JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1995, p. 322–328 Vol. 33, No. 2
0095-1137/95/$04.00+0

Detection of *Mycobacterium tuberculosis* Directly from Spiked Human Sputum by Q-Beta Replicase-Amplified Assay

JYOTSNA S. SHAH,* JING LIU, DEBRA BUXTON, BEN STONE, RAY NIETUPSKI, D. MICHAEL OLIVE, WALTER KING, AND JEFFERY D. KLINGER

GENE-TRAK, Framingham, Massachusetts 01701

Received 14 July 1994/Returned for modification 3 October 1994/Accepted 27 October 1994

We report on a rapid, sensitive, Q-Beta replicase-amplified nucleic acid hybridization assay for the detection of *Mycobacterium tuberculosis* directly from spiked human sputum. Specimens were processed by either an *N*-acetyl-L-cysteine–NaOH or a 2% NaOH digestion-decontamination method and then washed to neutralize the pH of the cell pellet. The washed sputum pellets were heated at 100°C to inactivate the *M. tuberculosis* organisms. The heat-inactivated samples were mechanically lysed at 5,000 rpm for 6 min in the GENE-TRAK Sample Processing Instrument in the presence of zirconium oxide beads and a buffer containing guanidine thiocyanate. The released nucleic acid was subjected to the GENE-TRAK Q-Beta replicase-amplified, dual-capture assay. The assay sensitivity was 10^3 purified rRNA targets or 1 CFU of *M. tuberculosis* spiked into *M. tuberculosis*-negative human sputum. There was a low level of noise because of the limitations of performing a signal amplification assay in an open system. High levels of other mycobacterial rRNA (approximately 10^7 organisms), including rRNAs of *Mycobacterium avium* and *Mycobacterium gordonae*, did not interfere with the sensitivity of the assay.

Approximately 1.7 billion people, or one-third of the world's population, are infected with *Mycobacterium tuberculosis* (27). In 1990, the estimated number of tuberculosis cases was 8 million, with 2.9 million deaths (27). Microscopic examination of stained smears of sputum is the most rapid method for detecting the bacilli. However, this method is neither sensitive nor specific. Current methods for the isolation and identification of *Mycobacterium* species include culture and biochemical and DNA probe procedures (30). Standard culture is a sensitive method for the detection of mycobacteria, but it takes anywhere from 3 to 8 weeks. It takes 7 to 20 days to achieve growth with the BACTEC system (Johnson Laboratories, BBL Microbiological Systems, Towson, Md.) or the biphasic Septi-Chek AFB system (Becton Dickinson Microbiological Systems, Cockeyville, Md.) and an additional 2 to 4 weeks for final identification by biochemical analysis. DNA-RNA hybridization assays specific for the rRNA of *M. tuberculosis* complex have been developed for the identification of mycobacteria from cultures (9, 20). These probe assays in combination with the BACTEC system have further reduced the time required for identification. The probe assays, however, lack the sensitivity required for the direct detection of *M. tuberculosis* from specimens. Therefore, there is a critical need for more rapid, specific, and sensitive methods for the detection and identification of mycobacteria.

Several investigators have reported the use of PCR as a non-culture-based method of detecting mycobacteria directly from respiratory specimens (2, 4–8, 10, 12, 19, 24, 25, 28). More recently, an rRNA nucleic acid-based amplification assay has been described for the direct detection and identification of *M. tuberculosis* complex bacteria from clinical samples (15).

We have previously described a highly specific and sensitive Q-Beta replicase-amplified nucleic acid hybridization assay for the detection of *Chlamydia trachomatis* directly from clinical specimens (23). We describe here a similar assay for the detection of *M. tuberculosis* complex organisms directly from spiked sputum. A simple sample processing protocol is used to release nucleic acid from cells concentrated from sputum by digestion and decontamination either by treatment with *N*-acetyl-L-cyteine–sodium hydroxide (Nalc-NaOH) or 2% NaOH. *M. tuberculosis* rRNA is specifically detected by an assay which includes elements of sandwich hybridization (21), dual capture, reversible target capture (23), and Q-Beta replicase amplification of the signal (3). The false-positive rates vary from 0 to 20% because the assay is performed in an open system (23). The assay fits into standard clinical laboratory work flow algorithms for preparing respiratory specimens. The sensitivity of the assay is 10^3 23S rRNA molecules, which corresponds to approximately 1 CFU. Like direct nucleic acid-based tests such as PCR and the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test, final results can be obtained in a single day. The Q-Beta replicase-amplified assay described here offers two major advantages over other DNA tests such as PCR: (i) the assay is not subject to sample inhibition and (ii) up to 10% of the sample can be assayed.

MATERIALS AND METHODS

Oligo$(dT)_{14}$ or oligo$(dC)_{25}$ magnetic particles. Carboxyl-terminated ferromagnetic particles (Advanced Magnetics, Inc., Cambridge, Mass.) were derivatized with either oligo(dT) or oligo(dC) by a modification of the method described by Morrissey et al. (18). 5′-Amino-terminated oligo$(dT)_{14}$ or oligo$(dC)_{25}$ was synthesized by standard phosphoramidite chemistry. Oligo$(dT)_{14}$ or oligo$(dC)_{25}$ was linked to carboxyl-terminated ferromagnetic particles as described below. Magnetic particles (final concentration, 4%), oligonucleotide (1 optical density unit per mg of magnetic particles), 0.1 M imidazole solution (pH 5.95), and 2% EDAC (a water-soluble carbodiimide [Sigma, St. Louis, Mo.]) were mixed for 65 ± 5 h at 2 to 8°C on a platform shaker. After coupling, the particles were washed once at room temperature with 0.1 M imidazole; this was followed by two washes with 0.1 M KCO_3. The beads were then washed twice with 0.1 M KCO_3 for 1 h each at 65°C; this was followed by two washes at room temperature with Milli Q-purified water. After the final wash, the particles were resuspended in Milli Q-purified water to a final concentration of 1% solids. Bead blocking buffer (4% bovine serum albumin [BSA], 20 mM EDTA, 0.5% Sarkosyl, 0.1 M Tris [pH 8.0], and 0.01% antifoam) was added to the washed particles to achieve a final concentration of 0.25% (wt/vol) solids, and the mixture was incubated for 4 h at 65°C with occasional vigorous agitation. The magnetic particles were washed

* Corresponding author. Mailing address: GENE-TRAK, 31 New York Avenue, Framingham, MA 01701. Phone: (508) 935-1300. Fax: (508) 879-6462.

