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Direct Genotypic Detection of *Mycobacterium tuberculosis* Rifampin Resistance in Clinical Specimens by Using Single-Tube Heminested PCR

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Recent analysis of the gene encoding the beta subunit of *Mycobacterium tuberculosis* RNA polymerase (*rpoB*) has demonstrated a small region that harbors the mutations most frequently associated with rifampin resistance. Earlier reports have described a high degree of sequence conservation of *rpoB* among mycobacteria other than *M. tuberculosis* and other GC-rich bacteria that can lead to false-positive amplification when applied directly to clinical specimens. We developed reagents for PCR amplification that are based on signature nucleotides discovered by comparative sequence analysis of the *rpoB* genes of organisms phylogenetically related to *M. tuberculosis*. The specificities of the reagents were challenged with 20 isolates of multiple-drug-resistant *M. tuberculosis* and more than 20 species of mycobacteria other than *M. tuberculosis* and other GC-rich organisms. A single-tube heminested PCR protocol was devised to obtain sensitivity equal to those of an IS*6110*-based PCR assay and culture in spiked sputum experiments. The assay correctly identified 21 of 24 (87.5%) culture-positive specimens, 13 of which were acid-fast smear-negative, in a panel of 51 clinical specimens. Three specimens that were false-positive initially were negative upon repeat testing when the assay was modified to eliminate the potential for aerosol carryover of the first-round amplification product during the open-tube addition of the second set of reaction reagents. This assay is the most sensitive and specific test to date for the direct detection of *M. tuberculosis rpoB* in clinical specimens. This rapid PCR-based assay can be used for the simultaneous identification of *M. tuberculosis* and its rifampin susceptibility genotype.

The three-decade decline in *Mycobacterium tuberculosis* infections in the United States was reversed in 1985 due, in part, to epidemic human immunodeficiency virus infection (17). Compounding this problem is the emergence of organisms resistant to many of the drugs used for antimicrobial therapy and prophylaxis (1, 13). Staining methods that lack sensitivity, slow mycobacterial growth rates, and prolonged and technically demanding susceptibility testing all contribute to the present shortcomings of conventional laboratory testing for *M. tuberculosis*. Recent advances in molecular methods for the detection of *M. tuberculosis* genomic targets, such as the use of 16S rRNA (2) or the insertion sequence IS*6110* (8), have approached the sensitivity of culture. Furthermore, the prospect of determining resistance in mycobacteria at the nucleic acid level, particularly to first-line drugs like rifampin (15, 27), isoniazid (6, 31), and streptomycin (4, 12), has provided a glimpse of the next generation of susceptibility tests for *M. tuberculosis.*

The genetic basis of rifampin resistance was first described in *Escherichia coli* (18, 24). Rifampin resistance in *M. tuberculosis* is largely associated with point mutations located in a 69-nucleotide region of the gene *rpoB*, which codes for the RNA polymerase beta subunit (19, 27, 29). Direct detection of *M. tuberculosis rpoB* by PCR has largely been limited to smear-positive specimens, and analysis of amplification products for rifampin resistance mutations has frequently been unsuccessful when few organisms are visualized (15, 28). Furthermore, nonspecific amplification because of sequence conservation among the GC-rich bacteria present in the respiratory tract interferes with amplification product (amplicon) analysis even when *M. tuberculosis* is present (15). Hunt et al. (15) used nucleic acid sequence information derived from the amplification of *rpoB* genes from various GC-rich organisms, including the mycobacteria, to describe five signature sequences comprising six nucleotides, the combination of which is present only in *M. tuberculosis*. Theoretically, these nucleotide positions could be exploited to enhance the specificity of amplification of the *rpoB* gene. In this report we describe a rapid test for prediction of the rifampin susceptibility of *M. tuberculosis* directly from clinical specimens.

Two requirements were met in the development of this assay, both of which are based on the need to subject the amplicon to further analysis. First, we needed a robust reaction to allow reliable detection and to yield sufficient product to determine the genotype. Second, the amplification had to be highly specific for *M. tuberculosis* since a mixture of products would likely complicate or preclude meaningful analysis. In the present study, an *M. tuberculosis rpoB*-specific protocol was developed on the basis of previously described signature sequences. We then designed a single-tube heminested PCR assay that avoids many of the problems associated with nested protocols and that specifically detects *M. tuberculosis* from seeded sputa and clinical specimens with a sensitivity approaching those of other methods. Subsequent screening of *rpoB*-specific amplification products by single-strand conformational polymorphism (SSCP) and then DNA sequence analysis if band migration was atypical (so as to avoid misinterpretation of silent mutations [10, 19]) allowed us to accurately predict rifampin susceptibility directly from clinical specimens.

