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Evaluation of the INNO-LiPA Rif. TB Assay, a Reverse Hybridization Assay for the Simultaneous Detection of *Mycobacterium tuberculosis* Complex and Its Resistance to Rifampin

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Mycobacterium tuberculosis **resistance to rifampin results from nucleotide changes in the gene encoding the β-subunit of the RNA polymerase (*rpoB*). We developed a reverse hybridization-based line probe assay (LiPA; the INNO-LiPA Rif. TB) carrying one oligonucleotide probe for the detection of *M. tuberculosis* complex strains and nine probes designed to detect nucleotide changes in the relevant part of *rpoB*. This assay was evaluated with 107 *M. tuberculosis* isolates with known *rpoB* sequences, 52 non-*M. tuberculosis* complex strains, and 61 and 203 clinical isolates found to be sensitive and resistant, respectively, by in vitro testing. The results indicated that (i) the *M. tuberculosis* complex probe was 100% specific, (ii) when compared to the results of nucleotide sequencing, no discrepancies with the results of INNO-LiPA Rif. TB were observed, (iii) all strains sensitive by in vitro susceptibility testing were correctly identified, and (iv) among the strains resistant by in vitro susceptibility testing, only 4 (2%) yielded conflicting results. The INNO-LiPA Rif. TB is therefore a reliable and widely applicable assay and a valuable tool for routine diagnostic use, given its simplicity and rapid performance.**

The appearance of multiple-drug-resistant (MDR) strains of *Mycobacterium tuberculosis* (6, 8) has stimulated the search for the molecular mechanisms responsible for resistance to antituberculosis drugs. This led to the elucidation of the mechanism responsible for resistance to rifampin (RMP) in *M. tuberculosis* (23).

In RMP-resistant (RMP^r) *M. tuberculosis* complex strains, resistance results from point mutations or from small deletions or insertions in a limited region of the gene encoding for the β-subunit of the RNA polymerase (*rpoB*). More than 30 different mutations have now been described in a region spanning 81 bp corresponding to a stretch of 27 amino acids (Fig. 1) (13). For most of these mutations, only circumstantial evidence with respect to their involvement in RMP resistance is available. However, since most of these mutations have never been documented in susceptible *M. tuberculosis* strains, it is generally accepted that a mutation found in a resistant strain is indeed responsible for the resistant phenotype.

Upon the elucidation of the mechanism of RMP^r, several publications describing various PCR-based molecular genetic techniques for the detection of RMP resistance have appeared. These techniques, including direct sequencing of PCR products (11, 12, 17–19, 22), single-strand conformation polymorphism analysis (7, 10, 14, 23–25), heteroduplexing (26), and dideoxy fingerprinting (7), produce results within a much shorter time than the time required for conventional culture techniques. In the present study, a recently described, reverse hybridization-based line probe assay (LiPA; the INNO-LiPA Rif. TB [5]) was evaluated with (i) 107 isolates of *M. tuberculosis* for which the nucleotide sequence in the *rpoB* region was known, (ii) 52 non-*M. tuberculosis* complex strains, and (iii) 264 clinical *M. tuberculosis* isolates analyzed by classical in vitro drug susceptibility tests.

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MATERIALS AND METHODS

Strains used. A total of 289 *M. tuberculosis* complex strains from different countries were analyzed by the INNO-LiPA Rif. TB. For 107 of these strains the nucleotide sequence of the relevant part of the *rpoB* gene was determined, and 264 strains were subjected to in vitro drug susceptibility testing. Thirty-five mycobacterial strains belonging to species other than the *M. tuberculosis* complex and 17 strains belonging to other genera were also included in this study (see Table 2).

Culture and susceptibility testing. Cultures were carried out on Löwenstein-Jensen (LJ) medium. RMP susceptibility was determined on LJ medium by the proportion method of Canetti et al. (3) (henceforth called in vitro susceptibility). For some strains, the MIC of RMP on 7H11 agar was also determined (9).