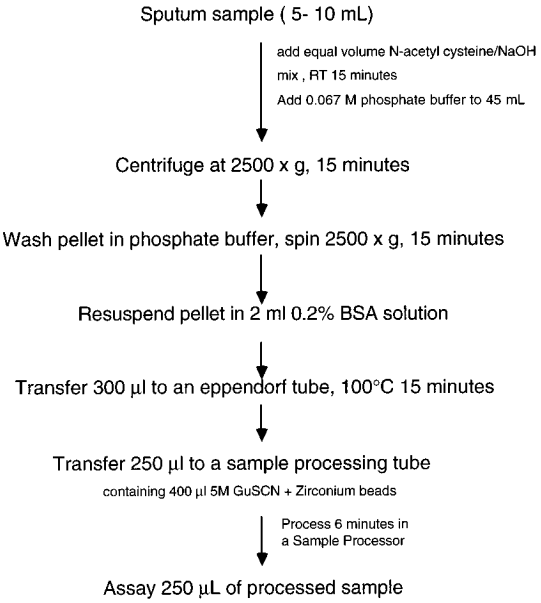

FIG. 1. Schematic diagram describing the sample processing methodology. RT, response time.

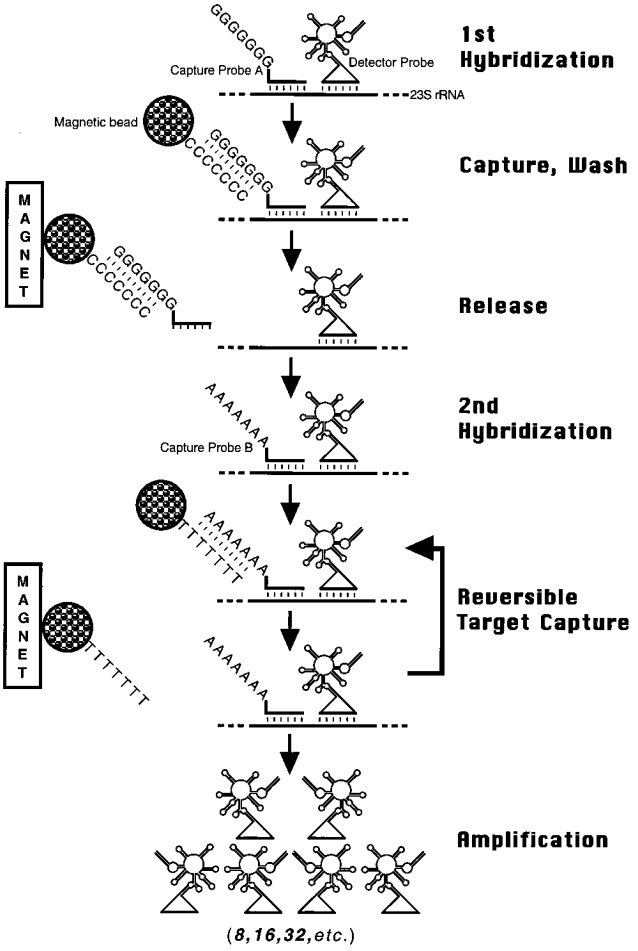

FIG. 2. Schematic diagram of the dual-capture reversible target capture assay format.

once with bead blocking buffer and were then suspended in fresh bead blocking buffer to a final concentration of 0.06% (wt/vol) solids. On the day of the experiment the particles were washed once with the bead blocking buffer and were then suspended in fresh bead blocking buffer to a final concentration of 0.06% (wt/vol) for oligo$(dC)_{25}$-derivatized beads and to a final concentration of 0.04% (wt/vol) for the oligo$(dT)_{14}$-derivatized beads.

Capture probes. Two single-stranded capture probes (A [26 mer] and B [28 mer]) complementary to the same target sequence in the 5′ region of the *M. tuberculosis* 23S rRNA were prepared by β-cyanoethyl phosphoramidite chemistry on a 380-B synthesizer (Applied Biosystems, Foster City, Calif.). The sequence of probe A is 5′-ACACGCCACTATTCACACGCGCGTAT. The sequence of probe B is 5′-GAACACGCCACTATTCACACGCGCGTAT. A tail of approximately 150 deoxydeazaguanosine (deaza-G) residues was added to the 3′ end of capture probe A, and a tail of approximately 150 dA residues was added to the 3′ end of capture probe B by a slight modification of the method described by Morrissey et al. (18). A tailing mixture was prepared; the mixture consisted of 0.01 mM oligonucleotide, 1× acetate buffer (10 mM KCOOH, 10 mM $MgCl_2$, 50 mM Tris acetate [pH 7.2] at 35°C, 50 mM Tris [pH 7.2] at 35°C), 2 mM nucleoside triphosphates (NTPs), 1 U of PPi per ml, and 1.1×10^6 U of terminal deoxynucleotidyl transferase. The reaction was performed overnight at 37°C.

Preparation of an RNA detector probe. A Q-Beta replicable RNA detector probe containing a probe sequence, complementary to a region of the *M. tuberculosis* 23S rRNA target a few bases upstream of the annealing site of the capture probes, was used in the examples described below as a midivariant (MDV) detector probe. MDV RNA is a small RNA molecule. It is the most extensively studied nonviral substrate for Q-Beta replicase into which a probe sequence has been cloned (17). Briefly, the probe sequence 5′-TGTGGGTCGCCCTATTCA GACTCGCTTTCGCTGCG was inserted into a recombinant plasmid containing the MDV sequence, at the unique *Mlu*I and *Nhe*I sites located between nucleotides 63 and 64 of the plus-strand MDV (3). In addition, it was flanked by 12-nucleotide spacer elements which improve the replication properties of the recombinant probe (3). The recombinant plasmid containing the probe sequence downstream of a T7 promoter was linearized by digestion with *Sma*I at the 3′ end of the MDV sequence. The RNA detector probe was prepared by transcription of this linearized recombinant plasmid with T7 RNA polymerase (Megascript kit; Ambion Inc., Austin, Tex.). The transcripts were checked for size and integrity by denaturing polyacrylamide gel electrophoresis. The RNA detector probe was diluted in 50 mM Tris (pH 8.0)–1 mM EDTA–0.5% Nonidet P-40 to a final concentration of 10 ng/µl (6.30×10^{10} molecules per µl).

Bacterial strains. The bacterial strains used in the study were obtained from the American Type Culture Collection and were grown by standard microbiological procedures (26).