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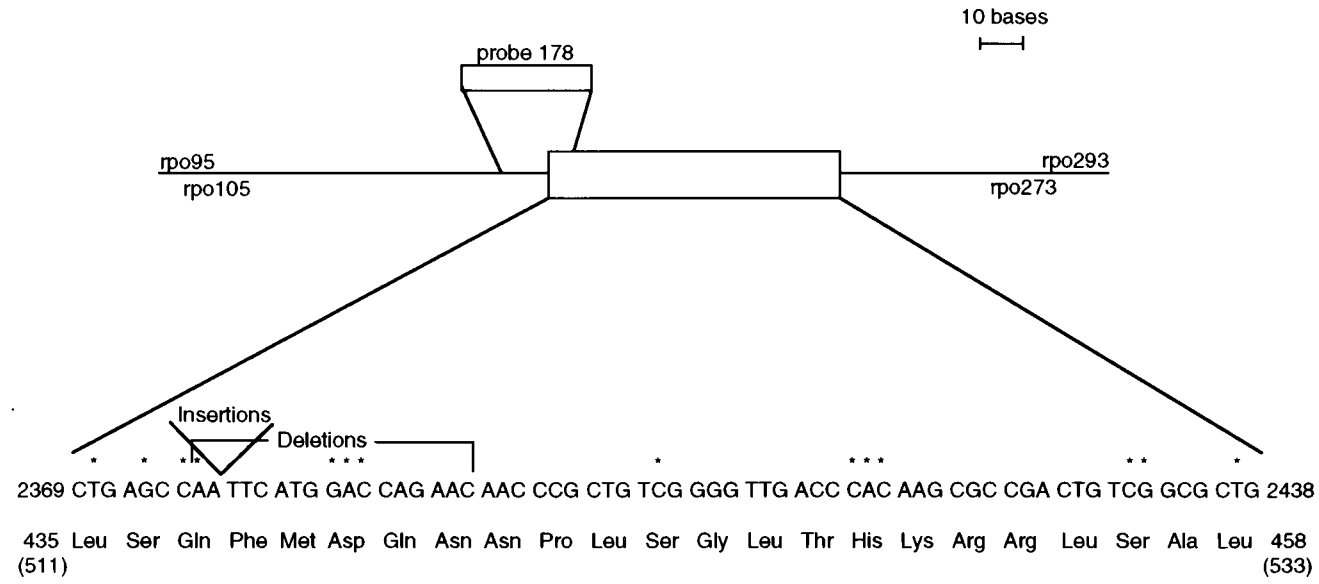

FIG. 1. Schematic orientation of oligonucleotides in heminested *rpoB* PCR assay. Asterisks identify bases where rifampin resistance mutations have been reported. Numbers indicate nucleotide and amino acid location within the *M. tuberculosis rpoB* gene (22); parentheses enclose the *E. coli* amino acid alignment (18, 27).

(This work was presented in part at the 1994 General Meeting, Las Vegas, Nev., 23 to 27 May 1994 [28a].)

MATERIALS AND METHODS

Specimen preparation. Three types of specimens were used in the study: pure cultures, processed sputa spiked with *M. tuberculosis*, and clinical specimens. Culture lysates, except multiple-drug-resistant (MDR) *M. tuberculosis*, were prepared by the bead-beat/boil or bead-beat/phenol extraction methods described by Hunt et al. (15). Briefly, a 10-μl loopful of culture was placed in a 2-ml screw-cap tube containing 0.1-mm zirconium beads and 1 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), oscillated on a Mini Bead Beater (Biospec Products, Bartlesville, Okla.) for 30 s, and boiled for 30 min (bead-beat/boil extraction). For bead-beat/phenol extraction, organisms were placed in a microcentrifuge tube containing 300 μl of phenol (equilibrated with TE), 0.1-mm zirconium beads, and 150 μl of TE buffer. Tubes were oscillated on a Mini Bead Beater for 30 s, incubated at 25°C for 30 min to ensure that the bacteria were killed, and centrifuged at 16,000 × *g* for 20 s to separate the phases. DNA was extracted from the aqueous phase with the Isoquick kit (Microprobe, Garden Grove, Calif.). Susceptibility testing of *M. tuberculosis* clinical isolates was performed by an agar dilution, 1% proportion method (16). MDR *M. tuberculosis* strains originally isolated from clinical specimens were cultured on Lowenstein-Jensen slants. The bacteria were scraped from the slants and were fixed in 1 ml of 70% ethanol (vol/vol in water) for 24 h. Pellets were obtained by centrifugation at 16,000 × *g* for 10 min and were resuspended in 1 ml of deionized water. Large clumps were allowed to settle for 2 min, and 100 μl of the supernatant was removed and subjected to three cycles of snap freeze-thaw (−164°C, 5 min; 94°C, 5 min).

