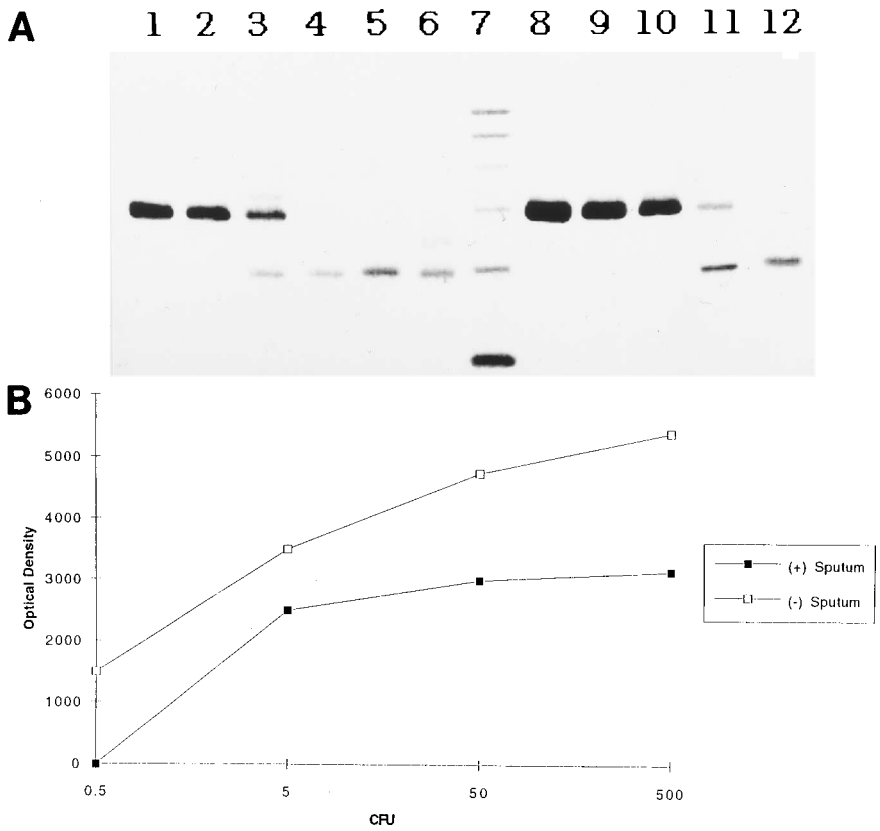

FIG. 8. Sensitivity of PCR amplification of *M. tuberculosis* in sputum following digestion and decontamination. Various numbers of *M. tuberculosis* CFU were added to *M. tuberculosis*-negative human sputum samples that had been digested and decontaminated with Nalc-NaOH, and the nucleic acids were released by the sample-processing procedure described in Materials and Methods. A set of samples containing identical numbers of bacteria were added to buffer and were lysed without digestion and decontamination. The released nucleic acids were detected by coamplification with 50 molecules of pIS247 followed by gel electrophoresis. The lower-level signals in specimens recovered from sputum reflect the combined losses due to PCR inhibition and the digestion-decontamination procedure. (A) Lanes 1 to 5 show amplifications of samples recovered from sputum. Lanes 8 to 12 show amplifications of samples in buffer lysed and amplified without digestion-decontamination. Lanes 1 and 8, 500 CFU; lanes 2 and 9, 50 CFU; lanes 3 and 10, 5 CFU; lanes 4 and 11, 0.5 CFU; lanes 5 and 12, 0.05 CFU; lane 6, pIS247 only; lane 7, marker DNA. (B) Graph of a densitometric scan of the gel in panel A, illustrating the relationship between the amount of amplicon produced (optical density) and the number of CFU of *M. tuberculosis* in the sample.

the amount of sample to 2 μl improved the result; however, two specimens, numbers 8 and 16, could not be amplified even after extensive extraction with phenol and chloroform. One of these specimens was positive for *M. tuberculosis* by culture, smear, and Qβ replicase assay. In addition, when 2 μl of sample was used for amplification, specimen 7, positive by culture and Qβ replicase assay, was found negative by PCR even though amplification was successful. The results of the PCR assay using 2 μl of sample for assessment were in agreement with the culture results for 15 (83%) of 18 samples.

DISCUSSION

We have examined the performance of two amplified assay formats for detection of *M. tuberculosis* nucleic acids. The Qβ replicase and PCR assays of sputum had practical lower limits of detection of 0.5 and 5 CFU of bacteria per assay point, respectively. The PCR assay was quantitative over a 500-fold range of target concentrations. We demonstrated that the Qβ replicase assay was quantitative over at least a 10^4-fold concentration of target. Others have demonstrated a 10^9-fold range of quantitation for Qβ replicase assays (19). The broad quantitative range of the Qβ replicase assay should be useful in both semiquantitative evaluation of clinical samples for the presence of *M. tuberculosis* and monitoring of drug therapy.

One of the concerns about amplified nucleic acid assays for the detection of *M. tuberculosis* in sputum is the effect of inhibitors on assay performance. We have shown here that the Qβ replicase assay is unaffected by inhibitors found in sputum which compromised PCR amplification. When *M. tuberculosis* spiked into sputum was tested in the Qβ replicase assay, the observed decrease in signal was due to the loss of organisms during the digestion and decontamination procedure. We used the Nalc-NaOH procedure which we and others have shown can routinely recover 30 to 80% of viable mycobacteria from sputum samples (14, 15, 17).