Sputum samples. Human sputum from patients with cystic fibrosis was obtained from Rainbow Babies & Children's Hospital, Case Western Reserve University School of Medicine, Cleveland, Ohio.

Sample processing. (i) *M. tuberculosis*-spiked sputum specimens. The method used to process the sputum samples is outlined in Fig. 1. Sputum aliquots of 5 to 10 ml were digested and decontaminated for 15 min with an equal volume of Nalc-NaOH or 2% NaOH, washed twice with 0.067 M phosphate buffer (pH 6.8), and suspended in 2 ml of 0.2% BSA (11). A 300-µl aliquot of the suspension was transferred to a cryovial, and the cryovial was heated at 100°C for 15 min. A 250-µl portion of this heat-inactivated material was added to a processing tube containing 0.5 g of zirconium oxide beads and 400 µl of 5 M GuSCN sample processing buffer (100 mM Tris-HCl [pH 7.8], 40 mM EDTA, 5 M GuSCN, 1.0% Sarkosyl) and was mechanically lysed by shaking at 5,000 rpm for 6 min in the GENE-TRAK Sample Processor.

(ii) Mycobacteria. Mycobacteria were grown in liquid cultures to between 10^7 and 10^8 CFU/ml. The mycobacterial cultures were homogenized in a Dounce tissue grinder (VWR Scientific, Boston, Mass.) to break up macroscopic clumps and were serially diluted to the appropriate concentration in either Middlebrook 7H9 broth (Remel, Lenexa, Kans.) or 0.2% BSA. A 300-µl aliquot of the suspension was transferred to a cryovial, and the cryovial was heated at 100°C for 15 min. A 250-µl aliquot of this heat-inactivated material was added to a processing tube containing 0.5 g of zirconium oxide beads and 400 µl of the 5 M GuSCN sample processing buffer and was lysed as described above. A slight modification of the method described above was used for *Mycobacterium avium* and *Mycobacterium gordonae*. A 400-µl aliquot of the suspension was transferred to a cryovial, and the cryovial was heated at 100°C for 15 min. A 350-µl aliquot of this heat-inactivated material was added to a processing tube containing 0.5 g of zirconium oxide beads and 550 µl of the 5 M GuSCN sample processing buffer and was lysed as described above.

(iii) Purified RNA. rRNA from bacteria was purified and quantitated as described by Barns et al. (1). A 250-µl aliquot of the RNA in probe dilution buffer (100 mM Tris-HCl [pH 7.8], 20 mM EDTA) was combined with 400 µl of 5 M GuSCN sample processing buffer, and the mixture was vortexed for 30 s. A 250-µl aliquot of the processed sample, containing RNA or lysed mycobacterial cells in buffer or processed sputum, was analyzed as described below.

Dual-capture reversible target capture assay. The dual-capture reversible target capture assay method is described schematically in Fig. 2. The ligand on the first capture probe was a poly(deaza-G) tail (described above), and the

antiligand on the first paramagnetic particles was poly(dC). The ligand on the second capture probe was a poly(dA) tail, and the antiligand on the second paramagnetic particles was poly(dT). Capture of the dG capture probe-target-detector probe complex onto the oligo$(dC)_{25}$ beads was performed in 2.20 M GuSCN, and the capture of the dA capture probe-target-detector probe complex onto the oligo$(dT)_{14}$ beads was performed in 0.875 m GuSCN or 4 M GuHCl. The hybridizations and reversible target capture reactions were carried out with a tube rack, a magnetic separator block, and an aspirator head specially designed to hold 96 1-ml Micronic tubes (Flow Laboratories, Huntsville, Ala.). A 250-μl portion of the sample, processed as described above, was added to 50 μl of the probe dilution buffer containing 30 ng each of capture probe A and MDV RNA detector probe. The probes were hybridized with the target for 30 min at 37°C. Following hybridization, 50 μl of a suspension of 0.06% (wt/vol) poly(dC) [oligo$(dC)_{25}$]-derivatized paramagnetic particles in bead blocking buffer was added to the hybridization mixture. The tubes were vortexed for 20 s and were incubated for 4 min at 37°C to capture the ternary hybrids (capture probe A-target-detector probe) on the paramagnetic particles via the oligo$(dC)_{25}$ tail. The particle-bound ternary complexes were collected on the sides of the reaction tubes by placing the rack of the tubes in a magnetic separator for 2 min, and the supernatants were removed by aspiration. To remove unbound detector probe, the particle-bound ternary complexes were washed four times with 200 μl of low-salt buffer (100 mM Tris [pH 8.1], 20 mM EDTA, 25 mM NaCl, 0.2% Sarkosyl, 0.05% BSA, 0.05% Bronopol) as described above. Following the washes, capture probe-target hybrids were disrupted by adding 3.5 M GuSCN release buffer (300 mM Tris-HCl [pH 7.8], 100 mM EDTA, 3.5 M GuSCN, 0.5% Sarkosyl, 0.5% BSA) and incubating the mixture for 4 min at 37°C. The target-detector probe complex was released, leaving the deaza-G tailed capture probe A on the oligo$(dC)_{25}$ beads. A 50-μl aliquot of the probe dilution buffer containing 15 ng of dA-tailed capture probe B was added to the eluted target-detector probe complex. The mixture was incubated for 30 min at 37°C. Following hybridization of the dA-tailed capture probe B to the target-detector probe complex, 250 μl of a 0.04% (wt/vol) suspension of oligo$(dT)_{14}$-derivatized paramagnetic particles in the bead blocking buffer was added. The ternary capture probe B-target-detector probe complex was captured onto the particles as described above. The particle-bound ternary complex was washed three times with 200 μl of high-salt buffer (100 mM Tris [pH 8.1], 20 mM EDTA, 300 mM NaCl, 0.5% Sarkosyl, 0.5% BSA, 0.5% Bronopol) and was then chemically eluted from the oligo$(dT)_{14}$ beads by the addition of 100 μl of the low-salt buffer as described above. The ternary complexes were subjected to two additional rounds of capture, washing, and elution from the oligo$(dT)_{14}$ particles. Following the fourth round of capture, the ternary complexes were washed with preamplification wash buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 300 mM KCl, 0.5% Nonidet P-40) and were then eluted in 200 μl of preamplification release buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% Nonidet P-40).