Sample preparation and PCR. A loopful of bacteria was suspended in 500 μl of TE (10 mM Tris, 1 mM EDTA [pH 8]), and the suspension was incubated for 10 min at 95°C. After centrifugation, 1 to 10 μl of the supernatant was used in the PCR, which was performed in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.2 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 0.01% gelatin, and 1 U of *AmpliTaq* polymerase (Perkin-Elmer, Norwalk, Conn.). The primers (IP1 and IP2), each used at a concentration of 20 pmol per reaction mixture, had the following respective sequences: 5′-GGTCGGCA TGTCGCGGATGG-3′ and 5′-GCACGTCGCGGACCTCCAGC-3′. They were biotinylated at their 5′ ends. The length of the amplicon was 256 bp. In the GeneAmp PCR System 9600 (Perkin-Elmer) or PHC-3 Thermal Cyclers (Techne, Cambridge, England), the following cycling protocol was applied: initial denaturation at 94°C for 3 min and 35 cycles consisting of 45 s at 94°C, 1 min at 64°C, and 45 s at 72°C, followed by a final cycle of 10 min at 72°C.

Nucleotide sequencing. The nucleotide sequences of both strands of the relevant part of the *rpoB* gene were determined by direct sequencing of the PCR product on an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, Calif.) with fluorescence-labelled dideoxynucleotide terminators (PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit; Applied Biosystems). The primers used for direct sequencing were identical to those used for the PCR amplification.

LiPA. LiPA is based on the reverse hybridization principle (20). Specific oligonucleotides are immobilized at known locations on a membrane strip and are hybridized under strictly controlled conditions with the biotin-labelled PCR product. The hybrids formed are subsequently detected colorimetrically (21). In the INNO-LiPA Rif. TB, the *rpoB* gene is the target for amplification and detection. Five partially overlapping probes (S1 to S5) were designed. The probes span the relevant region of the gene as indicated in Fig. 1. These S probes exclusively hybridize to the wild-type sequence. If a mutation is present in one of

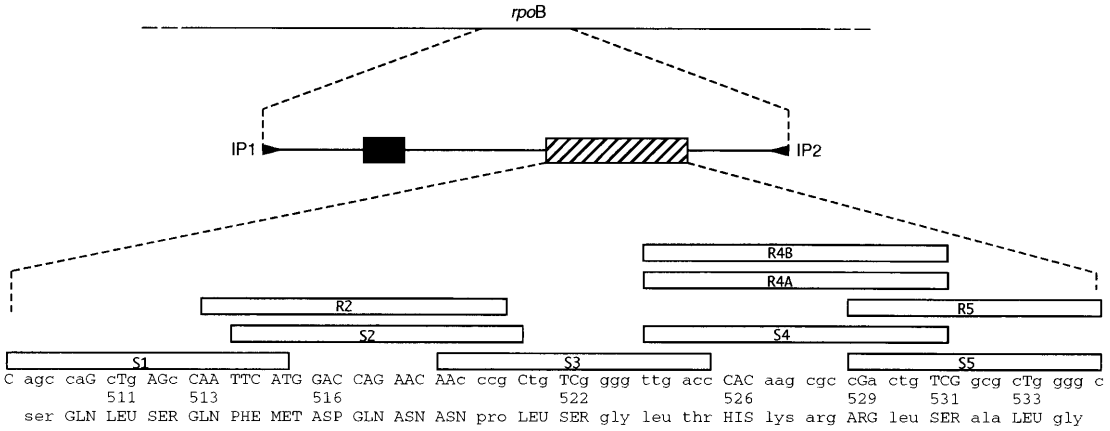

FIG. 1. Schematic representation of the *rpoB* gene and positions of primers and probes. The arrowheads indicate the positions of primers IP1 and IP2. The black box indicates the position of the *M. tuberculosis* complex probe. The hatched box corresponds to the area scanned by the S and R probes on the LiPA strips (indicated by horizontal bars). The wild-type nucleotide and amino acid sequences in the relevant part of the *rpoB* gene are presented as described by Telenti et al. (23). The nucleotide and amino acid positions indicated in capital letters have been found to be involved in RMP^r.

