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Detection of Rifampin-Resistant *Mycobacterium tuberculosis* in Sputa by Nested PCR-Linked Single-Strand Conformation Polymorphism and DNA Sequencing

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Either PCR-mediated single strand conformation polymorphism (SSCP) analysis or DNA sequencing of *rpoB* DNA (157 bp) can be used as a rapid screening method for the detection of mutations related to the rifampin resistance of *Mycobacterium tuberculosis*. However, due to the nonspecific amplification of *rpoB* DNA from nontuberculous mycobacteria these methods cannot be directly applied to clinical specimens such as sputa. We developed a nested PCR method that can specifically amplify the *rpoB* DNA of *M. tuberculosis* on the basis of *rpoB* DNA sequences of 44 mycobacteria. Nested PCR-linked SSCP analysis and the DNA sequencing method were applied directly in order to detect *M. tuberculosis* and determine its rifampin susceptibility in 56 sputa. The results obtained by nested PCR-SSCP and DNA sequencing were concordant with those of conventional drug susceptibility testing and DNA sequencing performed with culture isolates.

Mycobacterium tuberculosis is still regarded as a causative agent of high morbidity and mortality throughout the world (5). Due to the spread of human immunodeficiency virus infection, the decline in the incidence of tuberculosis which had been brought about by advanced antituberculosis chemotherapy and improved living conditions was reversed in the mid-1980s. Human immunodeficiency virus-related tuberculosis led to a rise in the frequency of multidrug-resistant *M. tuberculosis* (1, 7). The rapid detection of resistance to first-line drugs such as rifampin and isoniazid is essential for the efficient control of multidrug-resistant strains (3, 9, 16). Although the period required for culturing is shortened by the BACTEC system, drug susceptibility testing in a liquid medium still requires 1 to 2 weeks for final determination and report to the clinicians (19), calling for further reduction of the detection period.

Recently, molecular methods exploiting the genetic mechanism of drug resistance have markedly improved the diagnosis of drug-resistant tuberculosis (17, 20). Rifampin resistance of *M. tuberculosis* is largely associated with point mutations in a region of *rpoB* (20), mutations which cause rifampin resistance to a high level in *Escherichia coli* (10, 14). Various molecular methods have been applied to detect these unique mutations, including PCR-mediated single-strand conformation polymorphism (SSCP) analysis (12, 15, 21), single-tube heminested PCR-SSCP (23), the dideoxy fingerprinting method (6), line probe assay (4), and DNA sequence analysis (11, 20). For the rapid detection or determination of rifampin resistance, it is desirable to apply these methods directly to primary specimens, such as sputa, as well as cultures. Among these methods, PCR-SSCP may be the most cost-effective method for detecting point mutations within the 69-nucleotide region. However, the direct detection of rifampin-resistant *M. tuberculosis* in sputa by conventional PCR-SSCP has a drawback in that it lacks sensitivity. Nonspecific amplification due to the highly conserved sequences of *rpoB* DNA among GC-rich bacteria that may reside in the respiratory tract has led to difficulty in interpreting PCR-SSCP results (21). For this reason, earlier studies using this technique required the use of pure cultures of *M. tuberculosis*. To resolve these problems, a heminested PCR method based on *M. tuberculosis* signature nucleotides was introduced (23). Although heminested PCR was effective in amplifying *M. tuberculosis*-specific *rpoB* DNA directly from sputum samples, applying this method to SSCP analysis could not resolve mutations at codon 526 (in *E. coli* numbering). Furthermore, when compared with the conventional PCR-SSCP analysis of 157-bp DNA, this method required a longer electrophoresis time for the clear differentiation of bands in several resistant strains (6).

In this study, we developed a nested PCR that can specifically amplify the *rpoB* DNA of *M. tuberculosis*. Based on the 44 *rpoB* sequences of mycobacteria (11), we were able to design *M. tuberculosis*-specific primers for nested PCR. Nested PCR-linked SSCP analysis and a direct DNA sequencing method were applied to detect mutations of *M. tuberculosis* in sputa. This enabled us to detect mutations related to rifampin resistance occurring within the 69-nucleotide region of *rpoB* DNA derived from 56 sputa. The results were compared with those obtained by conventional PCR-SSCP, drug susceptibility test-

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TABLE 1. Mycobacteria and nonmycobacteria used to determine the specificity of the first-round PCR

Species	Type strains[a]	No. of clinical isolates[b]
Mycobacteria		
M. avium	ATCC 25291	6
M. fortuitum	ATCC 6841	4
M. gordonae	ATCC 14470	2
M. kansasii	ATCC 12478	5
M. nonchromogenicum	ATCC 19530	0
M. scrofulaceum	ATCC 19981	0
M. smegmatis	ATCC 19420	0
M. terrae	ATCC 15755	0
M. triviale	ATCC 23292	0
M. vaccae	ATCC 15483	0
M. chelonae	ATCC 35749	3
M. gastri	ATCC 15754	0
M. intracellulare	ATCC 13950	8
M. malmoense	ATCC 29571	0
M. phlei	ATCC 11758	0
M. simiae	ATCC 25275	0
M. szulgai	ATCC 35799	0
M. tuberculosis	ATCC 27294	20
M. ulcerans	ATCC 19423	0
Nonmycobacteria		
Rhodococcus equi	IMSNU20114	0
Rhodococcus erythropolis	IMSNU20115	0
Rhodococcus rhodochrous	IMSNU20349	0
Nocardia otitidiscaviarum	IMSNU21221	0
Nocardia nova	IMSNU21197	0
Corynebacterium diphtheriae	MSNU	0
Corynebacterium glutamicum	IMSNU21196	0
Neisseria meningitidis	MSNU	0
Haemophilus influenzae	MSNU	0

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[b] Clinical isolates were identified by biochemical tests and partial 16S rDNA sequencing.

ing, and the DNA sequence analysis of cultured *M. tuberculosis* from the same sputum samples.