Amplification with Q-Beta replicase. The released MDV detector probe was amplified with Q-Beta replicase (23). A 100-μl portion of released hybrid complex or purified detector probe, as indicated for individual experiments, was combined with 100 μl of Q-Beta replicase reaction buffer (220 mM Tris [pH 7.8], 40 mM $MgCl_2$, 1.2 mM NTPs [GTP, ATP, CTP, and UTP], 20 μg of propidium iodide per ml, and 25% glycerol containing about 110 μg of Q-Beta replicase per ml) in an 8-by-12 array of cuvettes, and the cuvettes were held at 4°C. Once all of the reaction mixtures were assembled, the rack of cuvettes was placed in a heater block in a proprietary fluorometer which maintains each reaction mixture at 37 ± 0.3°C. The fluorescence in each sample (615 nm) was measured at 40-s intervals through the optically clear flat bottom of the cuvette by a closely positioned fiber optic cable which moves on an x-y table positioned below the stationary array of cuvettes. The production of RNA in the amplification reactions was indicated by an increase in fluorescence because of the binding of the propidium iodide to the RNA. For each reaction a value termed the "response time" was calculated as the time at which fluorescence becomes detectable above the baseline fluorescence. In practice this corresponds to the amount of time that a particular reaction takes to produce a fixed amount of RNA (approximately 3.0×10^{11} molecules) and is inversely related to the logarithm of the number of RNA detector molecules present at the start of the amplification reaction (3).

Statistical analysis. The significance of differences in the mean response times between two sample sets was determined by the *t* test. If the *t* test value was greater than 2, the difference in the mean response time (signal) between the two sample sets was considered statistically significant.

RESULTS

Characterization of the *M. tuberculosis* detector probe. A plot of response time versus concentration of the *M. tuberculosis* detector probe is shown in Fig. 3A. The *M. tuberculosis* detector probe was serially diluted in the preamplification release buffer. One hundred-microliter aliquots of the diluted detector probe (ranging from 1 to 10^6 probe molecules per 100 μl) was mixed with 100 μl of the Q-Beta replicase reaction buffer, and the amplification reactions were monitored for 30 min as described above. The inverse log relationship between the concentration of the detector probe and the response time (16, 17) is apparent in Fig. 3A. It shows an approximately 2-min difference in response times between the logarithm input of the probe. At the single-molecule input level (log 0), only four of the six samples gave a response. This was not surprising because at the single-molecule input level there is expected Poisson failure because of sampling error (16, 17). The Poisson value (*N*) was 1.1 molecules per reaction mixture [$N = \ln (2/6) = 1.1$].

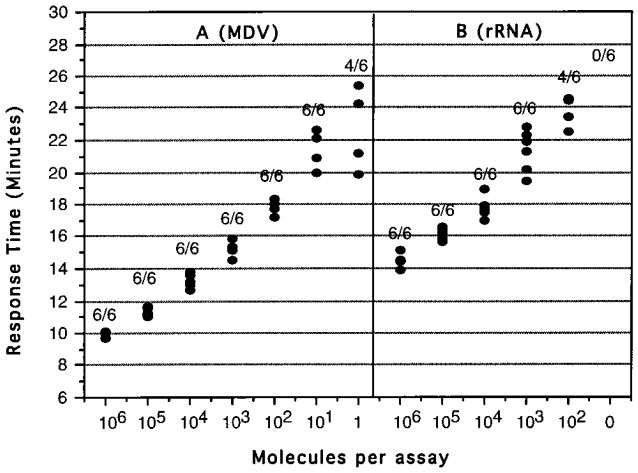

FIG. 3. (A) Replicability of the MDV detector probe. Serial dilutions of the probe (1 to 10^6 per assay) were amplified with Q-Beta replicase to generate a response time as described in the Materials and Methods section. (B) Response times of serially diluted *M. tuberculosis* rRNA. RNA was purified from *M. tuberculosis* ATCC 25177 CFU. Serial dilutions of purified RNA equivalent to 0 to 10^6 23S rRNA molecules per assay were analyzed as described in the Materials and Methods section.

Limit of detection of the Q-Beta replicase assay. The results of Q-Beta replicase-amplified dual-capture assays performed on various concentrations of purified *M. tuberculosis* rRNA are shown in Fig. 3B. The response time increased as the number of target molecules in the initial sample decreased (Fig. 3B). Samples at 10^3 targets per assay point and above gave response times of less than 30 min. At 10^2 targets input per assay point, four of the six samples gave response times of less than 30 min. This corresponds to approximately 1.1 molecules per assay point. Thus, the assay is about 1% efficient (one detector probe delivered, on average, per 10^2 input target molecules). The assay efficiency also can be inferred from a comparison of the response times shown in Fig. 3A and B. For example, the average response time for 10^2 molecules of rRNA, 23 min, corresponds to that of approximately 1 molecule of the detector probe (Fig. 3B). In the negative controls (zero target), no signal was measured by the fluorometer within the 30-min read time.

Reactivity of the *M. tuberculosis* assay on *M. tuberculosis* complex and non-*M. tuberculosis* complex organisms. The results of Q-Beta replicase-amplified dual-capture assays on purified RNA from a number of *M. tuberculosis* complex strains are shown in Table 1. At 10^6 and 10^5 targets input per assay point, all the *M. tuberculosis* complex organisms (*M. tuberculosis*, avirulent strain, H37A [ATCC 25177], *Mycobacterium africanum* ATCC 25420, *Mycobacterium microti* ATCC 19422, *M. bovis* ATCC 19210, and *M. tuberculosis*, virulent strain, H37RV [ATCC 25618]) gave similar response times. There was no statistical difference in the response times between

TABLE 1. Reactivity of the *M. tuberculosis* assay with members of the *M. tuberculosis* complex of organisms

Organism[a]	Mean ± STD RT[b] (min) for 10^5 rRNA targets/assay point	*t* test	Mean ± STD RT (min) for 10^6 rRNA targets/assay point	*t* test
M. tuberculosis H37A (ATCC 25177)	15.35 ± 0.90		13.99 ± 0.85	
M. africanum ATCC 25420	15.53 ± 0.42	1.83	14.08 ± 0.09	1.30
M. bovis ATCC 19210	14.49 ± 0.41	0.36	13.26 ± 0.68	0.21
M. microti ATCC 19422	15.68 ± 0.59	0.62	14.20 ± 0.58	0.41
M. tuberculosis H37RV (ATCC 25618)	14.84 ± 0.90	0.84	13.79 ± 0.33	0.44

[a] Four assay points were used for each organism.
[b] RT, response time.
[c] If the *t* value was greater than 2, the difference in response time (signal) between the two sample sets was considered significant.

different organisms at 10^6 (*t* test value = 0.21 to 1.31) and at 10^5 (*t* test value = 0.36 to 1.83) targets.