Spiked sputa were prepared by adding *M. tuberculosis* to pooled, processed sputum specimens from patients with nonmycobacterial lower respiratory disease (i.e., bacterial pneumonia) and were processed by a standard sodium hydroxide decontamination method (25). A fresh broth culture of drug-susceptible *M. tuberculosis* (≤4 weeks old) was serially diluted (10-fold) in both treated sputa and sterile, distilled water. DNA was extracted from 200 μl of spiked sputum and clinical specimens by the procedure used by Felmlee et al. (11). Briefly, L6 buffer (3) replaced TE buffer, phenol-chloroform-isoamyl alcohol (25:24:1) replaced phenol as the organic phase, bead-beat was carried out for 2 min instead of 30 s, and Isoquick DNA precipitation was facilitated by the addition of glycogen. Spiked water was used to determine *M. tuberculosis* concentrations by plate counting (without the interference of faster-growing respiratory flora). An aliquot of uninoculated, treated sputa was reserved as a negative control.

PCR. Oligonucleotides were synthesized with an Applied Biosystems model 394 synthesizer (Applied Biosystems, Foster City, Calif.). Upstream PCR primers included rpo95 (5′-CCA CCC AGG ACG TGG AGG CGA TCA CAC-3′) and rpo105 (5′-CGT GGA GGC GAT CAC ACC GCA GAC GT-3′); downstream primers were rpo273 (5′-GAC CTC CAG CCC GGC ACG CTC ACG T-3′) and rpo293 (5′-AGT GCG ACG GGT GCA CGT CGC GGA CCT-3′) (Fig. 1). The master mixture per 25-μl reaction mixture was standardized at 1 μM primers, 10% (vol/vol) glycerol, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate (in some reactions dUTP was used instead of dTTP for amplicon inactivation purposes), 0.25 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 1× PCR Buffer II (Perkin-Elmer Cetus). All PCRs were performed with 2 μl of target on a Perkin-Elmer Cetus model 9600 thermal cycler. Annealing temperatures were optimized for single primer pair reactions: rpo95 and rpo293, 72°C; rpo105 and rpo273, 74°C; rpo105 and rpo293, 72°C; rpo95 and rpo273, 72°C. Tenfold dilutions of cesium chloride-purified chromosomal DNA from *M. tuberculosis* ATCC 25177 (H37RA) were used. Conditions for amplification included an initial denaturation at 94°C for 4 min, 50 cycles of annealing at the optimal temperature (1 min) and 94°C (30 s), and a final incubation at 72°C for 5 min for strand elongation. PCR for the detection of IS*6110* was that of Felmlee et al. (11).

Primer combinations were tested in a four-primer nested PCR protocol (rpo95 and rpo293 [72°C] and then rpo105 and rpo273 [74°C]) and a three-primer heminested protocol (rpo105 and rpo293 [72°C] and then rpo105 and rpo273 [74°C]). Thermal cycling for the first-round reaction was optimized by using 10-fold dilutions of cesium chloride-purified chromosomal DNA from *M. tuberculosis* ATCC 25177 (H37RA). Conditions for both amplifications included an initial denaturation at 94°C for 4 min and a final incubation at 72°C for 5 min for strand elongation. Following the 25-μl first-round PCR at 72°C with or without Ampliwax (Perkin-Elmer Cetus), a 75-μl volume of the second-round master mixture was added directly to the reaction tube, and the resultant 100-μl volume was cycled 50 times using 74°C as the annealing temperature.