MATERIALS AND METHODS

Mycobacteria and DNA preparation. Twenty-eight reference strains (19 strains of mycobacteria and 9 strains of nonmycobacteria) and 48 clinical isolates of mycobacteria that had been isolated from patients with mycobacterial infections were used to determine the specificity of a first-round PCR (Table 1). Fifty-six sputum specimens from patients with suspected *M. tuberculosis* infections and *M. tuberculosis* isolates from culture of the same specimens were provided by the Korean Institute of Tuberculosis for the nested PCR-linked SSCP and direct sequencing. Amplification of *IS6110* DNAs (536 bp) and positive cultures also supported the presence of *M. tuberculosis* in all of the sputa. Clinical isolates were identified by conventional biochemical tests and partial 16S rDNA sequencing.

DNAs were purified by a previously described method (2, 11). A loopful culture of each strain was suspended with 200 μl of TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl [pH 8.0]) and placed in a 2.0-ml screw-cap microcentrifuge tube filled with 100 μl (packed volume) of glass beads (diameter, 0.1 mm; Biospec Products, Bartlesville, Okla.), and then 200 μl of phenol-chloroform-isoamylalcohol (50:49:1) was added to this mixture. The screw-cap tube filled with the mixture was oscillated on a Mini-Bead beater (Biospec Products) for 1 min to disrupt the bacteria. The tube was centrifuged (12,000 × *g*, 5 min), and the aqueous phase was transferred into another clean tube, to which 10 μl of 3 M sodium acetate and 130 μl of isopropyl alcohol were added. The DNA pellet was washed with 70% ethanol and solubilized with 60 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Two microliters of purified DNA was used as the template in the PCRs. The sputa were processed by 1% NaOH for liquefaction-decontamination and sedimentation (12,000 × *g*, 15 min) (18). The sediments were resuspended in 1.5 ml of phosphate buffer (pH 6.8) and 0.5 ml was inoculated onto Löwenstein-Jensen media. DNAs were isolated from the residual sediments (1.0 ml) of smear-positive sputa as above and dissolved in 60 μl of TE buffer (pH 8.0). Two microliters of prepared DNA in TE buffer was used as the template in both the conventional PCR and the nested PCR.

Identification and rifampin susceptibility testing of *M. tuberculosis*. Clinical isolates of *M. tuberculosis* were tested at the Korean Institute of Tuberculosis for drug susceptibility by agar dilution (1% proportion method) (13). Fifty-six sputa and the above-mentioned clinical isolates underwent nested PCR-SSCP and sequencing without prior information as to their rifampin susceptibility. Separate PCRs (22) were performed on these specimens to detect and identify *M. tuberculosis* in both sputa and cultures using an *IS6110* kit (catalog no. N5811; Bioneer, Chungbuk, Korea) according to the manufacturer's instruction.

Conventional PCR-linked SSCP and DNA sequencing. Conventional PCR-SSCP was performed as previously described, using TR9 and TR8 primers (12, 21). Separately, 56 culture isolates of *M. tuberculosis* derived from the same specimens were analyzed by conventional PCR-DNA sequencing. The amplified products were purified by QIAEX II gel elution system (QIAGEN, Hilden, Germany). Nucleotide sequences were directly determined from the purified PCR product (157 bp) with forward and reverse primers using an Applied Biosystems 373A automatic sequencer and BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Warrington, United Kingdom). For the sequencing reaction, 60 ng of PCR-amplified DNA, 3.2 pmol of either the forward or the reverse primer and 8 μl of BigDye Terminator RR mix (part no. 4303153; PE Applied Biosystems) were mixed and adjusted to a final volume of 20 μl by adding distilled water. The reaction was run using 5% (vol/vol) dimethylsulfoxide for 30 cycles of 15 s at 95°C, 10 s at 50°C, and 4 min at 60°C. Both strands were sequenced for cross check.

Nested PCR-linked SSCP and DNA sequencing. *M. tuberculosis*-specific nucleotides have been found in the *rpoB* sequences (8, 11). The outer primers TB1 (5′-**A**CGTGGAGGCGATCACACCGCAGACGT-3′) and TB2 (5′-TGCAC GTCGCGGACCTCCA**GCCCGGCA**-3′) were modified from *Rpo*105 (23) and TR8, respectively (21) (modifications are in bold). The inner primers for the second-round PCR were TB3 (5′-TCGCCGCGATCAAGGAGT**TCTTC**-3′), which was modified from TR9, and TR8 (21) (Fig. 1). The first-round PCR was performed in a 20-μl PCR mixture tube (AccuPower PCR PreMix; Bioneer) containing 2 U of *Taq* polymerase, 10 mM Tris-HCl (pH 8.3), and 1.5 mM $MgCl_2$, and 20 pmol of each primer was added. The volume was adjusted to 20 μl. The reaction mixture was subjected to 30 cycles of amplification (30 s at 95°C, 60 s at 78°C) followed by a 5-min extension at 78°C in a Perkin-Elmer Cetus Model 9600 thermalcycler. The first-round PCR product (205 bp) was diluted (100-fold) and used as a template for the second-round PCR (157 bp), which was performed in 30 cycles (30 s at 95°C, 1 min at 72°C) followed by a 5-min extension at 72°C. SSCP analysis was performed as described above, except that 0.1 μl (0.1 μCi) of [α-^{32}P]dCTP (Amersham International) was added to the second reaction mixture. DNA sequencing was directly performed with nested PCR products as above.