Competitor levels up to approximately 10^7 *M. avium* (clinical isolate) or *M. gordonae* ATCC 14470 organisms corresponding to ~10^{10} 23S rRNA molecules per assay point produced no signal (Table 2). In the same experiment all of the assay points containing 10^3 *M. tuberculosis* 23S rRNA molecules gave response times (mean, 19.32 ± 0.50 min) of less than 30 min (Table 2). At 10^{11} *M. avium* RNA molecules some competitor effect was detectable, but the response time was less than that measured against 10^3 target molecules of *M. tuberculosis* 23S rRNA (data not shown), which is the limit of detection of the assay. This corresponds to 10^7 CFU per assay point, a level which would be highly unexpected in a clinical specimen. In the presence of 10^6 CFU of the mycobacterial strains *M. fortutium*, *M. intracellulare*, *M. austroafricanum*, *M. gallinarum*, *M. lactis*, *M. acapulsensis*, *M. rhodesiae*, *M. haemophilium*, *M. chelonae*, *M. kansasi*, *M. xenopi*, and *M. senegalese* per ml, no interfering signal was generated (data not shown) by the *M. tuberculosis* Q-Beta replicase assay.

Effect of heat on *M. tuberculosis* viability. Aliquots of sputum were spiked with the avirulent strain of *M. tuberculosis* ATCC 25177, processed with Nalc-NaOH, and suspended in 0.067 M phosphate buffer (pH 6.8). The suspensions were heated at 100°C for various periods (0 to 30 min), plated on Middlebrook 7H10 agar (Remel), and incubated at 37°C in 10% CO_2 for 8 weeks. Results for sputum suspensions spiked with 7×10^7 and 7×10^5 CFU/per ml indicated that after 3 min of heating at 100°C no viable *M. tuberculosis* was recovered by quantitative plate counts (Table 3). In a parellel experiment, the spiked sputum suspensions were cultured in Middlebrook 7H9 broth following heat treatment. No organism was recovered after 5 min of the 100°C heat treatment. Even though our data indicated that *M. tuberculosis* in sputum was inactivated after 3 to 5 min of heating at 100°C, we chose a 15-min heat step to ensure the complete inactivation of viable *M. tuberculosis* and therefore to alleviate biohazard concerns during our assay.

TABLE 2. Reactivity of *M. tuberculosis* assay with non-*M. tuberculosis* complex mycobacteria

Organisms[a]	Mean ± STD RT[b] (min) for the following no. of rRNA targets/assay point:		
	10^3	~10^9	~10^{10}
M. tuberculosis, avirulent	19.32 ± 0.50	ND[c]	ND
M. avium	ND	NR[d]	NR
M. gordonae	ND	NR	NR

[a] Six assay points were used for each organism.
[b] RT, response time.
[c] ND, not done.
[d] NR, no response in 30 min.

TABLE 3. Effect of heat treatment on viability of *M. tuberculosis* organisms following digestion-decontamination of *M. tuberculosis*-spiked sputum with Nalc-NaOH

Time (min) at 100°C	No. of viable *M. tuberculosis* CFU/ml of sputum	
0	3×10^7	6×10^4
1	1×10^2	0
3	0	0
5	0	0

Effect of sputum matrix on signal and noise. Sputum was processed as described above (Fig. 1). Purified *M. tuberculosis* rRNA was spiked into the *M. tuberculosis*-negative processed sputum specimen, and the specimen was assayed. There were no significant differences between the response times generated by 10^5 purified rRNA molecules of *M. tuberculosis* in the absence (mean response time, 16.53 ± 0.78 min) or presence (mean response time, 15.94 ± 0.47 min) of the processed sputum (Fig. 4). One responder (an assay yielding a response time faster than 30 min) occurred in the no-sputum control, and no responders were observed in the presence of sputum matrix. Assay noise was defined as a signal in the absence of added *M. tuberculosis* targets and was unaffected by the presence of sputum. These results indicate that neither the specificity nor the sensitivity of the assay was affected by the presence of the sputum.

Effect of washing the processed sputum pellet on assay sensitivity and specificity. To study the effect of washing the processed sputum pellets, approximately 10^3 and 0 (as control) CFU of *M. tuberculosis* H37A (ATCC 25177) were added to 5-ml sputum samples in duplicate (sets A and B). Set A sam-

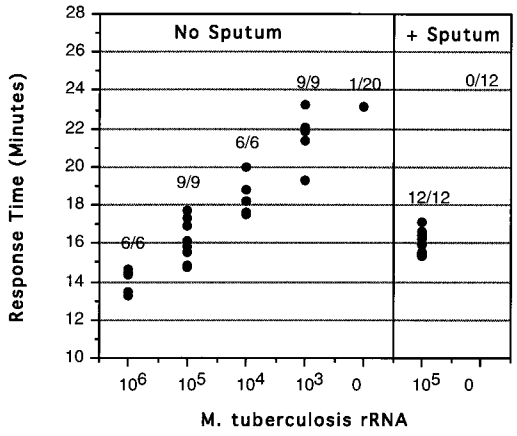

FIG. 4. Effect of sputum on the *M. tuberculosis* assay. Purified 23S rRNA molecules were assayed in the presence and absence of processed sputum. Purified *M. tuberculosis* rRNA molecules equivalent to 0 and 10^5 23S rRNA molecules per assay were spiked into processed sputum sample, and the samples were analyzed as described in the Materials and Methods section. The response time generated by the spiked samples was compared with the standard dose-response curve generated from 0 to 10^6 23S rRNA molecules analyzed at the same time.

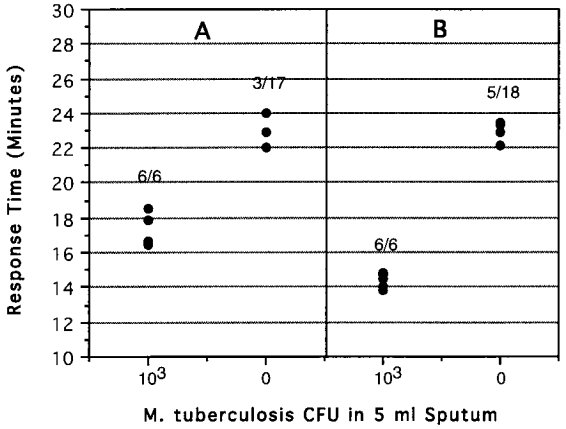

FIG. 5. Effect of washing processed sputum pellet on the assay signal and noise. All of the sputum samples were processed by the Nalc-NaOH method. Processed sputum pellets of set A were not washed prior to heating. Processed sputum pellets of set B were washed with phosphate buffer prior to heating. The pH of the unwashed pellets (set A) was between 10 and 13, and the pH of the washed pellets (set B) was approximately 7. The heat-inactivated mycobacteria were lysed and analyzed as described in the Materials and Methods section. The response times generated by the set A samples were compared with the response times generated by the set B samples.