Detection and analysis of PCR products. The products of the PCRs were detected by gel electrophoresis of 5 μl in 2.5% (wt/vol) agarose (Seakem GTG; FMC, Rockland, Maine) in 0.089 M Tris–0.089 M borate–2 mM EDTA (TBE); this was followed by staining with ethidium bromide.

Amplification products from clinical specimens were transferred to nylon membranes (Nytran 77593; Schleicher & Schuell, Keene, N.H.). The denatured, cross-linked amplicon was probed with Probe178, a ^{33}P-end-labeled oligonucleotide that included two more signature nucleotides (5′-ACC AGC CAG CTG AGC CAA-3′).

The amplicons generated from specimens that were culture negative for *M. tuberculosis* were purified with the Wizard PCR kit (Promega, Madison, Wis.), and 4 μl was used in automated dideoxyribonucleotide cycle sequencing reactions (Applied Biosystems 373A) with primer rpo105. Sequence alignment was performed using Genetics Computer Group programs (Madison, Wis.) run on the VAX computer system at the Mayo Clinic Research Computing Facility, Rochester, Minn.

Single-strand conformation polymorphism. Nested PCR was performed on clinical specimens, including a control specimen from a patient known to be infected with MDR *M. tuberculosis*, as described above except that half of the volume of dCTP in the second reaction mixture was replaced with [α-^{32}P]dCTP. To avoid direct detection PCR that involved radioactive substances, 1 μl of amplicon generated from clinical specimens could serve as the template in a 25-μl, 30-cycle PCR (28) with rpo105 and rpo273. Three microliters of the radiolabeled product was mixed with 3 μl of loading buffer, placed in a 100°C water bath for 3 min, immediately placed on ice, and loaded in 1.5-μl volumes onto a nondenaturing 0.5× MDE (Mutation Detection Enhancement) (Hy-

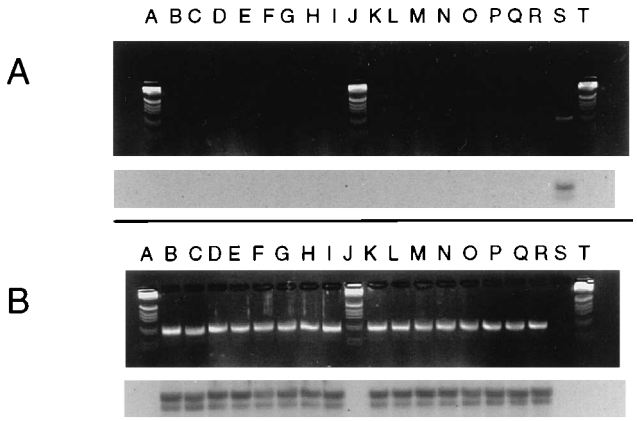

FIG. 2. Specificities of primers rpo105 and rpo273 in *rpoB* PCR shown by agarose gel electrophoresis and Southern blotting (upper and lower sections of each panel, respectively). (A) MOTT. Lanes: B, *M. chelonae*; C, *M. nonchromogenicum*; D, *M. smegmatis*; E, *M. scrofulaceum*, F, *M. phlei*; G, *M. simiae*; H, *M. asiaticum*; I, *M. szulgai*; K, *M. malmoense*; L, *M. triviale*; M, *M. marinum*, N, *M. xenopi*; O, *M. avium*; P, *M. avium-M. intracellulare-M. scrofulaceum*; Q, *M. kansasii*; R, *M. gordonii*; S, *M. tuberculosis*. (B) Sixteen separate MDR *M. tuberculosis* isolates which harbor mutations that encompass seven nucleotides and five amino acid positions. Lane S, negative control. (A and B) Lanes A, J, and T, 1-kb ladders.

drolink; AT Biochem Inc., Malvern, Pa.) gel with 0.6× TBE buffer (28). Constant-wattage (20 W) electrophoresis was performed at 18°C on a Pokerface sequencing apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The gels were dried and exposed for 2 to 6 h to X-ray film for autoradiography. Amplification product that demonstrated an atypical SSCP pattern was purified, sequenced, and analyzed as outlined above.

