

# Direct Detection of Multidrug-Resistant *Mycobacterium tuberculosis* in Clinical Specimens in Low- and High-Incidence Countries by Line Probe Assay

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**The INNO-LiPA Rif.TB assay is designed for the detection of *rpoB* gene mutations causing rifampin resistance in isolates. We applied the method directly to 60 Lithuanian and Danish clinical specimens to detect rifampin resistance rapidly. Results were obtained in 78.3% of clinical specimens, and all were concordant with those obtained by BACTEC 460. The assay could have major impact on the management of multidrug-resistant tuberculosis.**

Multidrug-resistant tuberculosis (MDR-TB) is defined as TB caused by bacteria resistant to at least rifampin and isoniazid. In two worldwide surveys conducted by the World Health Organization and the International Union Against Tuberculosis and Lung Disease covering the years 1994 to 1999, it has been documented that MDR-TB is a rapidly increasing health problem (5, 10). Evaluations of several outbreaks have shown that late recognition of drug resistance contributed considerably to the mortality and spread of MDR-TB, particularly among immunocompromised patients (3, 4).

Currently, resistance is detected with in vitro drug susceptibility testing methods, which require pure growth of *Mycobacterium tuberculosis* complex (MTC), thus delaying results up to 4 to 6 weeks.

Rifampin resistance is caused by mutations in the *rpoB* gene encoding the beta subunit of the RNA polymerase. These mutations diminish rifampin-binding affinity for the polymerase (9, 12, 16). As rifampin monoresistance is rare, detection of *rpoB* mutations offers a fast approach for detection of MDR-MTC. The INNO-LiPA Rif.TB (LiPA) assay (Innogenetics, Ghent, Belgium) is a commercially available kit-based method for use on isolates. It is based on reverse hybridization between *rpoB* amplicon and immobilized membrane-bound probes covering overlapping sequences of the wild-type sequence (S1-S5) and the most frequent mutations (R2:Asp516Val, R4a:His526Tyr, R4b:His526Asp, and R5:Ser531Leu). The absence of hybridization of one or more of the S probes indicates a mutation that may be identified by one of the R probes.

We aimed to evaluate the ability of this technique to detect MDR-MTC directly in clinical specimens. Furthermore, we validated the assay on isolates to characterize the mutations in the *rpoB* gene. LiPA results were compared to susceptibility

testing on BACTEC 460 system (Becton Dickinson) and to *rpoB* gene sequences.

The study was carried out at the International Reference Laboratory of Mycobacteriology at Statens Serum Institut, Denmark. Thirty-eight Lithuanian and 22 Danish pretreated clinical respiratory specimens, all collected during 2000, were included. Eighteen Danish MDR-MTC isolates collected from 1991 to 2000, 20 Lithuanian isolates from 1999, and 21 Danish rifampin-susceptible isolates were also included.

All specimens were processed by conventional mycobacterial procedures as described in detail elsewhere (7). Drug susceptibility testing for rifampin was carried out on BACTEC 460 (BACTEC 12B medium) at 2.0 µg/ml according to the manufacturer's instructions.

Aliquots (500 µl) of pretreated specimens were stored at –20°C until tested by the LiPA. Danish isolates were stored at –80°C, and Lithuanian isolates on Löwenstein-Jensen medium were recultured in BACTEC Mycobacteria Growth Indicator Tube 960 (Becton Dickinson, Sparks, Md.) before inclusion.