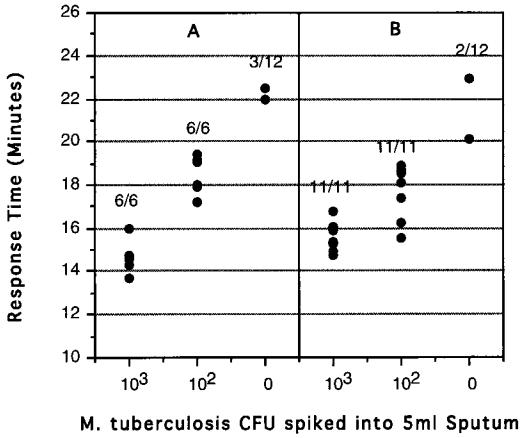

FIG. 6. Comparison of different digestion-decontamination procedures on the *M. tuberculosis* assay signal and noise. Set A samples were processed by the Nalc-NaOH method, and set B samples were processed by the 2% NaOH digestion-decontamination method.

ples were processed by the Nalc-NaOH method as described in Fig. 1, except that the processed pellet was not washed prior to the heat step, and 250 μl of the heat-inactivated material was added to a processing tube containing 1 g of zirconium oxide beads and 1,250 μl of 5 M GuSCN sample processing buffer. Set B samples were processed as described in Fig. 1, except that 250 μl of the heat-inactivated material was added to a processing tube containing 1 g of zirconium oxide beads and 1,250 μl of 5 M GuSCN sample processing buffer. As shown in Fig. 5, the average response time of the washed sputum pellet spiked with 10^3 CFU was 2.67 min earlier than that of the unwashed sputum pellet spiked with the same level of *M. tuberculosis* organisms. The mean response times of the washed and the unwashed pellet were 14.44 ± 0.38 and 17.11 ± 0.87 min, respectively. There was a statistical difference in the response times between the washed and the unwashed pellets (*t* test value = 6.89). Under both conditions, in the absence of the *M. tuberculosis* targets, a few assay points (3 of 17 for set A and 5 of 18 for set B) gave response times of less than 30 min (Fig. 5). A response in the absence of a target is referred to as the false-positive rate. In this experiment the occurrence of false-positive responses was slightly higher (8 of 35) than that which is normally seen (e.g., see Fig. 4). For all subsequent experiments, processed sputum pellets were washed with phosphate buffer prior to the heat step.

Effect of different digestion-decontamination methods on assay sensitivity. To further examine the effects of different digestion-decontamination procedures on the Q-Beta assay, spiked and unspiked samples were processed by either the Nalc-NaOH (set A) or the 2% NaOH (set B) methods. Sputum was spiked with either 10^2 or 10^3 CFU of *M. tuberculosis* H37A (ATCC 25177) in Middlebrook 7H9 broth or Middlebrook 7H9 broth without *M. tuberculosis* and was subjected to the digestion-decontamination procedures. The volumes of processed sputum pellets were adjusted to 2 ml with 0.2% BSA, and 300-μl portions were heated at 100°C for 15 min. Heat-inactivated samples were combined with sample processing buffer and were lysed as described above. Assay results are shown in Fig. 6. Sensitivities for *M. tuberculosis*-spiked sputum samples at 10^2 and 10^3 CFU were statistically equivalent (*t* test values = 1.32 and 1.54, respectively) when they were digested and decontaminated by either method. False-positivity rates and assay noise were likewise equivalent.

Assay sensitivity in sputum matrix. To determine the overall sensitivity of the assay, two sets of samples were prepared. Set A was for *M. tuberculosis* CFU (without matrix) and set B was for *M. tuberculosis* CFU spiked into the sputum matrix. In set A, five 0.2% BSA samples were spiked with 0, 10^1, 10^2, 10^3, and 10^4 CFU of *M. tuberculosis* H37A (ATCC 25177), respectively. The samples were then processed and assayed as described in the Materials and Methods section. In set B, five sputum samples were spiked with 0, 10^1, 10^2, 10^3, and 10^4 CFU of *M. tuberculosis* H37A (ATCC 25177), respectively. The spiked samples were processed and assayed by the Nalc-NaOH digestion-decontamination procedure, which was followed by a phosphate buffer wash prior to heat inactivation, as described in the Materials and Methods section. All of the samples containing 10^2, 10^3, and 10^4 CFU of *M. tuberculosis* (per spike) in the absence (set A) or the presence (set B) of 5-ml sputum samples gave response times of less than 30 min (Fig. 7). All of

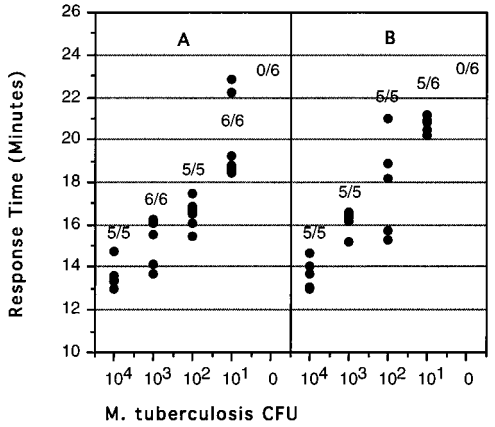

FIG. 7. Limit of detection of the Q-Beta replicase *M. tuberculosis* assay on cells spiked into the sputum matrix. Set A samples (no sputum matrix) did not go through the digestion-decontamination step. They were heat inactivated, lysed, and assayed in the same way as set B samples. Set B samples were processed by the Nalc-NaOH digestion-decontamination method; this was followed by heat inactivation and lysis.

the samples of *M. tuberculosis* containing 10^1 CFU in the absence of the sputum matrix (set A) gave response times of less than 30 min. Five of six of the sputum samples spiked with 10^1 CFU per 5 ml of sputum gave response times of less than 30 min (Fig. 7). None of the *M. tuberculosis*-negative sputum samples gave amplification response times within the 30-min reading interval.

