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Evaluation of a Line Probe Assay Kit for Characterization of *rpoB* Mutations in Rifampin-Resistant *Mycobacterium tuberculosis* Isolates from New York City

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A commercial line probe assay kit (Inno-LiPA Rif.TB) for rapid identification of mutations in the *rpoB* gene associated with rifampin resistance in *Mycobacterium tuberculosis* was evaluated with a collection of 51 rifampin-resistant strains. Nine distinct *rpoB* mutations were identified. Concordances with automated sequence results for five wild-type kit probes and four probes for specific mutations were 94.1 and 100%, respectively. Overall concordance of the line probe assay kit with phenotypic rifampin susceptibility testing results was 90.2%.

The resurgence of tuberculosis in the United States since 1984 was exacerbated by an alarming emergence of *Mycobacterium tuberculosis* strains resistant to primary and secondary antituberculosis drugs (1). This epidemic was dramatized in New York City, which experienced an increase of nearly 300% in the number of tuberculosis patients from 1978 through 1992. Approximately 20% of these patients harbored multidrug-resistant strains. Intensive control measures in institutional settings and improvements in therapy have contributed to a sharp (21%) decrease in tuberculosis cases in New York City from 1992 through 1994 (5). Appropriate choices for alternative therapeutic regimens for drug-resistant *M. tuberculosis* may be impeded by lengthy delays in the availability of drug susceptibility results by conventional methods. Improved methodologies, such as the BACTEC system, which quantitates respiratory rates of bacilli based upon the production of [^{14}C]palmitic acid, have reduced turnaround times for susceptibility results for some primary tuberculosis drugs to as few as 4 days (9).

Identification of mutations by using amplification or probe hybridization methods has been proposed, subject to certain limitations, as a rapid means to screen for antimicrobial drug resistance (13). Foremost among these limitations are the inability of these methods to semiquantify clinically important resistant mutants within a predominantly wild-type susceptible culture population or to identify heterogeneous novel resistance mechanisms and the requirement for multiple amplifications or hybridizations if resistance is conferred by more than a single genetic region. We previously demonstrated, however, that for at least one primary antituberculosis drug, streptomycin, clinically important high-level resistance (MIC >10 μg/ml) may be associated with a single mutation in *rpsL*, the gene encoding ribosomal S12 protein, and that mutant populations as low as 1% of the total may be identified by nonradioactive (cold) single-strand conformation polymorphism electrophoresis (2, 6). Rifampin resistance in *M. tuberculosis*, however, is conferred by a diverse group of mutations within a 69-nucleotide-pair hypervariable (hot spot) region of the *rpoB* gene, which codes for the beta subunit of DNA-dependent RNA polymerase (13). More than 35 mutations within this region have been reported (11). Approximately 95% of Rif^r isolates from the United States (7) and other countries (14) possess mutations within the 69-bp *rpoB* hypervariable region. Although these mutations have been unambiguously characterized by automated DNA sequencing of amplified PCR products, a variety of other rapid methods such as ^{32}P-single-strand conformation polymorphism (4, 14), dideoxy fingerprinting (4), heminested PCR (15), line probe hybridization (3), and PCR heteroduplex analysis (16) have been successfully applied to this task. In a search for practical and convenient methods for screening larger collections of isolates for *rpoB* mutations, we evaluated the line probe assay which is available as a kit for research purposes.

Bacterial isolates. We received 367 viable cultures of *M. tuberculosis* for susceptibility testing in support of a 1-month (April 1994) survey of tuberculosis in New York City; 53 of these (14.4%) were resistant to 1 μg of rifampin per ml as determined by Middlebrook 7H10 agar proportion susceptibility tests (8). At least 15 different IS*6110* fingerprint patterns and 31 patterns of susceptibility to eight primary and secondary antituberculosis drugs were reported among Rif^r isolates. Two of the 53 Rif^r isolates were nonviable upon retrieval from storage, leaving 51 isolates available for study. We previously evaluated 27 of these strains which were additionally resistant to at least 2 μg of streptomycin per ml for mechanisms of resistance to this drug (2).

