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Newly Developed Primers for Comprehensive Amplification of the *rpoB* Gene and Detection of Rifampin Resistance in *Mycobacterium tuberculosis*▽

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New *rpoB* gene primers for detecting Rif^r in *Mycobacterium tuberculosis* complex bacteria achieved 100% specificity and 88% (fresh sputa) and 92% (ethanol-preserved sputa) diagnostic sensitivity and detected up to 4 CFU/sample. Of the 99 Rif^r isolates examined, 97% had mutations within cluster I, 2% at codon 176, and 1% at codon 497.

Molecular detection of rifampin-resistant (Rif^r) *Mycobacterium tuberculosis* usually relies on amplification of the hotspot zone for resistance-conferring mutations (cluster I, covering codons 432 to 458 according to the *M. tuberculosis* nomenclature) (7) of the *rpoB* gene (4, 8, 10, 12, 13, 17). Previous studies have shown that 94 to 98% of Rif^r *M. tuberculosis* strains show a mutation in cluster I (10, 11, 14, 15). Resistance-associated mutations have also been described for cluster II (codons 496 and 497) and for codons 176, 486, 558, and 598 (1, 6, 7).

We describe and evaluate new primers, covering the entire region with all currently known significant mutations in a single assay.

Oligonucleotides were designed on the basis of an alignment of the *rpoB* gene sequence from the H37Rv *M. tuberculosis* reference strain (NC 000962; NCBI bank), some relevant nontuberculous mycobacteria (NTM), and nonmycobacterial species using ClustalX (version 1.83.1) software. Amplify software (version 1.2; University of Wisconsin—Madison) was used to estimate the stabilities and binding capacities of the selected oligonucleotides and to simulate PCRs. Figure 1 shows the relative locations of the selected primers.

A single PCR with primers *rpoB*geneSA (5′-GGTTCGCCGC GCTGGCGCGAAT-3′) and *rpoB*geneRB (5′-GACCTCCTCG ATGACGCCGCTTTCT-3′) was used for bacterial suspensions, whereas a nested PCR with primers *rpoB*geneSAnew (5′-GCAA AACAGCCGCTAGTCCTAGTCCGA-3′) and *rpoB*geneRA (5′-GCGCCATCTCGCCGTCGTCAGTACAG-3′) for the first run, and *rpoB*geneSA and *rpoB*geneRB as inner primers, was used to amplify clinical specimens.

The first run of the nested PCR was performed with a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.65 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 12.5 pmol of each primer, 1.5 U *Taq* polymerase (Promega, Madison, WI), and 5 μl of DNA extract from clinical specimens. PCR was performed using a PTC 100 MJResearch thermocycler (Whaltham, MA) as follows: a hot start (90°C) followed by 5 min at 94°C; 45 cycles of 45 s at 94°C, 1 min 30 s at 66°C, and 45 s at 72°C; and a final extension of 10 min at 72°C. The second run was performed with a final volume of 25 μl enzyme mixture with 0.5 U *Taq* polymerase and 0.25 μl of the first PCR amplicon as follows: a hot start (90°C) followed by 5 min at 94°C, 29 cycles of 45 s at 94°C, 1 min 45 s at 72°C (annealing and extension), and a final extension of 10 min at 72°C. For bacterial suspensions, a single PCR was run under similar conditions but using a 50-μl enzyme mixture and 45 cycles. Amplicons were analyzed with a 2% (wt/vol) agarose gel.

DNA was extracted from sputum by an adapted Boom extraction method (16) and from bacterial suspensions in 1× TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) by boiling it for 5 min. The extracted DNA was analyzed immediately or stored at <-18°C.

A set of well-documented mycobacterial isolates (4 *M. tuberculosis* complex isolates and 67 NTM isolates belonging to 20 different species) was used to evaluate the specificities of the primers (Table 1). In addition, 99 Rif^r and 117 rifampin-susceptible (Rif^s) *M. tuberculosis* isolates, which will form part of the World Health Organization-Tropical Disease Research (WHO-TDR) *M. tuberculosis* strain bank in November 2006, were included. A set of 18 nonmycobacterial isolates of genera closely related to the genus *Mycobacterium* was tested as well. None of the nonmycobacterial or NTM strains tested were amplified, whereas all *M. tuberculosis* complex isolates yielded good-quality amplicons (Table 1).

The detection sensitivities as determined by analysis of the logarithmic dilutions of the positive PCR controls reached 0.08 picograms of DNA or about 10 acid-fast bacilli (AFB)/reaction. The performance of the nested PCR on a set of 12 spiked sputum specimens collected from the 2005 round of the Qual-

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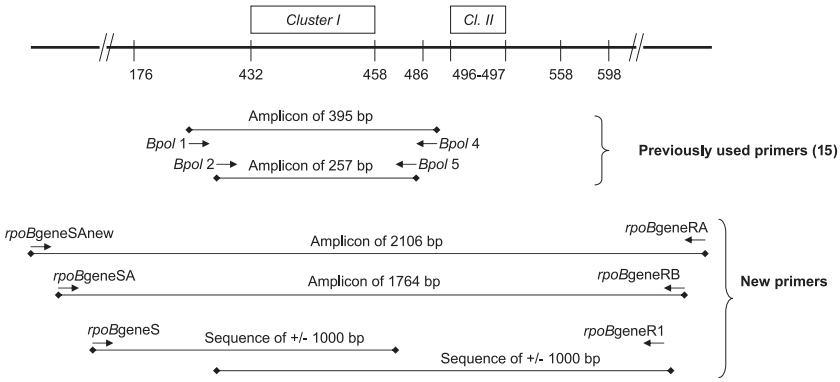

FIG. 1. Location of the newly developed primers on the *M. tuberculosis rpoB* gene relevant to previously used primers for nested PCR (15) and relevant to the currently known mutations conferring rifampin resistance. Codon designation is in accordance with the *M. tuberculosis* nomenclature (7). Cl., cluster.

ity Control for Molecular Diagnosis organization (QCMD MTBDNA05; European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases) showed a detection limit of 4.0 CFU/sample.