Southern blotting. First-round PCR products were electrophoresed on a 1.5% (wt/vol) agarose gel (Sigma, Steinheim, Germany). The DNA on agarose gel was denatured with a solution of 1.5 M NaCl and 0.5 N NaOH and neutralized with a solution of 1 M Tris (pH 7.4) and 1.5 M NaCl. Subsequently, the denatured DNA was transferred to nylon membranes (Nytran 77593; Schleicher & Schuell, Inc., Keene, N.H.) by the capillary transfer method. The membranes were hybridized with ^{32}P-labeled 157-bp *rpoB* fragment probes, which were amplified by PCR using the TR9-TR8 primer set from *Mycobacterium avium* (ATCC 25291). The random primer labeling kit (Amersham, Arlington Heights, Ill.) was used for radiolabeling. Hybridization was performed at 68°C with a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 0.5% sodium dodecyl sulfate, 100 μg of salmon sperm DNA per ml, and 5× Denhardt solution. The membrane was washed twice at 65°C with a solution of 2× SSC and 0.1% sodium dodecyl sulfate for 30 min.

RESULTS

Amplification of *rpoB* DNA by nested PCR. The PCR product amplified by the first-round PCR (TB1-TB2 primer set) was 205-bp *rpoB* DNA comprising a 157-bp fragment which was previously used for SSCP analysis (12, 15, 21). The specificity of the first-round PCR was tested with the DNAs from the reference strains of 19 mycobacteria and 9 nonmycobacteria and 48 clinical isolates of mycobacteria. The products were

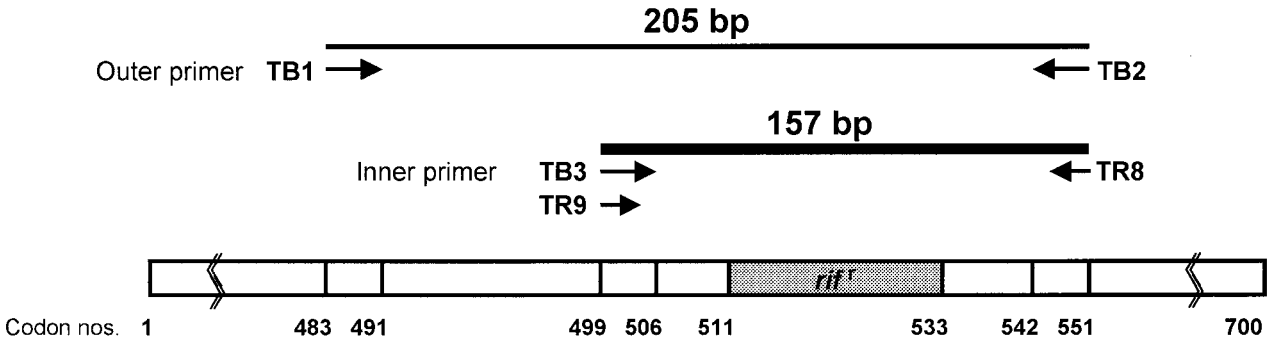

FIG. 1. Primers used for PCR and nested PCR. Conventional PCR was performed with the TR9-TR8 primer set as previously described. The first-round PCR was performed with the TB1-TB2 primer set amplifying 205-bp *rpoB* DNA. The second-round PCR was performed with the TB3-TR8 primer set to amplify 157-bp DNA from the first-round PCR product. Numbers indicate *rpoB* codons of *E. coli*.

amplified only from the type strain and 20 clinical isolates of *M. tuberculosis*. The specificity of the first-round PCR was verified by Southern blotting (Fig. 2). Nothing was amplified when the template DNAs were diluted to less than 10 fg. Thus, the sensitivity of nested PCR assay in terms of DNA amount in a reaction could be determined as 10 fg of DNA in ethidium bromide-stained gels (Fig. 3).