the target regions, the mismatch created will prevent the amplicon from hybridizing with the corresponding probe under the stringent conditions applied. Consequently, the absence of a hybridization signal with one of the probes (probes S1 to S5) is indicative of the presence of a mutation and hence implies a resistant genotype. In addition, four probes (R probes) which specifically hybridize with amplicons carrying a particular mutation are present. These R probes (probes R2, R4a, R4b, and R5) hybridize with amplicons carrying the following mutations: D516V, H526Y, H526D, and S531L, respectively. An additional probe, specific for organisms belonging to the *M. tuberculosis* complex and situated approximately 82 bp upstream from the RMP^r-determining region in the *rpoB* gene, allows for the unequivocal identification of *M. tuberculosis* complex strains. The key to the interpretation of the strips is presented in Table 1.

INNO-LiPA Rif. TB kits were produced by Innogenetics N.V. (Zwijndrecht, Belgium). The assay was performed manually or by using an automated system (AUTO-LiPA; Innogenetics), according to the manufacturer's instructions.

RESULTS

PCR and *M. tuberculosis* complex probe. For all *M. tuberculosis* complex strains studied (n = 289), the primer set used generated an amplified product of the expected length (approximately 256 bp). Most other organisms tested yielded products of different lengths or were not amplified to a level detectable in agarose gels (Table 2). The *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum* isolates tested hybridized with the *M. tuberculosis* complex probe. Cross-reactivity of the probe with non-*M. tuberculosis* complex strains was not observed. Most of the S and R probes were also specific for the *M. tuberculosis* complex. Only probe S2 reacted with strains of some other *Mycobacterium* species such as *M. avium*, *M. kansasii*, *M. malmoense*, and *M. scrofulaceum* (data not shown).

LiPA versus nucleotide sequencing. For 91 RMP^r and 16 RMP^s strains isolated from samples from different countries, LiPA was performed and the relevant part of the *rpoB* gene sequenced. The comparison of the results obtained by LiPA and by sequencing is presented in Table 3. All LiPA results for wild-type strains corresponded to the wild-type sequence. Among the 91 RMP^r strains sequenced, 27 different mutations were detected, all of them resulting in an amino acid change. Among these mutations, five had not been described earlier. Four of these (513GAA, 513-515ΔAATTCA, 522CAG, and 526ACC) were the only mutations found in the relevant part of the *rpoB* gene and are thus probably the cause of RMP resis-

TABLE 1. Key to the interpretation of the INNO-LiPA Rif. TB strips[a]

Result for the following probes:										LiPA pattern	Mutation[b]	Interpretation
M. tuberculosis	S1	S2	S3	S4	S5	R2	R4a	R4b	R5			
−	−	±[c]	−	−	−	−	−	−	−	0		No *M. tuberculosis* complex
+	+	+	+	+	+	−	−	−	−	Wild type		Sensitive
+	−	+	+	+	+	−	−	−	−	ΔS1	In S1	Resistant
+	+	−	+	+	+	−	−	−	−	ΔS2	In S2	Resistant
+	+	−	+	+	+	+	−	−	−	R2	D516V	Resistant
+	+	+	−	+	+	−	−	−	−	ΔS3	In S3	Resistant
+	+	+	+	−	+	−	−	−	−	ΔS4	In S4	Resistant
+	+	+	+	−	+	−	+	−	−	R4a	H526Y	Resistant
+	+	+	+	−	+	−	−	+	−	R4b	H526D	Resistant
+	+	+	+	+	−	−	−[d]	−[d]	−	ΔS5	In S5	Resistant
+	+	+	+	+	−	−	−[d]	−[d]	+	R5	S531L	Resistant

[a] According to the reactivities of the probes, 10 regular LiPA patterns can be expected for *M. tuberculosis* complex strains. If all R probes are negative and all S probes are positive, a wild-type sequence is present. When one of the S probes is missing and no R probe hybridizes, this pattern is described with a Δ preceding the missing probe (e.g., ΔS1). If one of the S probes is negative and the corresponding R probe is positive, the pattern is described according to the R probe observed (e.g., R2). Occasionally, deviating patterns may be observed, as outlined in the text (and Fig. 3).