RESULTS

Design of PCR primers for specific detection of *rpoB*. Nucleotide sequence conservation in the *M. tuberculosis rpoB* gene that flanked the 69-bp region associated with rifampin resistance was exploited in the design of our primers (Fig. 1). However, because this high degree of sequence conservation existed among several distinct taxonomic groups, we found that other GC-rich organisms produced false-positive reactions unless oligonucleotides were selected carefully. Subsequent analysis of the mycobacteria and related bacteria found in the respiratory tract revealed several nucleotide positions in this region that were unique to *M. tuberculosis*. We used two of these signature nucleotides to anchor the 3′-hydroxyl termini of primers rpo105 and rpo273 and tested them against a variety of phylogenetically related organisms and GC-rich respiratory bacteria at high PCR annealing stringencies. The culture lysates tested included those of the following mycobacteria: *M. avium-M. intracellulare-M. scrofulaceum* ($n = 3$), *M. chelonae* ($n = 3$), *M. fortuitum* ($n = 3$), *M. xenopi* ($n = 2$), *M. gordonii* (2), and mycobacteria other than *M. tuberculosis* (MOTT) ($n = 11$) *M. smegmatis*, *M. nonchromogenicum*, *M. phlei*, *M. simiae*, *M. asiaticum*, *M. triviale*, *M. malmoense*, *M. marinum*, *M. kansasii*, *M. scrofulaceum*, and *M. szulgai*. Culture lysates of the following other organisms were also tested: *Rhodococcus* spp. ($n = 4$), *Nocardia* spp. ($n = 4$), *Corynebacterium* spp. ($n = 2$), *Actinomyces* spp. ($n = 2$), *Propionibacterium* spp. ($n = 2$), and aerobic actinomycetes ($n = 2$). None of the 15 species of MOTT or members of the 7 other GC-rich genera were amplified with these two primers (Fig. 2). All lysates amplified with the less specific primer rpo95 or sequence-degenerate primers, which ensured the adequacy of the initial reactions and reagents for PCR amplification. *M. tuberculosis* lysates all yielded the expected 193-bp product, including 19 of a panel of 20 isolates that were resistant to isoniazid and rifampin (Fig. 2). Fourteen of those isolates were also resistant to streptomycin, and six were resistant to ethambutol. Point mutations in the 69-bp region of the *rpoB* gene encompassed seven nucleotide positions within five codons (29, 30). The one lysate that did not amplify was also PCR negative at two other targets: 16S (20) and *katG* (6). Probed Southern blots provided additional evidence of specificity, but they were not necessary because the same information was provided by the gel.

Sensitivity of *rpoB* detection. Although primers rpo105 and rpo273 were specific for *M. tuberculosis*, their sensitivity for detection *M. tuberculosis* ATCC 25177 (H37RA) chromosomal DNA in serial 10-fold dilutions were 3 to 4 $\log_{10}$ units lower than that of our IS*6110* PCR assay. Because locations of the 3′ hydroxyl group were fixed, we devised a single-tube heminested protocol with the goal of increasing sensitivity without compromising specificity (Fig. 3).

We determined by serial dilution of target DNA that primer pairs rpo95 and rpo293 or rpo105 and rpo293 amplified *M. tuberculosis* best at 72°C, whereas the optimal temperature for rpo105 and rpo273 was 74°C. Consequently, these respective annealing temperatures were used in the four-primer conventional nested and three-primer heminested PCR protocols. Both assays were equivalent in sensitivity, yielding a detectable product at dilutions of 10^{-6} (about 8 pg) of *M. tuberculosis* chromosomal DNA (Fig. 4). A live culture of *M. tuberculosis* was then serially diluted in pooled, processed sputum to optimize the first round of amplification and to determine if the substances present in respiratory tract samples would interfere with the assay. The cycle number for the first-round amplification was reduced from 30 to 25 c to eliminate the presence of a visible product seen in experiments with spiked sputa at