PCR- and nested PCR-SSCP analysis for sputa. When conventional PCR-SSCP using the TR9-TR8 primer set was directly applied to sputa, rifampin susceptibility of 11 of 56 samples could not be determined (Table 2). The targeted *rpoB* DNAs were rarely amplified from eight sputum specimens (N2895, N2994, N3440, N3448, N3903, N3912, N3990, and N4064). Three sputum specimens (N3104, N3928, and N3983) showed ambiguous SSCP patterns displaying more than three bands, which was possibly caused by simultaneous amplification of the *rpoB* DNA from both *M. tuberculosis* and other bacteria. Furthermore, one (N3928) of these three sputum specimens showed a false-positive PCR-SSCP result. Because the SSCP pattern differed from that of the rifampin-susceptible reference strain, it was identified as a rifampin-resistant strain (Fig. 4). In contrast, the nested PCR successfully produced a 157-bp DNA directly from all of the specimens. Therefore, the presence of mutations in the amplified *rpoB* DNA from all specimens could be determined easily by SSCP analysis (Tables 2 and 3). Typical SSCP patterns of *M. tuberculosis rpoB* DNA were observed (Fig. 4). The three sputum specimens that had shown ambiguous results by conventional PCR-SSCP analysis were clearly determined to be two rifampin-resistant strains (N3104 and N3983) and one susceptible strain (N3928) of *M. tuberculosis* by nested PCR-SSCP analysis (Fig. 4), susceptibility testing, and PCR direct sequencing of *rpoB* DNA from the culture (Table 3). The results of the nested PCR-SSCP method performed on sputum specimens were entirely concordant with those of cultures analyzed by drug susceptibility testing and conventional PCR-SSCP. Based on the result of susceptibility testing performed with cultures, conventional PCR-SSCP analysis for sputa showed only 75.8% sensitivity and 87% specificity (Table 2).

Nested PCR-linked DNA sequencing for sputa. We also applied both the conventional and the nested PCR sequencing methods directly to sputa. The advantage of nested PCR-linked sequencing was revealed when *rpoB* DNA from the three specimens (N3104, N3928, and N3983) which had shown ambiguous results in conventional PCR-SSCP was selectively amplified by nested PCR. Thus, mutations related to rifampin resistance were definitely determined by direct sequencing. It was interesting to note that the results of conventional PCR sequencing did not coincide with those of nested PCR sequencing. The peaks on the electropherogram of conventional PCR sequencing were confusing in one sample (N3983) (Fig. 5, upper panel). The third nucleotide (CG**A**) at codon 529, which is a specific nucleotide of *M. tuberculosis* (8, 11), was not

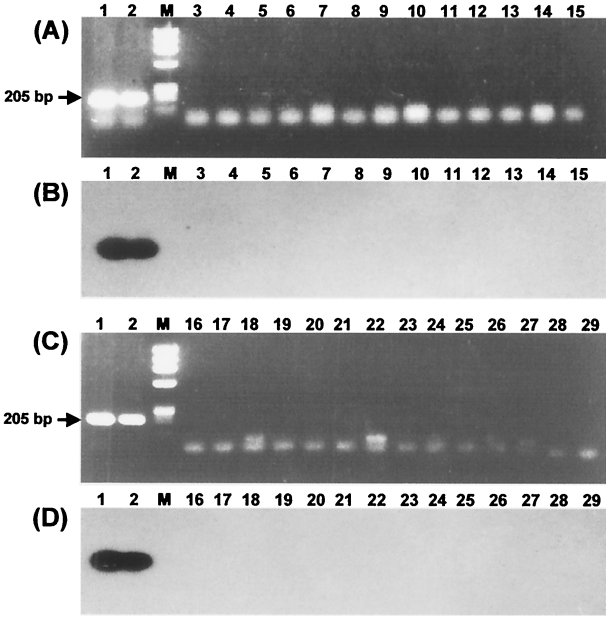

FIG. 2. Specific amplification of *M. tuberculosis rpoB* DNA by first-round PCR (TB1-TB2 primer set). The Southern blot shows that the PCR product (205 bp) was amplified from only *M. tuberculosis* by the first-round PCR. (A and B) Lanes: 1, *M. tuberculosis* H37Rv; 2, clinical isolate of *M. tuberculosis*; M, ϕX174/RF DNA/*Hae*III digest; 3, *M. avium*; 4, *M. fortuitum*; 5, *M. gastri*; 6, *M. gordonae*; 7, *M. intracellulare*; 8, *M. kansasii*; 9; *M. malmoense*; 10, *M. nonchromogenicum*; 11, *M. phlei*; 12, *M. scrofulaceum*; 13, *M. simiae*; 14, *M. smegmatis*; 15, *M. terrae*. (C and D) Lanes 16, *M. triviale*; 17, *M. vaccae*; 18, *M. chelonae*; 19, *M. szulgai*; 20, *M. ulcerans*; 21, *Rhodococcus equi*; 22, *Rhodococcus erythropolis*; 23, *Rhodococcus rhodochrous*; 24, *Nocardia otitidiscaviarum*; 25, *Nocardia nova*; 26, *Corynebacterium glutamicum*; 27, *Corynebacterium diphtheriae*; 28, *Neisseria meningitidis*; 29, *Haemophilus influenzae*.