[b] Mutations are described as follows: first, the amino acid residue in the wild-type sequence (one-letter International Union of Pure and Applied Chemistry code, e.g., D for aspartic acid) then the codon position in Fig. 1 (e.g., 516), and finally, the amino acid residue in the mutated sequence (e.g., V for valine).

[c] ±, occasionally positive with non-*M. tuberculosis* complex strains.

[d] These probes react weakly with *M. tuberculosis* complex strains when S5 is negative.

TABLE 2. Sensitivity and specificity of the *M. tuberculosis* complex probe

Species	No. of strains tested	PCR result[a]	*M. tuberculosis* probe result
Mycobacterium tuberculosis	285	+	+
Mycobacterium bovis	2	+	+
Mycobacterium africanum	1	+	+
Mycobacterium microti	1	+	+
Mycobacterium avium	5	+	−
Mycobacterium paratuberculosis	2	+	−
Mycobacterium silvaticum	1	+	−
Mycobacterium intracellulare	4	a	−
Mycobacterium avium complex	1	+/a	−
Mycobacterium scrofulaceum	3	+	−
Mycobacterium kansasii	1	+	−
Mycobacterium marinum	1	a	−
Mycobacterium ulcerans	1	a	−
Mycobacterium gordonae	1	a	−
Mycobacterium xenopi	1	+	−
Mycobacterium genavense	1	a	−
Mycobacterium genavense-like	1	−	−
Mycobacterium simiae	2	a	−
Mycobacterium celatum	1	+/a	−
Mycobacterium malmoense	1	+/a	−
Mycobacterium haemophilum	1	+	−
Mycobacterium smegmatis	1	a	−
Mycobacterium gastri	1	a	−
Mycobacterium asiaticum	1	−	−
Mycobacterium chelonae chelonae	1	a	−
Mycobacterium chelonae abscessus	1	a	−
Mycobacterium chelonae-like	1	+/a	−
Mycobacterium fortuitum	1	a	−
Bordetella pertussis	1	a	−
Bordetella parapertussis	1	a	−
Bordetella bronchiseptica	1	a	−
Moraxella catarrhalis	4	a	−
Haemophilus influenzae	1	a	−
Streptococcus pneumoniae	3	−	−
Acinetobacter calcoaceticus	1	−	−
Staphylococcus aureus	2	−	−
Pseudomonas aeruginosa	2	+	−
Burkholderia cepacia	1	a	−

[a] The PCR result is positive (+), negative (−), generates one or more aspecific fragments of different lengths (a), or is positive with other aspecific fragments (+/a).

TABLE 3. Comparison of results obtained by LiPA and nucleotide sequencing

LiPA profile	No. of strains analyzed	Mutation(s) identified by sequencing[a]
Wild type	16	None (16)
ΔS1	8	511cCg (6), 513cCa (1), 513Gaa* (1)
ΔS2	11	516Tac (3), 516-517ΔGACCAG (6), 518ΔAAC (2)
ΔS3	7	522tTg (5), 522CAg* (2)
ΔS4	21	526Aac (2), 526ACc* (1), 526cCc (4), 526cGc (8), 526cTc (3), 526TGc (3)
ΔS5	11	531tGg (4), 533cCg (7)
R2	6	516gTc (6)
R4A	5	526Tac (5)
R4B	5	526Gac (5)
R5	9	531tTg (9)
ΔS1/ΔS2	3	511cCg+516gCc (1), 513-515ΔAATTCA* (1), 513-516ΔAATTCATGG (1)
ΔS2/ΔS4	1	516gGc+529cAa* (1)
ΔS1/ΔS4	1	511cGc+526cGc (1)
ΔS1/ΔS2/ΔS4	1	515Gtg+526Aac (1)
ΔS1/R2	1	511cGg+516gTc (1)
R2/ΔS4	1	516gTc+526caG (1)
Total	107	

[a] Mutations not previously described are indicated with an asterisk. Capital letters in the codon indicate the changes in nucleotide sequence compared to the wild-type sequence. The region taken into account was from codon 508 to 535, as shown in Fig. 1. The numbers of strains with the corresponding sequence are indicated in parentheses.

tance. One new mutation (529CAA) was found together with an earlier described mutation. No discrepancies were found between the sequencing results and the LiPA results. When the LiPA predicted the presence of a mutation, this was always confirmed by sequencing. Each mutation prevented the corresponding S probe(s) from hybridizing (represented by ΔS in Table 1), although some residual background signal was observed for two mutations (R529Q and L533P) at the levels of probes S4 and S5, respectively. When an R probe was also visible, it always corresponded to the expected mutation.