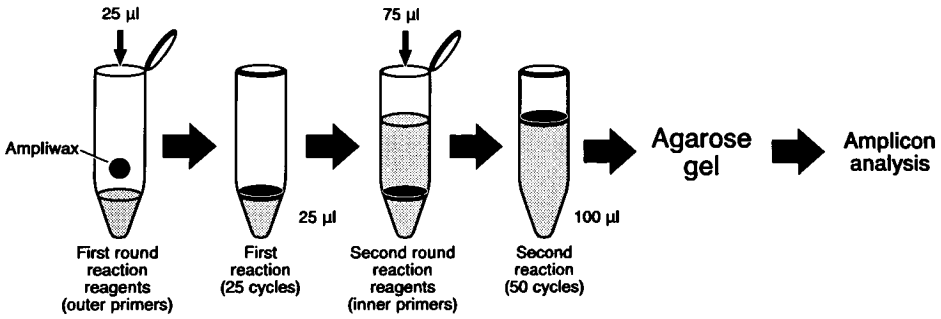

FIG. 3. Single-tube heminested PCR assay. Ampliwax is included in the first 25-µl limited-cycle reaction as an effective barrier to aerosol contamination during the addition of the second master mixture. The 75-µl mixture containing *M. tuberculosis*-specific primers dilute first-round primer(s) and product so that the second-round reaction at the higher annealing temperature is favored.

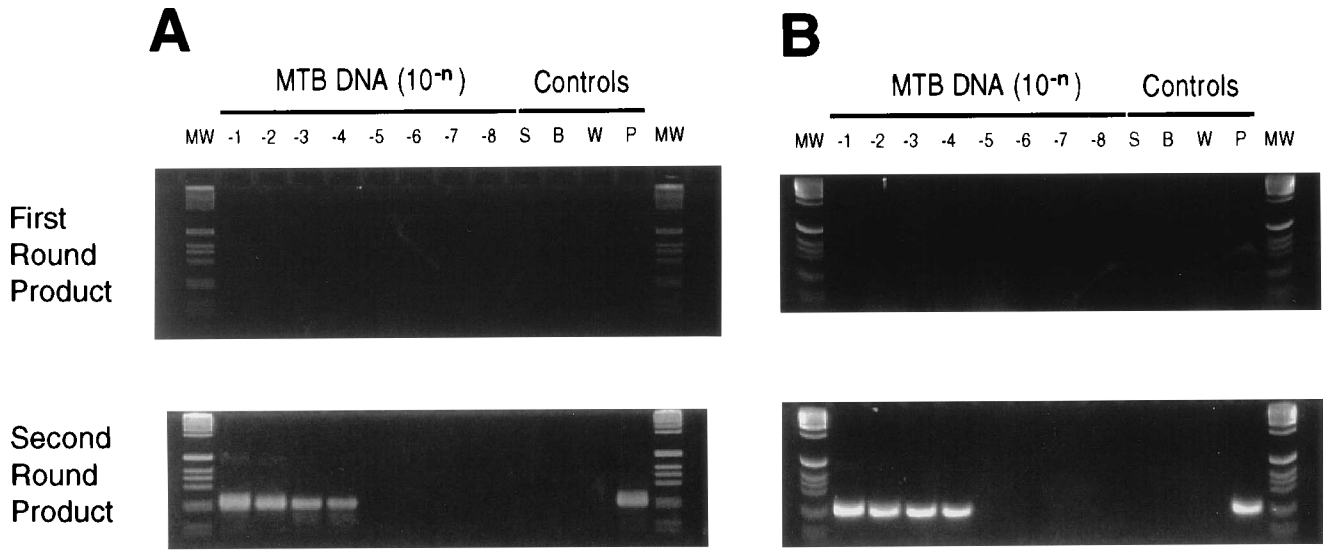

FIG. 4. Comparison of the four-primer nested (A) and three-primer heminested (B) protocols. With limited first-round thermocycling, both methods could detect ~8 pg of *M. tuberculosis* (MTB) chromosomal DNA spiked into pooled, processed sputa without compromising specificity. The endpoint dilution (sensitivity) was the same for IS*6110* PCR and culture (<10 colonies) in parallel testing of the spiked sputa. Southern blots (data not shown) were identical to the gels. MW, molecular weight markers. Lanes: S, sputum; B, lysis buffer; W, water; P, positive.

high dilutions and in negative control sputa when rpo95 (a less specific oligonucleotide) was used in the four-primer conventional nested protocol. When the three-primer nest was used, the first-round product was inapparent except for a faint band seen only on blotting (data not shown) at high concentrations of *M. tuberculosis* target DNA. Colony counts of the corresponding dilutions of *M. tuberculosis* indicated that the *rpoB* assay can detect <10 organisms, which was comparable to the sensitivity of culture and an assay based on detection of IS*6110* (i.e., all tests had the same dilution endpoint). All 20 lysates in the MDR *M. tuberculosis* panel were positive at a 1:10 dilution with the three-primer heminested protocol, including the one that had been negative with primers rpo105 and rpo273 alone, as well as with the primers for the other two genomic targets.