DISCUSSION

In the present study we demonstrated that the Q-Beta replicase-amplified *M. tuberculosis* assay can be performed on sputum samples processed by either of the two most commonly used digestion-decontamination procedures. The assay uses a simple three-step sample processing protocol consisting of neutralization of the digested decontaminated pellet by washing, inactivation of the viable microorganisms by heating for 15 min, and combined mechanical-chemical lysis of the *M. tuberculosis* organisms for 6 min. The analytical sensitivity of the assay was 10^3 purified 23S rRNA targets (Fig. 3). This translates to less than one *M. tuberculosis* CFU per assay point, assuming that there are 2.0×10^3 targets per CFU (15). In the present study, 4.8% of the initial sample was analyzed. On the basis of the results of spiking experiments in human sputum, the assay had a lower limit of detection, i.e., between 10^1 and 10^2 CFU per 5 ml of sputum (Fig. 7) or between 0.48 and 4.8 *M. tuberculosis* CFU per assay point. The loss during digestion-decontamination was about 50%. Therefore, the calculated assay sensitivity is 1 CFU per assay point.

Two elements control the performance of the dual-capture reversible target capture chemistry. First, the first capture probe is more easily dissociated from its target sequence than is either the detector probe-target hybrid or the deaza-G–dC interactions between the tail of the capture probe and the dC on the solid support. Second, deaza-G has no affinity for the second-round dT-tailed solid support. The first design element allows for two sequential, specific hybridizations to be imposed, decreasing the competitor effects of closely related organisms such as *M. avium* and *M. gordonae* and improving the specificity for *M. tuberculosis* complex bacteria. The second design element effectively excludes the small amount of nonspecific carryover of the first capture probe-detector probe complex from subsequent rounds of capture, therefore reducing the background noise (23).

In the present study (Fig. 4 to 7), the false-positive rate (FP) varied from 0 to 23% (Fig. 3 and 7, FP = 0%; Fig. 4, FP = 3%; Fig. 5, FP = 23%; Fig. 6, FP = 21%), with an average FP of 14% (14 of 103 samples). When the final elution reactions of the false-positive samples were diluted 10-fold prior to being amplified by Q-Beta replicase, no signal was observed, indicating that the detector probe was present at a level of, at most, one to a few molecules. This low level of noise is indicative of the limitations of performing a signal amplification assay in an open system. In an open system, many of the manual manipulations such as pipetting and aspirations in the presence of high initial concentrations of detector probe molecules create aerosols which can cross-contaminate samples, thus giving false-positive results (23).

The Q-Beta replicase assay may offer certain advantages over PCR. First, whereas PCR performed on DNA cannot differentiate live organisms from dead organisms, the Q-Beta replicase assay performed on RNA is strongly suggestive of the persistence of live organisms (13). DNA and RNA can persist well beyond the period of detection by culture (13). RNA is more labile than DNA and is less likely to survive intact in dead organisms for any appreciable length of time. It may be advantageous to follow RNA levels rather than DNA levels for the purpose of monitoring active infections (13). A limited study on patients on treatment with rifampin (an RNA chain initiation inhibitor) has shown that the antibiotic treatment does not affect the results of an *M. tuberculosis* probe assay directed to rRNA targets (20). On the basis of these studies, the Q-Beta replicase assay may be more useful than traditional culture for following patients with tuberculosis on antibiotic treatment.

Second, it is well documented that PCR inhibitors are found in some sputum specimens (2, 4, 25, 29). Several methods have been described for the extraction of sufficient quantities of clean DNA free of PCR-inhibitory substances from clinical specimens (2, 5, 8, 12, 24). However, these methods are lengthy, time-consuming, and subject to target loss during the extraction procedure. In the present study, neither the signal nor the noise was affected by the presence of the sputum matrix (Fig. 4). This is not surprising because the probe-target complex is extensively purified by reversible target capture prior to the amplification. This method has been reported to decrease interfering substances by 10^{12}- to 10^{16}-fold following four rounds of cycling (14, 18). Thus, the Q-Beta replicase assay can be performed directly on processed sputum sample pellets.

The two most common digestion-decontamination methods are the Nalc-NaOH and 2% NaOH methods. Both methods of processing sputum are compatible with the Q-Beta replicase assay. Digestion-decontamination methods eliminate up to 80 to 90% of the mycobacteria present in sputum (22). At 0.48 CFU per assay point, 100% of the nondigested *M. tuberculosis*-spiked samples gave response times of less than 30 min (Fig. 7). However, at the equivalent of 0.48 CFU per assay, five of the six digested-decontaminated samples gave response times of less than 30 min (Fig. 7). As discussed above, the Q-Beta replicase assay is not inhibited by the presence of matrix (Fig. 4). Thus, the loss in signal (approximately 50%), and therefore the loss in *M. tuberculosis* CFU, is attributable to the digestion-decontamination procedure.

The procedures used for performing digestion-decontamination with Nalc-NaOH and 2% NaOH differ significantly from laboratory to laboratory. The most serious issue that we noted is the lack of pH neutralization of the processed sputum pellets prior to culturing the specimens. All mycobacteria are nonviable after 5 min of heat treatment at 100°C (Table 3). Thus, in order to facilitate the safe handling of specimens, we used a 15-min heat treatment prior to mechanical lysis of the *M. tuberculosis* bacteria. However, without pH neutralization prior to the heating, the assay sensitivity is significantly reduced (Fig. 5). In the presence of high pH, the heat treatment will result in significant degradation of the 23S rRNA. The simple wash step prior to heat inactivation uniformly neutralizes the pH of the samples and preserves the target nucleic acid.

In summary, because of its speed, accuracy, and sensitivity, the Q-Beta replicase assay provides a potentially powerful new tool for the detection of active *M. tuberculosis* infection and may facilitate more effective disease management. This test could aid in the diagnosis of partially treated patients, especially those who are acid-fast stain negative.

ACKNOWLEDGMENTS

We thank D. J. Lane for critically reviewing the manuscript and drawing Fig. 2, S. Cohen for preparing the oligo$(dT)_{14}$ and oligo$(dC)_{25}$ magnetic particles, L. Burg for cloning the detector probe sequence into a recombinant plasmid containing the MDV sequence and preparing the RNA detector probe, and Rainbow Babies & Children's

Hospital, Cleveland, Ohio, for providing the sputum from patients with cystic fibrosis.