In six cases, sequencing data revealed two mutations in different codons of the *rpoB* gene. These were also detected by LiPA. These double mutations generated patterns different from those presented in Table 1. At least two S probes were absent, and occasionally, an R probe was positive. A deletion of 6 or 9 nucleotides at codons 513 to 515 also resulted in an irregular pattern: both the S1 and S2 probes failed to hybridize. Several examples of the results obtained by LiPA are presented in Fig. 2 and 3.

Detection of mixtures. Although the LiPA analysis was performed with bacterial cultures starting from isolated colonies, LiPA revealed the presence of mixtures in three cases: a wild type and a resistant (R) organism (wild type plus an R5 strain, wild type plus an R4 strain) in two cases and a mixture of the wild type with two different resistant organisms in another case (Fig. 3). Mixtures could easily be recognized since they gave rise to superimposed profiles. In all these cases, subculturing confirmed the LiPA results.

LiPA results compared with in vitro susceptibility results. Two hundred sixty-four strains for which the results of the in vitro RMP susceptibility tests were available were analyzed with INNO-LiPA Rif. TB strips. All available RMP^r *M. tuberculosis* strains (n = 203) as well as 61 randomly chosen RMP^s *M. tuberculosis* strains were included. All in vitro RMP^s strains yielded an RMP^s LiPA profile, and 199 of the 203 in vitro RMP^r strains yielded an RMP^r LiPA profile. Only four strains produced discrepant results (in vitro RMP^r and LiPA RMP^s); this accounted for 1.97% of the total number of RMP^r strains tested.

Mutation frequencies. In Table 4, 193 RMP^r strains are classified according to their origins and LiPA profiles. The R types (141 strains) accounted for 73% of the mutations detected. The R5 mutation (S531L) was predominant (44.5%), followed by R4b (H526D) (10.9%) and R4a (H526Y) (10.05%). Mutations giving rise to a ΔS profile occurred in 27% (n = 52) of the strains.

DISCUSSION

The resistance of *M. tuberculosis* to one or more of the first-line drugs currently in use is on the increase, and several

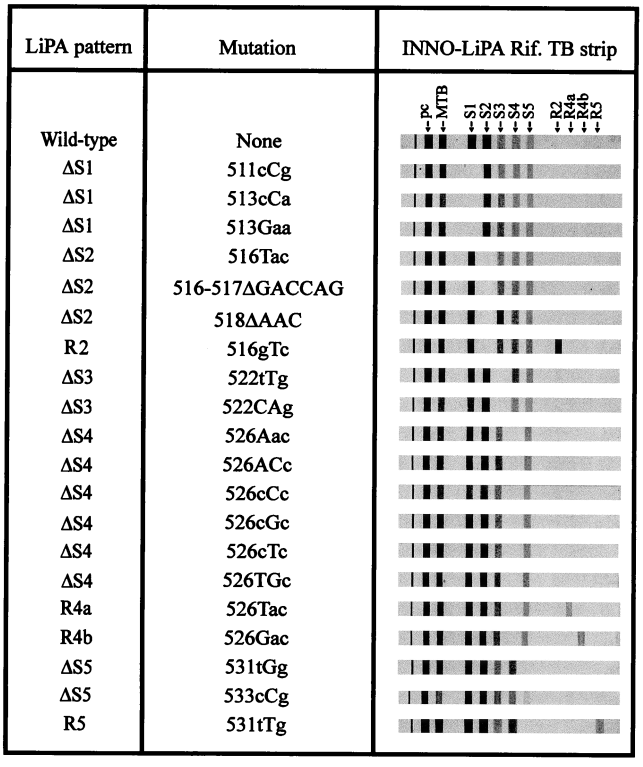

FIG. 2. Regular patterns obtained with the INNO-LiPA Rif. TB and the mutations found. The capital letters in the codons indicate the change in nucleotide sequence compared to the wild-type sequence. The arrows indicate the positions of the different probes on the strips. pc, positive conjugate control; MTB, *M. tuberculosis*.

outbreaks caused by MDR strains have been reported recently (6, 8). This emphasizes the need for fast and reliable methods for the detection of *M. tuberculosis* and the determination of its drug susceptibility both for optimal patient treatment and for control of the disease.