Detection of *M. tuberculosis rpoB* directly from clinical specimens. Fifty-one clinical specimens representing a variety of anatomical sources composed a blinded challenge panel (Table 1). The assay detected 21 of 24 culture-positive specimens (87.5%), including 13 that were auramine-rhodamine smear negative and one that was IS*6110* negative. However, 3 of 27 specimens (11.1%) that were IS*6110* and culture negative for *M. tuberculosis* were positive for *rpoB*. The amplicons were sequenced, and all of the *M. tuberculosis* signature nucleotides were present, indicating that the amplification was not due to false priming. We suspected that the most likely source of false-positive results in this assay was the requirement for opening the reaction vessels after first-round PCR and the aerosol-prone addition of the second-round reagents. When PCR was repeated with Ampliwax to contain the first-round products (Fig. 3), culture-positive specimens remained PCR positive and all of the culture-negative specimens were PCR negative, raising the specificity from 88.9 to 100% (data not shown).

In order to determine the *rpoB* genotypes of those specimens that were PCR positive, SSCP analysis and/or automated cycle sequencing was performed. SSCP patterns were indicative of the wild-type, rifampin-susceptible genotype, which correlated with in vitro susceptibility results (Fig. 5). One specimen from a patient infected with *M. tuberculosis* resistant to rifampin and five other antimycobacterial agents gave rise to an amplification product demonstrating a subtle upward shift of the largest SSCP species typical of the serine-to-leucine mutation at amino acid residue 531 of *rpoB* (27). Sequence analysis of the *rpoB* product proved this mutation to be present (7).

DISCUSSION

The mapping and sequencing of mutations in the *Escherichia coli rpoB* gene associated with rifampin resistance by Ovchinnikov et al. (24) and more recently by Jin and Gross (18)

TABLE 1. Direct detection of *M. tuberculosis rpoB* in 51 clinical specimens

Specimen source (no. of specimens)[a]	Result[b]			
	rpoB	IS*6110*	Smear	Culture
Respiratory (8)	+	+	+	TB
Respiratory (11)	+	+	−	TB
Respiratory (1)	+	−	−	TB
Respiratory (2)	−	−	−	TB
Respiratory (6)	−	−	−	Neg
Respiratory (3)	−	−	+	MOTT
Respiratory (12)	−	−	−	MOTT
Respiratory (1)	+[c]	−	−	MOTT
Respiratory (1)	+[c]	−	+	MOTT
Respiratory (1)	+[c]	−	−	Neg
Nonrespiratory-GW (1)	+	+	−	TB
Nonrespiratory-U (1)	−	−	−	TB
Nonrespiratory-A (1)	−	−	+	Neg
Nonrespiratory-G, GW (2)	−	−	−	MOTT

[a] Respiratory specimens include sputum, induced sputum, and bronchial wash/lavage specimens. GW, gastric wash; U, urine; A, ankle; G, groin.

[b] TB, *M. tuberculosis*; Neg, negative; MOTT, mycobacteria other than *M. tuberculosis*.

[c] False-positive result that was most likely due to aerosol contamination since the amplicon contained signature sequences and repeat *rpoB* PCR with Ampliwax was negative.

FIG. 5. SSCP analysis of *rpoB* amplicon from clinical specimens. All amplification products except those in the two rightmost lanes exhibited the rifampin-susceptible genotype, which correlated with in vitro susceptibility tests. The last two lanes contain amplicon (note the subtle upward shift of the top band) from a specimen that grew *M. tuberculosis* that was resistant to isoniazid, rifampin, pyrazinamide, ethanbutol, ethionamide, and cycloserine and that displayed a Ser-to-Leu (TCG to TTG) mutation at *rpoB* amino acid position 456 (position 531 by *E. coli* alignment).

facilitated the discovery of *rpoB* mutations that are associated with rifampin resistance in *M. tuberculosis* (27). The mutations identified thusfar occur in a 69-bp region and predominate within the histidine and serine codons at positions 526 and 531, respectively, relative to the *E. coli rpoB* sequence alignment. Miller et al. (22) described the entire sequence of *rpoB* in *M. tuberculosis*, and they have appropriately numbered these sites independently of the numbering in *E. coli*; i.e., serine-531 is now serine-456. Recently, additional polymorphisms in *rpoB* have been described in rifampin-resistant isolates from New York City and Texas (19, 29); however, with few exceptions, rifampin resistance-associated mutations are located in the 69-bp core region.