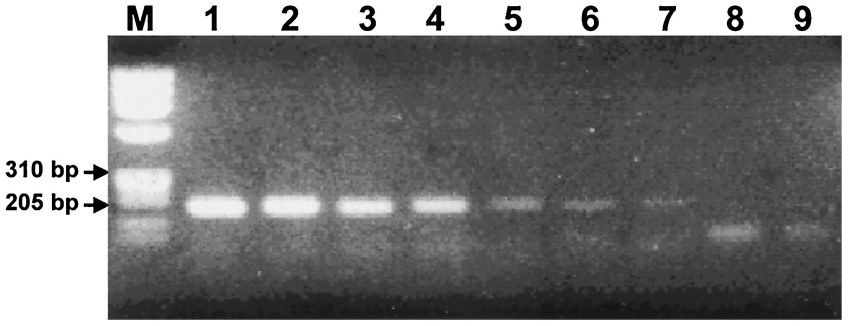

FIG. 3. Amplification of *rpoB* DNA by nested PCR using serially diluted *M. tuberculosis* DNA as templates. Amplified products are observed only in the lanes that used more than 10 fg of template DNA. Lanes: M, ϕX174/RF DNA/*Hae*III digest; 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, 10 fg; 8, 1 fg; 9, negative control without DNA.

clearly determined by conventional PCR direct sequencing. Furthermore, PCR sequencing misidentified codon 531 as T**C**G, which reflects the rifampin-susceptible strain. However, the second nucleotide at codon 531, which is the most frequent site of mutation related to rifampin resistance, was determined as T**T**G by nested PCR sequencing (Fig. 5, lower panel). The sequence analysis of *rpoB* DNA performed on a culture isolate from the same sputum (N3983) showed a mutation at Ser_{531} (TCG→T**T**G).

Rifampin susceptibility of culture isolates. Culture isolates of *M. tuberculosis* from sputa were analyzed in order to determine their rifampin susceptibility using the agar dilution method and PCR direct sequencing. Results from the phenotypic and genotypic analyses were identical. All the sequences determined by PCR sequencing of culture isolates were concordant with those of sputa determined by nested PCR sequencing but not with those determined by conventional PCR sequencing.

Twenty-three strains displayed point mutations that are correlated with rifampin resistance as determined by the agar dilution method. Mutations in each codon of eight amino acids (Gln_{513}, Met_{515}, Asp_{516}, Gla_{517}, Asn_{518}, His_{526}, Ser_{531}, and Leu_{533}) were found. The highest frequency (61%) was observed at Ser_{531}. Resistant strains were also easily differentiated in a sequence-specific manner with the PCR-SSCP analysis (Fig. 6). Interestingly, novel genotypes were observed in two specimens. One strain (N3471) harbored 5 mutations (AT**G** G**AC** **CAG** **AAC**→AT**C** G**CC** **AAC** **TAC**) at four consecutive codons, which led to changes of four amino acids (Met_{515} $Asp_{516}Gla_{517}Asn_{518}$→$Ile_{515}Ala_{516}Asn_{517}Tyr_{518}$). The other (N3522) harbored a double mutation (**TC**G→**AT**G) at Ser_{531}. These two strains also showed novel SSCP patterns (Fig. 6).

TABLE 2. Rifampin susceptibility of *M. tuberculosis* in sputa determined by conventional PCR-SSCP and nested PCR-SSCP analyses

Method	Result	No. of sputum samples found by rifampin susceptibility testing[a] to be:	
		Susceptible (n = 33)	Resistant (n = 23)
PCR-SSCP	Susceptible	25	0
	Resistant	0	20
	Not determined[b]	8	3
Nested PCR-SSCP and DNA sequencing	Susceptible	33	0
	Resistant	0	23
	Not determined	0	0

[a] Agar dilution method performed with cultures from the same sputa used for SSCP analysis.
[b] SSCP patterns could not be interpreted due to no amplification or more than three bands.

DISCUSSION

Missense mutations, usually confined to the 69-nucleotide region of the *rpoB* gene, are related to the rifampin resistance of *M. tuberculosis*. Using molecular techniques to detect these

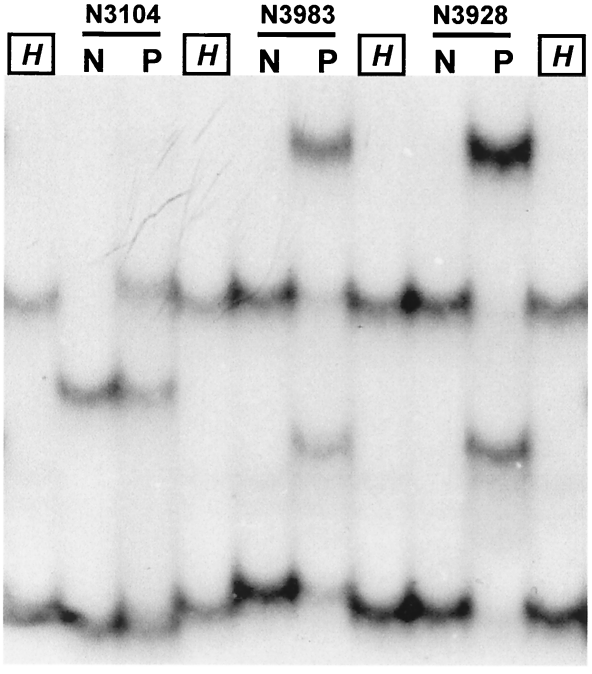

FIG. 4. Comparison of PCR-SSCP and nested PCR-SSCP performed with three sputum specimens, N3104 (His_{526} [C**A**C]→Arg [C**G**C]), N3928 (wild type), and N3983 (Ser_{531} [**TT**G]→Leu [**TC**G]). Patterns of conventional PCR-SSCP using the TR9-TR8 primer set showed more than two bands (lanes P). They were unusual in PCR-SSCP analysis performed with culture. However, nested PCR-SSCP showed only two bands by which the rifampin resistance of *M. tuberculosis* could be determined as other reports (lanes N). H, *M. tuberculosis* H37Rv.