The INNO-LiPA Rif. TB used in this study may help meet this need. This PCR-based hybridization assay is able to detect and identify *M. tuberculosis* complex strains and simultaneously provide information with respect to the susceptibility of the strain to RMP. This drug is a key element in the treatment of tuberculosis and a marker for MDR (2, 15).

The basic principle of the INNO-LiPA Rif. TB is that each nucleotide change should impede the hybridization of the target and the corresponding wild-type probes. This was the case for 25 of the 27 mutations examined. For two mutations, slight deviations from this rule were observed. (i) In the isolate with the R529Q mutation, a weak hybridization signal appeared at the level of the S4 probe (Fig. 3). This was not entirely unexpected since codon 529 is located close to the 3′ end of the S4 probe (Fig. 1). However, this mutation is probably not clinically relevant since it was never reported before and was detected in a strain harboring another, more frequent mutation (D516G). (ii) The L533P mutation also did not entirely disrupt the hybrid formation with the S5 probe; however, the hybridization signal obtained was clearly weaker than the signal obtained with a wild-type sequence (Fig. 2). Recently, it was demonstrated (4) that the infrequent TTC insert mutation at position 514 cannot be distinguished from the wild-type sequence.

The most clinically relevant level of interpretation of the LiPA is the recognition of an RMP-resistant genotype (Table 1). Since profiles deviating from a normally observed wild-type profile are indicative of the presence of a nucleotide change, every profile deviating from the normally observed profile should be considered as originating from a resistant strain. In addition, the responsible mutation can be inferred (R profiles), or when a ΔS profile is observed, the location of the mutation in the *rpoB* gene can be predicted. *M. tuberculosis* complex strains can produce the 10 probe patterns presented in Table 1. Although infrequent, aberrant profiles may result from mixtures of *M. tuberculosis* complex strains or other *Mycobacterium* strains reacting with the S2 probe, from strains harboring two or more point mutations, or from strains carrying some of the described deletions (Fig. 3). Usually, the interpretation remains straightforward, and the presence of a mutation causing RMP^r will almost always be readily recognized. The pattern obtained may be indistinguishable from that of a normal wild-type strain for mixtures of two strains with a ΔS profile or strains with a ΔS and a wild-type profile. However, this possibility is an exception. First, most mixtures will be composed of the most frequent mutations for which a confirmatory mutation probe (R probe) is present on the strip. Second, the pattern generated can be that of a wild type only for mixtures in a 1:1 ratio; in all other cases, at least one of the bands will have a modified signal intensity relative to the intensities of the other bands on the strip. Among the 264 strains studied, three mixtures were detected, and in each of these, one of the four R probes was involved. Mixtures of two ΔS profile strains or one wild-type and one ΔS strain were not observed and probably did not occur in the panel investigated. It is highly unlikely that such mixtures would have gone undetected because they would have resulted in a discrepancy between LiPA and in vitro susceptibility results (LiPA sensitive and in vitro susceptibility testing resistant).

Initially, 16 LiPA-resistant in vitro-sensitive strains were found. The 16 strains were retested on 7H11 medium. RMP MIC were greater than 40 μg/ml for 15 of the strains, and the RMP MIC was 2 μg/ml for 1 strain. Subsequently, all 16 strains were retested on a new batch of LJ medium, and all were