Molecular methods. Template preparation and other conditions for PCR amplification and automated DNA sequence analyses of sense and antisense strands of PCR products were described previously (2). Sequences for oligonucleotide primers flanking the 69-bp *rpoB* hypervariable region were ATCA ACATCCGGCCGGTGGT (polB5a) (10) and TACACCGAC AGCGAGCCGAT (*rpoB*2659r); these primers were used to generate 255-bp amplimers for sequence analyses.

Line probe assay. The *rpoB* line probe assay kits (Inno-LiPA Rif.TB; Innogenetics N.V., Zwijnaarde, Belgium) were supplied by the manufacturer and used according to kit instructions (3). Cellular extracts containing nucleic acid templates for amplification with kit components were prepared from active broth cultures (Middlebrook 7H9 broth containing 10%

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TABLE 1. Identification of mutations in *rpoB* (nucleotides 2316 to 2571) (10) among 51 *M. tuberculosis* isolates by line probe (Inno-LiPA Rif.TB) assay

Mutation	No. of isolates (%)	No. correctly identified by line probe[c]:	
		ΔS	R
His_{526}→Tyr	20 (39)	20	20
Ser_{531}→Leu	17 (33)	17	17
His_{526}→Asp	2 (4)	2	2
Asp_{516}→Val	2 (4)	2	2
TTC insert (Phe_{514})	2 (4)	0	ND[a]
Leu_{533}→Pro	2 (4)	2	ND
Asp_{516}→Tyr	1 (2)	1	ND
Gln_{513}→Leu	1 (2)	1	ND
AAC deletion (Asn_{518})	1 (2)	1	ND
None	3 (6)	3	NA[b]

[a] ND, no probe for this mutation available in line probe kit.
[b] NA, not applicable.
[c] ΔS, hybridization of wild-type probe in region where mutation was found; R, hybridization of probe specific for mutation.

Middlebrook ADC enrichment; Carr Scarborough Microbiologicals, Inc., Decatur, Ga.) by the minibead method previously described (12). In essence, the line probe kit consists of 10 oligonucleotide probes (one specific for *M. tuberculosis* complex, five overlapping wild-type probes that encompass the entire 69-bp hypervariable region, and four for specific *rpoB* mutations) immobilized on nitrocellulose paper strips. Biotinylated PCR products from test samples are hybridized with the immobilized probes, and results are determined by colorimetric development involving streptavidin, alkaline phosphatase, and a chromogenic substrate. The kit includes a protocol for extracting and testing nucleic acids directly from clinical samples which was not evaluated in this study.

Agar proportion testing for susceptibility to 1 μg of rifampin per ml revealed that 47 of 51 *M. tuberculosis* isolates had no rifampin-susceptible subpopulations as determined by comparing the numbers of colonies on drug-containing media with drug-free controls, and four isolates had susceptible subpopulations ranging from 50 to 75% of the total inoculum. Fifteen of 51 isolates were susceptible to 2 μg of rifabutin per ml. Seven distinct single-nucleotide substitutions were found by automated sequencing among 45 isolates. A 3-nucleotide insertion (coding for Phe_{514}) was found in two isolates, a 3-nucleotide deletion (coding for Asn_{518}) was found in one isolate, and no mutation in the 69-bp region was found in three isolates (Table 1). Visual examination of sequence chromatograms did not reveal the presence of wild-type minor peaks superimposed with mutations for the four isolates determined to have susceptible subpopulations by agar proportion testing.