TABLE 3. Comparison of results determined by three different methods to determine rifampin susceptibility of *M. tuberculosis* in sputa and their culture isolates

Specimen no.	Clinical specimen designation	Resistance to[a]:				Result from:		
		RIF	INH	STR	EMB	Conventional PCR-SSCP or direct sequencing[b]	Nested PCR-SSCP or direct sequencing[b]	DNA sequencing
1	N2838	S	S	S	S	Wild type	Wild type	No mutation
2	N2872	S	S	S	S	Wild type	Wild type	No mutation
3	N2877	R	R	S	R	Abnormal	Abnormal	Mutation
4	N2893	R	R	S	R	Abnormal	Abnormal	Mutation
5	N2895	R	R	S	S	Not determined[c]	Abnormal	Mutation
6	N2902	R	R	S	R	Abnormal	Abnormal	Mutation
7	N2903	R	R	S	R	Abnormal	Abnormal	Mutation
8	N2904	S	S	S	S	Wild type	Wild type	No mutation
9	N2910	R	R	R	R	Abnormal	Abnormal	Mutation
10	N2984	R	R	S	S	Abnormal	Abnormal	Mutation
11	N2994	S	S	S	S	Not determined	Wild type	No mutation
12	N2999	S	S	S	S	Wild type	Wild type	No mutation
13	N3000	S	S	S	S	Wild type	Wild type	No mutation
14	N3012	S	S	S	S	Wild type	Wild type	No mutation
15	N3085	S	S	S	S	Wild type	Wild type	No mutation
16	N3096	S	S	S	S	Wild type	Wild type	No mutation
17	N3104	R	R	R	S	Ambiguous[d]	Abnormal	Mutation
18	N3105	S	S	S	S	Wild type	Wild type	No mutation
19	N3106	S	S	S	S	Wild type	Wild type	No mutation
20	N3426	S	S	S	S	Wild type	Wild type	No mutation
21	N3428	S	S	S	S	Wild type	Wild type	No mutation
22	N3440	R	R	S	R	Not determined	Abnormal	Mutation
23	N3448	S	S	S	S	Not determined	Wild type	No mutation
24	N3471	R	R	S	S	Abnormal	Abnormal	Mutation
25	N3474	R	R	S	S	Abnormal	Abnormal	Mutation
26	N3496	R	R	R	R	Abnormal	Abnormal	Mutation
27	N3500	S	S	S	S	Wild type	Wild type	No mutation
28	N3501	R	R	S	S	Abnormal	Abnormal	Mutation
29	N3507	S	S	S	S	Wild type	Wild type	No mutation
30	N3522	R	R	S	R	Abnormal	Abnormal	Mutation
31	N3523	R	R	S	S	Abnormal	Abnormal	Mutation
32	N3524	R	R	S	R	Abnormal	Abnormal	Mutation
33	N3536	R	R	S	S	Abnormal	Abnormal	Mutation
34	N3606	S	S	S	S	Wild type	Wild type	No mutation
35	N3874	R	R	S	S	Abnormal	Abnormal	Mutation
36	N3878	S	S	S	S	Wild type	Wild type	No mutation
37	N3903	S	S	S	S	Not determined	Wild type	No mutation
38	N3912	S	S	S	S	Not determined	Wild type	No mutation
39	N3914	S	S	S	S	Wild type	Wild type	No mutation
40	N3924	S	S	S	S	Wild type	Wild type	No mutation
41	N3928	S	S	S	S	Ambiguous	Wild type	No mutation
42	N3983	R	R	S	S	Ambiguous	Abnormal	Mutation
43	N3986	R	R	S	R	Abnormal	Abnormal	Mutation
44	N3990	S	S	S	S	Not determined	Wild type	No mutation
45	N3992	S	S	S	S	Wild type	Wild type	No mutation
46	N3993	S	S	S	S	Wild type	Wild type	No mutation
47	N4025	R	R	S	R	Abnormal	Abnormal	Mutation
48	N4030	R	R	R	R	Abnormal	Abnormal	Mutation
49	N4062	S	S	S	S	Wild type	Wild type	No mutation
50	N4064	S	S	S	S	Not determined	Wild type	No mutation
51	N4069	S	R	S	R	Wild type	Wild type	No mutation
52	N4115	R	R	S	S	Abnormal	Abnormal	Mutation
53	N4116	S	S	S	S	Wild type	Wild type	No mutation
54	N4123	S	S	S	S	Wild type	Wild type	No mutation
55	N4159	S	S	S	S	Wild type	Wild type	No mutation
56	N4165	S	S	S	S	Wild type	Wild type	No mutation

[a] RIF, rifampin; INH, isoniazid; STR, streptomycin; EMB, ethambutol; S, susceptible; R, resistant.
[b] Wild type or Abnormal, identical SSCP patterns and sequences or different from that of reference strain, respectively.
[c] Products were poorly amplified for the SSCP analysis.
[d] The extra bands originating from other bacteria which may coexist with *M. tuberculosis* interfered with SSCP analysis.