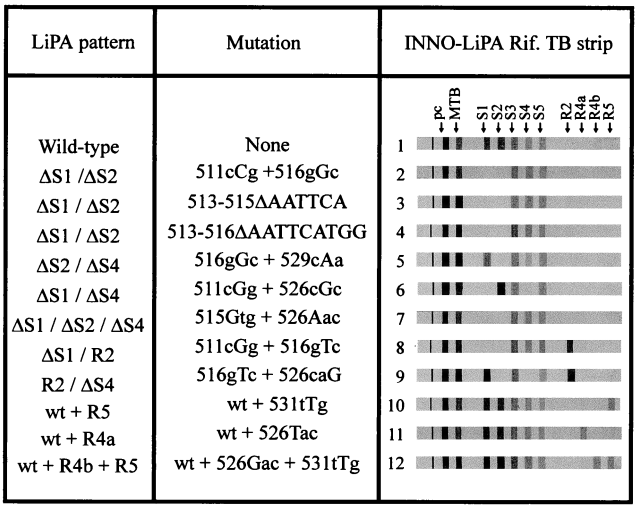

FIG. 3. Aberrant LiPA patterns observed with strains harboring double mutations or deletions (△) (strips 2 to 9) or observed in case of mixed cultures (strips 10 to 12). The capital letters in the codons indicate the change in nucleotide sequence compared to the wild-type sequence. The arrows indicate the positions of the different probes on the strips. pc, positive conjugate control; MTB, *M. tuberculosis*; wt, wild type.

TABLE 4. Number of samples with the different LiPA profiles in different countries[a]

Country	No. of samples									
	ΔS1	ΔS2	ΔS3	ΔS4	ΔS5	R2	R4a	R4b	R5	Total
Algeria	1		1		1			3	4	10
Azerbaijan		1	2	2	2	1	1	2	5	16
Bangladesh		1	1	2	6	2	4		9	25
Belgium			1			3	1		13	18
Benin							1		1	2
Burkina Faso		1	2				2	1		6
Canada[b]	1			1		1		2	8	13
Egypt				1	1		2	3	1	8
Guinea					2				1	3
Pakistan				1	1	1			6	9
Peru				1	1		3		2	7
Romania		1		2	1	1	1	3	8	17
Rwanda		1		8	1		2	1	22	35
Tunisia	1			2	1	6	2	6	6	24
Total	3	5	7	20	17	15	19	21	86	193

[a] Samples not included are four RMP^r strains with the wild-type LiPA pattern, three mixtures, and three samples of unclear origin.
[b] Reference strains were obtained from A. Laszlo (National Reference Centre for Tuberculosis, Ottawa, Canada) and were used in an international quality control study (16).

found to be resistant. The initial results on LJ medium were an error, presumably due to the presence of drugs in the eggs used to prepare the LJ medium (16).

Four LiPA-sensitive in vitro-resistant strains were found. These four strains exhibited a low-level resistance to RMP (MIC, about 4 μg/ml) and were isolated from four patients infected with MDR strains. Repeated LiPAs, including tests run with colonies taken from RMP-containing culture medium, produced identical results. A wild-type sequence was found in the sequences of the three strains sequenced. Either these strains carried a mutation in another part of the *rpoB* gene or a different mechanism of resistance was active in these strains.

The mutation frequencies reported by others (reviewed by Kapur et al. [13]) are in agreement with those reported here. Worldwide, the most frequent mutations are S531L, H526Y, and H526D. All three can be reliably detected and differentiated by the INNO-LiPA Rif. TB. The results also indicate that the majority of the mutations (84%) occur in the region from codons 526 to 533.

Despite the high number of different RMP resistance-inducing mutations possibly present in a very short stretch of the *rpoB* gene, the INNO-LiPA Rif. TB proved to be a simple, convenient, and highly reliable tool both for the identification of *M. tuberculosis* complex strains and for the detection of their resistance to RMP. In comparison with DNA sequencing, the "gold standard" for the detection of mutations, discrepancies were not observed in our ad hoc collection. However, an insertion mutation not recognized by the LiPA was recently reported (4). The accordance with the results of the in vitro susceptibility test was also high. The LiPA did not diagnose resistance in only 4 of the 203 (1.97%) strains resistant by in vitro susceptibility testing. In vitro-sensitive strains were always correctly recognized by the LiPA. A resistant pattern by LiPA always corresponded to in vitro resistance.

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