The PCR assay, in contrast, showed significant inhibition by sputum. The presence of inhibitors in pooled sputum decreased overall assay sensitivity by greater than 10-fold. Inhibition was still evident even after multiple phenol extractions. Monitoring of PCR inhibition is usually based on the presence or absence of an amplicon derived from the amplification of an internal control (6, 7, 23). This procedure is generally done by an all-or-none approach in which it is assumed that PCR inhibition will result in complete abolition of the internal control product. However, as we have shown here, this may be an erroneous assumption.

The deleterious effects of sputum on the PCR assay were even more striking when sputum samples from individual patients were examined. The results reported here were not in-

TABLE 1. Analysis of the effects of specimens from a patient on Qβ replicase and PCR assay performance[a]

Sample no.	Result of:			Estimated CFU/100 μl[b]	PCR result	
	Culture	Smear[c]	Qβ assay[d]		48-μl sample	2-μl sample
1	+	1+	5.5×10^5	8.4×10^1	+	+
2	−	-[e]	-	-	I[f]	−
3	+	1+	5.9×10^6	9.1×10^2	+	+
4	+	2+	9.7×10^6	1.5×10^3	+	ND[g]
5	−	-	-	-	I	−
6	+	1+	2.0×10^4	3.1×10^0	+	ND
7	+	-	1.5×10^4	2.3×10^0	I	−
8	−	-	-	-	I	I
9	+	1+	1.5×10^6	2.3×10^2	+	+
10	−	-	-	-	I	−
11	+	1+	3.9×10^5	6.0×10^1	+	+
12	−	-	-	-	I	−
13	−	-	-	-	I	−
14	+	3+	6.4×10^7	9.8×10^3	+	+
15	−	-	-	-	−	−
16	+	1+	7.9×10^5	1.2×10^2	I	I
17	−	-	-	-	I	−
18	−	-	-	-	I	−
19	−	-	-	-	I	−
20	+	2+	9.9×10^6	1.5×10^3	I	+

[a] The culture, smear, and Qβ replicase assays and the 48- and 2-μl PCR samples utilized 5, 2.5, and 5.6% and 6 and 0.25% of the original sputum sample, respectively.

[b] Estimations of the number of CFU per 100 μl of sample were based on a calculated 6.5×10^3 23S rRNA molecules per CFU.

[c] Smear results were obtained by fluorochrome staining and are denoted as follows: 1+, 1 to 9 acid-fast bacilli per 10 oil immersion fields; 2+, 1 to 9 acid-fast bacilli per oil immersion field; 3+, 10 to 90 acid-fast bacilli per oil immersion field.

[d] The number of 23S rRNA molecules detected per 100 μl of the original sample.

[e] -, negative result.

[f] I, inhibited, yielding an uninterpretable result.

[g] ND, not done.

tended to assess clinical assay performance in terms of sensitivity or specificity, since a statistically significant number of specimens were not tested. Rather, the assay performance with clinical specimens was intended only to examine the variable effects that individual samples might have on the assay efficiencies and sample utilization. The results of the Qβ replicase assay were in 100% agreement with the culture results for 20 clinical specimens previously characterized for the presence of *M. tuberculosis* and showed no evidence of inhibition by the various samples. The Qβ replicase assay also produced quantitative results in agreement with the smear results and detected the single smear-negative culture-positive sample tested.

In contrast to the Qβ replicase assay, the PCR assay showed variable performance with the clinical samples. For a volume of sample (48 μl) equivalent to the volume of the sample utilized by the Qβ replicase assay, 9 of the 20 samples showed significant PCR inhibition. When the volume of sample amplified was reduced 24-fold to a volume of sample equivalent to that used by most investigators (6, 7, 23), seven of the nine PCR-inhibited specimens showed amplification of the internal control plasmid IS*247*; however, data for two specimens were still uninterpretable because of inhibition. Specimen 16, which was positive by smear, culture, and Qβ replicase assay but yielded an uninterpretable PCR result, contained approximately 1,000 CFU/ml of sputum, well within the detectable limits of the PCR assay. While other researchers have reported similar inhibitory effects at somewhat variable levels, all agree that inhibition is a significant problem (5, 7, 23).

A second limitation to new amplified nucleic acid assay methods may be the occurrence of false-negative results due to sampling errors with specimens containing few organisms. For *M. tuberculosis* detection, this problem will be most acute in detecting smear-negative culture-positive specimens. The false-negative PCR result for sample 7 (Table 1) is indicative of what is probably an error due to inadequate sample utilization. On the basis of the Qβ replicase assay quantitation, there were only 2 CFU of *M. tuberculosis* in 48 μl of sputum sediment. Therefore, the number of bacilli per 2-μl sample assayed would be below the detection limits of the PCR assay. The ability to use a larger sample will be clearly advantageous in detecting the presence of *M. tuberculosis* in sputum.

These data indicate that the absolute presence or absence of a control target amplicon may be an insufficient criterion to judge the efficacy of PCR of a clinical sample. As shown here (Fig. 8), PCR amplification can occur at decreased efficiency in the presence of significant inhibition, resulting in a less-sensitive assay. The predicted effect in terms of clinical analysis would be an increase in false-negative results for samples containing both small numbers of organisms and PCR inhibitors. For *M. tuberculosis* detection, the problem would be most acute when analyzing smear-negative culture-positive samples. Detection of a decrease in assay sensitivity will require monitoring of actual assay efficiency as well as the losses due to digestion and decontamination of the sample. The *Mycobacterium smegmatis* strain described by Kolk et al. (13) offers promise in this regard.

The Qβ replicase assay for *M. tuberculosis* in sputum samples is both sensitive and semiquantitative. The assay is not subject to sample-inhibitory effects, allowing greater amounts of sample to be assayed. The Qβ replicase assay should therefore be especially useful in detecting smear-negative, *M. tuberculosis* culture-positive specimens.

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