mutations is more rapid than rifampin susceptibility testing which depends on culture and therefore requires an additional 4 to 6 weeks after the primary isolation to obtain the results. Although problems due to silent mutations were recently described (12), rifampin resistance in *M. tuberculosis* was successfully determined by PCR-SSCP (12, 15, 21) and PCR direct sequencing (11, 20) when the DNA was prepared from cultures. However, it has not been easy to apply these methods directly to sputa because of the poor sensitivity and specificity of PCR (8, 20). If PCR could be performed with sputa as well

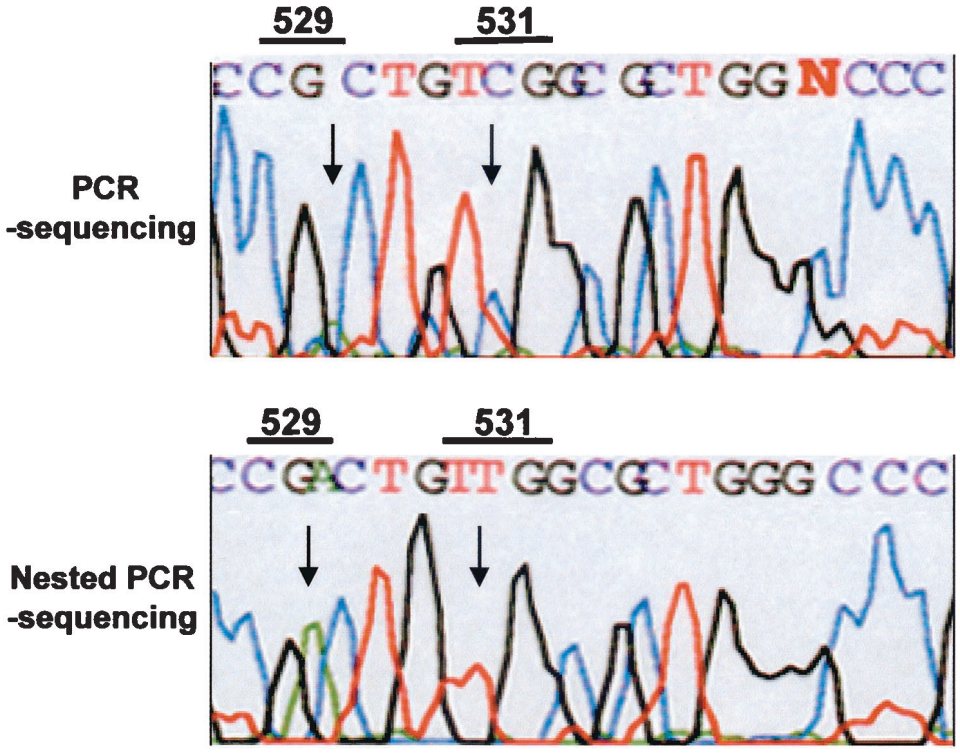

FIG. 5. Electropherograms of automatic DNA sequencing after the amplification of *rpoB* DNA by PCR and nested PCR which were directly performed with the DNA from sputum sample N3983. The PCR DNA sequencing (upper panel) showed ambiguous results due to nonspecific amplification of *rpoB* DNA. However, nested PCR DNA sequencing (lower panel) revealed a signature nucleotide at codon 529 (CG**A**) and a mutation at codon 531 (**TTG**) in the *rpoB* DNA of *M. tuberculosis*. N3983 was confirmed by susceptibility testing and sequence analysis of culture isolate as a rifampin-resistant strain harboring a mutation at Ser_{531} (**TCG**→**TTG**).

as culture isolates, the entire identification process for *M. tuberculosis* would be shortened to several days.

For this purpose, a line probe assay (4) and single-tube heminested PCR protocol (23) have been developed. Although there are several advantages to the line probe assay, it is more expensive than other methods and requires several probes for reverse hybridization to determine the mutation. Heminested PCR uses signature nucleotides of *M. tuberculosis* to avoid