REFERENCES

1. **Barns, S. M., D. J. Lane, M. L. Sogin, C. Bibeau, and W. G. Weisburg.** 1991. Evolutionary relationship among pathogenic *Candida* species and relatives. J. Bacteriol. **173:**2250–2255.
2. **Brisson-Noel, A., B. Gicquel, D. Lecossier, V. Levy-Frebault, X. Nassif, and A. J. Hance.** 1989. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet **ii:**1069–1071.
3. **Burg, J. L.** Personal communication.
4. **Clarridge, J. E., III, R. B. Shawar, T. M. Shinnick, and B. B. Plikaytis.** 1993. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine microbiology laboratory. J. Clin. Microbiol. **31:**2049–2056.
5. **Cousins, D. V., S. D. Wilton, B. R. Francis, and B. L. Gow.** 1992. Use of the polymerase chain reaction for rapid diagnosis of tuberculosis. J. Clin. Microbiol. **30:**255–258.
6. **De Wit, D., L. Steyn, S. Shoemaker, and M. Sogin.** 1990. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. J. Clin. Microbiol. **28:**2437–2441.
7. **Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford.** 1990. Polymerase chain reaction amplification of a repetitive sequence specific for *Mycobacterium tuberculosis*. J. Infect. Dis. **161:**977–981.
8. **Eisennach, K. D., M. D. Gifford, M. D. Cave, J. H. Bates and J. T. Crawford.** 1991. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. Am. Rev. Respir. Dis. **144:**1160–1163.
9. **Ellner, P. D., T. E. Kiehn, R. Cammarata, and M. Hosmer.** 1988. Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods. J. Clin. Microbiol. **26:**1349–1352.
10. **Forbes, B. A., and K. E. S. Hicks.** 1993. Direct detection of *Mycobacterium tuberculosis* in respiratory specimens in a clinical laboratory by polymerase chain reaction. J. Clin. Microbiol. **31:**1688–1694.
11. **Gullans, C. R., Sr.** 1992. Digestion-decontamination procedures, p. 3.4.1–3.4.7. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.
12. **Hermans, P. W. M., A. R. J. Schuitema, D. van Sooligen, C. P. H. J. Verstynen, E. M. Bik, J. E. R. Thole, A. H. J. Kolk, and J. D. A. van Embden.** 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J. Clin. Microbiol. **28:**2051–2058.
13. **Holland, S. M., A. P. Hudson, L. Bobo, J. A. Whittum-Hudson, R. P. Viscidi, T. C. Quinn, and H. R. Taylor.** 1992. Demonstration of chlamydial RNA and DNA during a culture-negative state. J. Clin. Microbiol. **60:**2040–2047.
14. **Hunsaker, W. R., H. Badri, M. Lombardo, and M. L. Collins.** 1989. Nucleic acid hybridization assays employing dA-tailed capture probes. II. Advanced capture methods. Anal. Biochem. **181:**360–370.
15. **Jonas, V., M. J. Alden, I. Curry, K. Kamisango, C. A. Knott, R. Lankford, J. M. Wolfe, and D. F. Moore.** 1993. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. **31:**2410–2416.
16. **Kramer, F., and P. Lizardi.** 1989. Replicatable RNA reporters. Nature (London) **339:**401–402.
17. **Lizardi, P. M., C. Guerra, H. Lomeli, I. Tussie-Luna, and F. Kramer.** 1988. Exponential amplification of recombinant-RNA hybridization probes. Bio/Technology **6:**1197–1202.
18. **Morrissey, D. V., M. Lombardo, J. K. Eldridge, K. R. Kearney, E. P. Groody, and M. L. Collins.** 1989. Nucleic acid hybridization assays employing dA-tailed capture probes. Anal. Biochem. **181:**345–359.
19. **Noordhoek, G. T., A. H. J. Kolk, G. Bjune, D. Catty, J. W. Dale, P. E. M. Fine, P. Godfrrey-Faussett, S. Cho, T. Shinnick, S. B. Svenson, S. Wilson, and J. D. A. van Embden.** 1994. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J. Clin. Microbiol. **32:**227–284.
20. **Peterson, E. M., R. Lu, C. Floyd, A. Nakasone, G. Friedly, and L. M. de la Maza.** 1989. Direct identification of *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare* from amplified primary cultures in BACTEC media using DNA probes. J. Clin. Microbiol. **27:**1543–1547.
21. **Ranki, M., A. Palva, M. Virtanen, M. Laaksonen, and H. Soderlund.** 1983. Sandwich hybridization as a convenient method for the detection of nucleic acids in crude samples. Gene **21:**77–85.
22. **Roberts, G. D., E. W. Koneman, and Y. K. Kim.** 1991. *Mycobacterium*, p. 304–339. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
23. **Shah, J., J. Liu, J. Smith, S. Poppof, G. Serpe, and W. King.** 1994. A novel ultra sensitive, Q-beta replicase amplified hybridization assay for the detection of *Chlamydia trachomatis*. J. Clin. Microbiol. **32:**2718–2724.
24. **Sjobring, V., M. Mecklenburg, A. B. Anderson, and H. Miorner.** 1990. Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28:**2200–2204.
25. **Soini, H., M. Skurnil, K. Liippo, E. Tala, and M. K. Vilganen.** 1992. Detection and identification of mycobacteria by amplification of a segment of the gene coding for the 32-kilodalton protein. J. Clin. Microbiol. **30:**2025–2028.
26. **Sommers, H. M., and R. C. Good.** 1985. *Mycobacterium*, p. 216–248. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of Clinical Microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
27. **Sudre, P., G. en Dam, and A. Kochi.** 1992. Tuberculosis: a global overview of the situation today. Bull. W. H. O. **70:**149–159.
28. **Veringa, E., B. van Harrelaar, and P. Hermans.** 1992. Polymerase chain reaction to detect *Mycobacterium tuberculosis* in a clinical microbiology laboratory. J. Microbiol. Methods **16:**139–147.
29. **Victor, T., R. du Toit, and P. D. van Helden.** 1992. Purification of sputum samples through sucrose improves detection of *Mycobacterium tuberculosis* by polymerase chain reaction. J. Clin. Microbiol. **30:**1515–1517.
30. **Whittier, P. S., K. Westfall, S. Setterquist, and R. L. Hopfer.** 1992. Evaluation of the Septi-Chek AFB System in the recovery of mycobacteria. Eur. J. Clin. Microbiol. Infect. Dis. **11:**915–918.