A probe specific for *M. tuberculosis* complex nucleotide sequences included on each strip of the line probe kit was clearly positive for each of the 51 rifampin-resistant isolates and an antimicrobic-susceptible *M. tuberculosis* control strain, $H_{37}Rv$ (Fig. 1). Hybridization patterns of wild-type probes correlated with sequence results for 49 of 51 isolates and the control isolate. Two isolates, possessing a 3-nucleotide insertion (TTC, coding for Phe_{514}), hybridized with all five wild-type probes. Four distinct nucleotide substitutions accounting for resistance in 41 of 51 isolates (80.4%) were correctly identified by kit probes specific for these mutations (Table 1). Concordance with phenotypic rifampin susceptibility testing results was 90.2% (46 of 51 isolates) since two isolates with codon insertions and three isolates which had no mutations in the 69-bp region were identified as Rif^s by line probe results.

While more than 35 distinct missense mutations within the 69-bp hypervariable region of *rpoB* accounting for rifampin resistance in *M. tuberculosis* have been reported (11), two of these mutations (His_{526}→Tyr and Ser_{531}→Leu) account for ≥65% (73% in our study) of rifampin resistance. The line probe kit includes probes for these mutations as well as two additional mutations, and results for these four specific mutations completely agreed with sequencing data. Mutations in the four isolates which demonstrated resistant subpopulations (50 to 75% of the total) by phenotypic susceptibility testing were correctly identified by the line probe assay. The ability of the kit to detect less frequent mutant subpopulations (e.g., <50%) is unknown. Kit probes for wild-type sequences agreed with sequence results except for nucleotide insertions which are infrequent (11). Since ~5% of all Rif^r *M. tuberculosis* strains evaluated have no mutation within the 69-bp hypervariable region, other mechanisms of resistance or *rpoB* mutations external to this region are indicated. Although the inability of the line probe kit to detect insertional mutants and isolates with wild-type sequence in this region may preclude its usefulness as a substitute for phenotypic rifampin susceptibility testing, it may serve an important role as a rapid and convenient screen for rifampin resistance in *M. tuberculosis.*

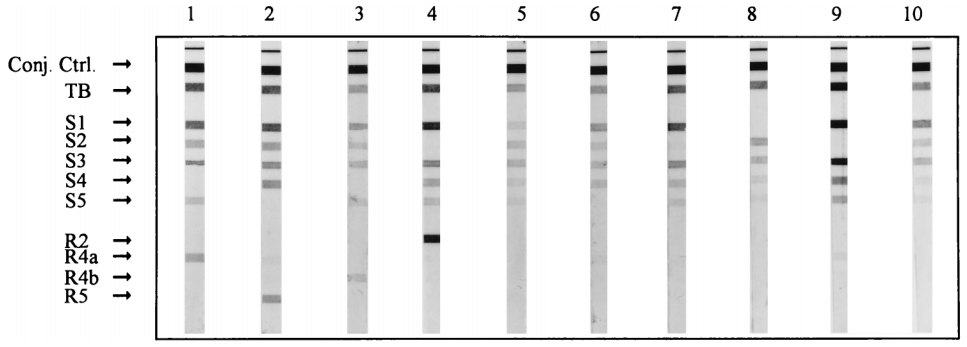

FIG. 1. Line probe kit (Inno-LiPA Rif.TB) hybridization results for rifampin-resistant *M. tuberculosis* isolates. Lanes: 1, 94-3047 (His_{526}→Tyr); 2, 94-2850 (Ser_{531}→Leu); 3, 94-3070 (His_{526}→Asp); 4, 94-2856 (Asp_{516}→Val); 5, 94-2812 (Phe_{514} insert); 6, 94-3013 (Leu_{533}→Pro); 7, 94-2990 (Asp_{516}→Tyr); 8, 94-2953 (Gln_{513}→Leu); 9, 94-2868 (Asn_{518} deletion); 10, $H_{37}Rv$ (wild type). Abbreviations: Conj. Ctrl., conjugate control; TB, probe for *M. tuberculosis* complex; S1 through S5, probes for wild-type sequences in 69-bp hypervariable region of *rpoB*. Probes for specific *rpoB* mutations: R2, Asp_{516}→Val; R4a, His_{526}→Tyr; R4b, His_{526}→Asp; R5, Ser_{531}→Leu.

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