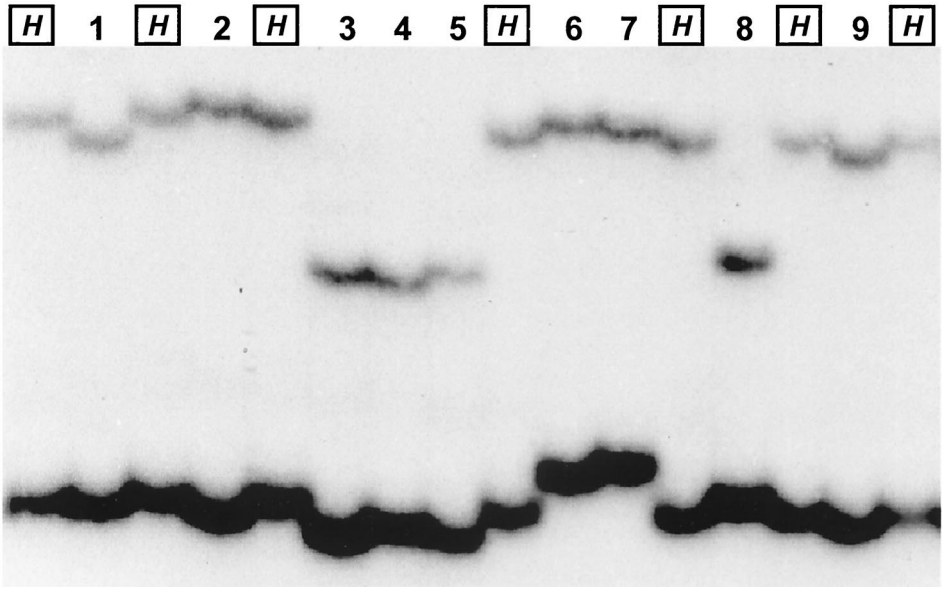

FIG. 6. Nested PCR-SSCP patterns of rifampin-resistant strains (lanes 1 to 9) identified in this study. Lanes: H, *M. tuberculosis* H37Rv; 1, N3523 (Gln_{513}→Pro); 2, N2893 (Asp_{516}→Val); 3, N2910 (His_{526}→Tyr); 4, N3501 (His_{526}→Asp); 5, N2902 (His_{526}→Arg); 6, N2895 (Ser_{531}→Leu); 7, N3522 (Ser_{531}→Met); 8, N2877 (Leu_{533}→Pro); 9, N3471 (Met_{515}, Asp_{516}, Gla_{517}, Asn_{518}→Ile, Ala, Asn, Tyr).

amplification of DNA from other GC-rich bacteria. It is a powerful method to detect *rpoB* DNA of *M. tuberculosis* directly from sputa. However, when this method was applied to detect *M. tuberculosis* rifampin resistance, further analysis such as sequencing or dideoxy fingerprinting after the amplification of 193-bp *rpoB* DNA by heminested PCR was required. This method can be linked to SSCP analysis performed directly on a DNA sample without a postamplification step but, compared to SSCP analysis which targets the 157-bp *rpoB* DNA, this method has problems in detecting several specific mutations. In order to detect the most frequent mutation at codon 531 (Ser [**T**CG]→Leu [**TT**G]), it required longer electrophoresis. Furthermore, the C-to-T transition mutation in codon 526 (His [**C**AC]→Tyr [**T**AC]) was not differentiated (6).

In this study, we used a nested PCR strategy targeting the 157-bp *rpoB* DNA, which has been most widely used for PCR-SSCP analysis and sequencing in order to detect mutations related to rifampin resistance. This approach confers a higher sensitivity and specificity than conventional PCR on SSCP and sequence analysis. Unlike heminested PCR, which is based on limited numbers of mycobacterial species (8, 23), the *M. tuberculosis*-specific primers for nested PCR can be selected from the signature nucleotides on the basis of *rpoB* DNA sequences of 44 mycobacteria (11). Therefore, the possibility of nonspecific amplification of *rpoB* DNA from NTM or nonmycobacteria should be greatly reduced. The signature nucleotides for *M. tuberculosis* were located at the 3′ hydroxyl termini of each primer as previously described (23). Thus, amplification of *rpoB* DNA from NTM or nonmycobacteria could be avoided by first-round PCR performed at a high annealing temperature and direct detection and determination of the rifampin susceptibility of *M. tuberculosis* in sputum were possible. Misidentification of *rpoB* sequences (codons 529 and 531 of N3983) by PCR sequencing may have resulted from simultaneous amplification of the *rpoB* DNA from *M. tuberculosis* and other bacteria, but this problem was solved by the nested PCR.

Conventional PCR-SSCP did not detect the *rpoB* sequences in 11 of 56 sputa (19.6%). Although the *rpoB* DNAs were not amplified (or detected) by conventional PCR in eight specimens, *IS6110* PCR and cultures identified *M. tuberculosis* in all cases. Moreover, nested PCR amplified *rpoB* DNA from these specimens, and thus permitted rifampin susceptibilities to be determined by SSCP and sequencing. Another example of the high specificity of nested PCR-SSCP was found in the results for 3 of the 11 specimens which showed ambiguous results by conventional PCR-SSCP. Each had three bands that may have been caused by the simultaneous amplification of the *rpoB* DNA from both *M. tuberculosis* and other bacteria. Judging from SSCP patterns that were clearly different from that of the rifampin-susceptible reference strain (*M. tuberculosis* H37Rv), they might be regarded as rifampin resistant. Considering that NTM and nonmycobacteria have sequence variations in the corresponding region (157 bp) of amplified *rpoB* DNA, these bands may also represent false-positive results. Nested PCR-SSCP and DNA sequencing definitively identified these strains to be either rifampin-resistant or rifampin-susceptible *M. tuberculosis*. In contrast, specimen N3928 was quite interesting in that it was identified as a rifampin-resistant strain by PCR-SSCP but was proven to be rifampin susceptible by nested PCR-SSCP, susceptibility testing, and PCR-direct sequencing of *rpoB* DNA from pure culture. These results suggest that *rpoB* amplification from bacteria other than *M. tuberculosis*, causing a false-positive result, may be excluded by the nested PCR protocol used in this study.

The results of the nested PCR-SSCP method applied to sputa were entirely concordant with those of culture and conventional drug susceptibility testing as well as those obtained by conventional PCR-SSCP analysis. Furthermore, nested PCR-SSCP analysis enabled the direct detection of rifampin resistance from primary clinical specimens, such as sputa. Although the nested PCR-SSCP has a great advantage in reducing the time required for the primary culture and specific identification of *M. tuberculosis* in sputa, it still is limited by the requirements of radiolabeled PCR products, extensive labor, and a level of technical expertise not found in most clinical laboratories. It does, however, provide a significant advance in the rapid detection of multidrug-resistant *M. tuberculosis* directly from a clinical specimen.

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