***rpoB* sequencing.** One thousand microliters of positive culture medium was centrifuged (at 12,000 rpm for 15 min in an Eppendorf [Hamburg, Germany] centrifuge 5415D), and the pellet was resuspended in 100 µl of glass beads and 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). After a brief vortex and centrifugation (12,000 rpm, 10 min), 5 µl of lysate was transferred to the PCR amplification mixture (2 mM MgCl<sub>2</sub>, 100 µM each dNTP, 0.2 µM primer [Rp1 {5'-GGGA GCGGATGAACCACCCA-3'} and Rp2 {5'-GCGGTACGG CGTTTCGATGAAC-3'}], 1 U AmpliTaq GOLD [Perkin Elmer] in PCR buffer II [Perkin Elmer]). The cycling protocol was as follows: initial denaturation at 95°C for 12 min; 45 PCR cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1 min and 40 s; and final extension at 72°C for 5 min. Sequence analysis was carried out after purification through QIAquick columns (Qiagen). *M. tuberculosis* *rpoB*-specific primers used for sequencing were Rs1 (5'-TCGCCGCGATCAAGGAGT-3') and Rs2 (5'-TGCACGTCGCGGACCTCC-3'). The sequence re-

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TABLE 1. LiPA assay results obtained in clinical specimens compared to results by microscopy and the BACTEC 460 system

LiPA assay result	No. of isolates with the indicated BACTEC 460 in vitro susceptibility results and microscopy smear results			
	Rifampin-susceptible		Rifampin-resistant	
	Smear positive	Smear negative	Smear positive	Smear negative
Rifampin-susceptible	20	1	0	0
Rifampin-resistant	0	0	21	5
Inconclusive <sup>a</sup>	0	0	6	7
Total (n = 60)	20	1	27	12

<sup>a</sup> Inconclusive due to lack of PCR amplification. The influence of inhibition could not be monitored directly due to lack of internal amplification control.

actions were performed in 20  $\mu$ l using 8  $\mu$ l of ABI PRISM dye terminator cycle sequencing ready reaction mixture (Perkin Elmer), 0.25  $\mu$ M each primer, and 1 to 5  $\mu$ l of PCR product. The sequence reaction was performed for 25 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The products were precipitated and sequenced with an ABI373A automated sequencer.

**INNO-LiPA Rif. TB assay.** Test kits were used in accordance with the manufacturer's instructions for isolates and for clinical specimens, as described below.

Five hundred microliters of decontaminated sample was centrifuged (13,000 rpm, 15 min), and the pellet was resuspended in 100  $\mu$ l of TE buffer. After a brief vortex and centrifugation (13,000 rpm, 15 min), the pellet was resuspended in 50  $\mu$ l of TE buffer. The suspension was heated (95°C, 30 min) followed by freezing (-20°C, 30 min). Five microliters of lysate was transferred to the PCR amplification mixture (10  $\mu$ l of amplification buffer, 10  $\mu$ l of outer primers, 10  $\mu$ l of MgCl<sub>2</sub> solution, 1 U Taq polymerase). Each run contained a negative control. After initial denaturation (95°C, 1 min) and 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s, a final extension (72°C, 5 min) was applied. Successful amplification of isolates and specimens was verified by a band of 260 bp on agarose gel electrophoresis. If amplification of specimens was unsuccessful, 1  $\mu$ l of the first PCR was used for a second amplification by applying the primer mix and the cycling protocol used for isolates.

The fully automated hybridization was carried out at 62°C as described in detail elsewhere (7). Successful amplification was achieved for 25 of 38 Lithuanian specimens and for all 22 Danish specimens (Table 1). Fourteen of 25 Lithuanian resistant specimens had a Ser531Leu mutation, 6 specimens had a Asp516Val mutation, and 2 specimens had a His526Tyr mutation. Hybridization to the S4 probe failed in one specimen, and one specimen showed no hybridization to probe S5, revealing rare mutations in the corresponding codons. The last specimen had Asp516Val and Ser531Leu mutations and hybridization to all S probes and thus probably contained more than one strain. The Danish resistant specimen had a Ser531Leu mutation.

LiPA was inconclusive in 13 Lithuanian MDR-MTC specimens due to lack of PCR amplification. LiPA results were obtained on specimens in 1 to 2 days.