In previous reports, detection and characterization of *rpoB* have been limited to organisms isolated in culture or specimens that were acid-fast smear positive (19, 28). Hunt et al. (15) used degenerate priming to generate amplicons from *M. tuberculosis* and a variety of other GC-rich organisms including MOTT. Sequence analysis of those amplification products led to the identification of nucleotides that were unique to *M. tuberculosis*. By exploiting signature nucleotides to ensure primer specificity, we developed a single-tube heminested protocol to improve the sensitivity of detection of *M. tuberculosis* in specimens that were smear negative. This is a potentially important advantage, since the accepted sensitivity of acid-fast staining is in the range of 30 to 40%. Nucleic acid amplification tests directed at other targets, such as our IS*6110* assay, have similar sensitivities; however, it is not possible to evaluate the product for direct evidence of drug resistance. Indirect evidence for resistance has been reported by using novel approaches such as mixed-linker PCR of IS*6110* and then restriction fragment length polymorphism analysis (14).

On an operational basis, genotypic detection of rifampin susceptibility should be as sensitive as the primary assay used for direct PCR-based detection. Otherwise, determination of drug-resistant *M. tuberculosis* in many PCR-positive specimens will be delayed by the need to recover the organism in culture. Potential approaches in a laboratory setting might include this *rpoB* PCR assay as a stand-alone method for both the direct detection of *M. tuberculosis* and the determination of rifampin susceptibility or resistance or its use in combination with another direct detection assay (16S rRNA, IS*6110*, etc.). Because single-drug rifampin resistance occurs rarely in the United States (about 0.4% of *M. tuberculosis* isolated from January to March 1991 [1]), *rpoB* mutations may serve as surrogate markers for MDR *M. tuberculosis.*

Three specimens were positive only by culture, which remains the "gold standard" for samples that contain few organisms. Cultures that were positive for only a few colonies of *M. tuberculosis*, such as these, represent the total number of viable organisms in 2.5 ml of sputum lysate. This is approximately 200 times the volume used for our PCR after adjusting for the cell lysis procedure and the extraction and precipitation of DNA. Thus, the simple absence of target DNA in this small aliquot might easily explain the negative result for these three specimens. Concentration methods, such as magnetic particle separation (23), may help to overcome this limitation.

Contamination control is critical to any nucleic acid amplification and is particularly important in nested protocols. Methods that have been successfully used include substitution of dTTP with dUTP, which makes amplification products susceptible to uracil *N*-glycosylase degradation (9, 21, 26), and UV inactivation in the presence of isopsoralen (5, 9, 26). Both techniques have resulted in amplicon inactivation in this system (data not shown); however, methods that use isopsoralen have limited utility in the system described here because they constrain the methods by which amplicons can be analyzed, especially sequencing reactions.

The most contamination-prone step in nested protocols is the open transfer of first-round amplification products into a second tube for the final reaction. We eliminated the need for open transfer by using a single-tube approach that diluted the first-round reaction components and were careful to ensure that only one tube was open at a time over a bleach-soaked surface. Even with these precautions, we experienced three false-positive results in our first evaluation of the blinded clinical panel. They were most likely due to the aerosols created during the opening of the tubes after the first round of amplification to add second-round components. By adding Ampliwax to each tube before the first reaction, we established a solid barrier that effectively isolated the first-round amplicon and that allowed for the safe addition of the second reaction mixture and the elimination of false-positive results. Experiments in our laboratory have shown that complete isolation of the first-round reaction components can be achieved by this method (data not shown). Theoretically, this novel approach to nested amplification can be adapted to a wide variety of diagnostic protocols for which high degrees of sensitivity and high yields are requirements.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Williams et al. (30) reported previously undescribed mutations found in rifampin-resistant isolates of *M. tuberculosis* that are not shown in Fig. 1. These mutations are located at nucleotide positions 2364 (G to C), 2376 (A to C), 2385 (A to G), 2399 (C to A), and 2417 (A to C).

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