The likely field performance of the PCR was evaluated for 211 smear-positive sputum specimens from Bangladesh (Table 2) that were preserved in ethanol (final concentration, 50%; total volume, 1.5 ml) at an ambient temperature for 5 to 24 months. Of the 211 specimens tested, 195 (92.4%) yielded good-quality amplicons with the nested PCR. This is comparable to the sensitivity reported for the Rifoligotyping assay using stained sputum slides (16) and the 95% sensitivity for smear-positive sputum specimens using the Genotype MTBDR assay (2). The INNO-LIPA Rif TB assay showed a 98.3% sensitivity for culture-positive specimens (5) and 92.9% for all sputum specimens (15), using a nested-PCR system, whereas it reached only 78.3% for both smear-negative and smear-positive specimens after a single PCR (9). The negative results in our study might be due to the long-term storage at the ambient temperature, with the gradual breakdown of our large target sequence into smaller fragments, as suggested by the fact that 12 (75%) of the 16 negative specimens reacted positive in a nested PCR using the old β-*pol* primers targeting cluster I of the *rpoB* gene (amplicon size of 257 bp) (15) and/or our in-house 16S diagnostic PCR (amplicon size of 271 bp).

For the WHO-TDR strain bank, phenotypic resistance to RIF was determined by the proportion method (3) with Löwenstein-Jensen medium, using multiple drug concentrations (10, 20, 30, 40, and 80 μg/ml; cutoff at 40 μg/ml). Direct double-strand sequencing of the *rpoB* amplicons was done using a capillary sequencer (Applied Biosystems 3730 DNA analyzer) in combination with an ABI PRISM BigDye Terminator cycle sequencing kit. The innermost primers, *rpoB*geneS (5′-ATGACGTACGCGGCTCCACTG-3′) and *rpoB*geneR1 (5′-CAGCGGGGCCTCGCTAC-3′), were used for sense and reverse sequencing, respectively. The ClustalX program (ver-

TABLE 1. *M. tuberculosis* complex, NTM, and nonmycobacterial isolates tested with the new *rpoB* primer combinations

Species	Remark	No. of isolates tested	No. of isolates with a positive PCR using:	
			Outer primers	Inner primers
M. abscessus		1	0	0
M. africanum	*M. tuberculosis* complex	1	1	1
M. avium		4	0	0
M. bovis	*M. tuberculosis* complex	1	1	1
M. canetti	*M. tuberculosis* complex	1	1	1
M. celatum		1	0	0
M. chelonae		4	0	0
M. fortuitum		1	0	0
M. gastri		1	0	0
M. genavense		4	0	0
M. gordonae		4	0	0
M. haempohilum		5	0	0
M. intracellulare		10	0	0
M. kansasii		1	0	0
M. malmoense		5	0	0
M. marinum		5	0	0
M. microti		1	0	0
M. peregrinum		5	0	0
M. scrofulaceum		2	0	0
M. simiae		5	0	0
M. smegmatis		2	0	0
M. tuberculosis	*M. tuberculosis* complex	1	1	1
		216	NT[a]	216
M. ulcerans		5	0	0
M. xenopi		1	0	0
Nocardia spp.	*N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova*	6	0	0
Rhodococcus equi		1	0	0
Corynebacterium spp.	*C. diphtheriae*, *C. equi*, *C. flaccumfaciens*, *C. huayni*, *C. pyogenes*, *C. xerosis*	11	0	0

[a] NT, not tested.

TABLE 2. Nested-PCR results for ethanol-preserved sputum specimens broken down by quantitative smear microscopy results

Smear microscopy group	No. of specimens with indicated nested-PCR result		Total
	rpoB positive[a]	*rpoB* negative	
1–9 AFB/100 fields	9 (90)	1	10
10–99 AFB/100 fields	35 (87.5)	5	40
1–10 AFB/field	70 (89.7)	8	78
>10 AFB/field	81 (97.6)	2	83
Total	195 (92.4)	16	211

[a] Values in parentheses are percentages.

sion 1.83.1) and Genedoc software (version 2.100) were used to analyze the final nucleotide sequences in comparison to the *M. tuberculosis* H37Rv wild-type sequence. All *M. tuberculosis* isolates resulted in nucleotide sequences between 750 and 1,000 bp. Sequencing agreed with the phenotypic results for all isolates tested. Only single-nucleotide mutations were identified. Two of the Rif^r isolates showed mutations at codon 176 (GTC176TTC resulting in Val176Phe), and one isolate had a mutation at codon 497 (ATC497TTC or Ile497Phe), whereas the remaining 96 Rif^r isolates had mutations within cluster I.

Of the amplicons obtained from sputum specimens for which cultures were not available, 34 showed Rif^r mutations, in agreement with the clinical picture (treatment failure) of the respective patients.

Since the primer combinations described herein proved to be *M. tuberculosis* complex-specific, with good sensitivities and clear sequencing results from clinical specimens (using nested PCR), our assay is suitable for direct application on smear-positive sputum specimens. This allows rapid individual diagnosis of Rif^r *M. tuberculosis* complex diseases as well as epidemiological studies of RIF resistance in settings where culture and DST are not widely available. If needed, storage at room temperature and referral to a distant laboratory are easy for ethanol-preserved sputum specimens, offering important additional advantages.

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