LiPA results were concordant with BACTEC 460 results in

TABLE 2. Comparison of results obtained by LiPA and DNA sequencing on 36 MDR-MTC isolates

LiPA pattern <sup>a</sup>	Mutation(s) identified by DNA sequencing	No. of isolates <sup>b</sup>
R5 (Ser531Leu)	TCG→TTG (Ser531Leu)	11/11
R2 (Asp516Val)	GAC→GTC (Asp516Val)	1/4
R4a (His526Tyr)	CAC→TAC (His526Tyr)	2/0
R4b (His526Asp)	CAC→GAC (His526Asp)	1/0
$\Delta$ S1	CAA→CCA (Gln513Pro)	1/0
$\Delta$ S2	ATG→GAC (Met515Leu), ATC→TAC (Asp516Tyr)	1/0
$\Delta$ S4	CAC→CCC (His526Pro), CAC→CTC (His526Leu)	1/0
		0/1
$\Delta$ S5	Not interpretable	0/1
Wild type	No mutation	0/1

<sup>a</sup> The LiPA assay provided the exact type of mutation in 85% of both Danish and Lithuanian isolates.

<sup>b</sup> Values are numbers of isolates from Denmark/numbers of isolates from Lithuania.

all 39 Danish isolates and in 19 of 20 (17 resistant and 3 susceptible) Lithuanian isolates. The isolate with discordant result was found to be wild type by both LiPA and sequencing. The isolate remained rifampin- and rifabutin-resistant upon retesting by BACTEC 460.

LiPA results were compared to DNA sequencing that was performed on all 36 MDR-MTC isolates (Table 2).

Time-consuming drug susceptibility testing postpones effective treatment of patients infected with MDR-TB, as treatment is normally initiated with standard short-course chemotherapy. Additionally, transmission of MDR-MTC in the community may persist, as patients with pulmonary smear-positive TB are isolated only during the first weeks of treatment unless resistance or noncompliance is suspected. For MDR-TB patients, this procedure is not sufficient. Turett and coworkers reported that rapid initiation of appropriate therapy significantly affected survival among HIV-positive patients (14). Fast, reliable determination of susceptibility is crucial to overcome these problems.

The LiPA assay has shown good performance when used on isolates (1, 6, 11, 13), but bacterial growth must be awaited for 2 to 6 weeks. We demonstrated the ability of the LiPA assay to provide rapid and reliable detection of rifampin resistance in 78.3% of clinical specimens, thereby establishing the MDR-TB diagnosis within a few days of sample collection. This offers improvement in the management of MDR-TB, as these vulnerable patients can commence treatment with second-line drugs while still in isolation. Due to the cost, it might seem reasonable to restrict the procedure to smear-positive patients with suspected MDR-TB (patients originating from high-incidence areas and immunocompromised and previously treated patients).

The sensitivity of the assay may be improved if used on fresh samples only. Deep freezing could damage the mycobacteria, causing release of DNA that might be washed away. It would be of great interest to test the performance in specimens before and after freezing in a subsequent study.

Previous studies have shown regional variation in the *rpoB* gene mutations responsible for the rifampin resistance (1, 2, 6, 8, 9, 12, 16). In Denmark, the most frequent mutation was

Ser531Leu mutation, followed by His526Tyr, in line with findings worldwide (6, 9, 12, 16). Lithuania is the Baltic state where MDR-TB is most prevalent (17). This study is the first dealing with the distribution of mutations in the *rpoB* gene in Lithuania. The Ser531Leu mutation was the most frequent, followed by the Asp516Val mutation. This is in contrast to previous reports on the distribution worldwide. However, the Asp516Val mutation recently has been shown to be the most frequent in Hungary (2).

Mutations in codon 526 and 531 correlate with high-level resistance and have shown cross-resistance between rifabutin and rifampin, whereas mutations in codon 516 yield low-level resistance and are correlated with rifabutin susceptibility (15, 18). All five isolates carrying the 516 mutations in our study were rifabutin susceptible.

In conclusion, the LiPA assay is easy to perform and allows rapid detection of rifampin resistance. If MDR-TB is suspected, the assay can be used for rapid screening to initiate treatment that targets MDR-TB, thereby limiting transmission of the disease in the community. Furthermore, the assay provides epidemiological information that may have direct clinical implications.

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