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Characterization of Streptomycin Resistance Mechanisms among *Mycobacterium tuberculosis* Isolates from Patients in New York City

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From a collection of 367 isolates of *Mycobacterium tuberculosis* from patients in New York City in 1994, 45 isolates (12.3%) were resistant in vitro to 2 μg or more of streptomycin (SM) per ml. We further evaluated these isolates for levels of SM resistance and for mutations previously associated with resistance in the *rpsL* (S12 ribosomal protein) gene and the *rrs* (16S rRNA)-coding region. Twenty-four isolates, representing nine distinct patterns of susceptibility to antituberculosis drugs, were resistant to 500 μg of SM per ml and shared a common point mutation at nucleotide 128 in the *rpsL* gene. This mutation, which substitutes lysine for arginine in the S12 ribosomal binding protein, was not present in isolates with low-level SM resistance or in SM-susceptible control isolates. Among 20 isolates with low-level SM resistance, one possessed a substitution ($C \rightarrow G_{865}$) in the 912 loop of the *rrs* gene. No mutations in the 530 loop of the *rrs* coding region were detected, suggesting the presence of an alternative SM resistance mechanism in 19 isolates. Single-strand conformation polymorphisms of mutants were readily detected by a nonradioactive gel screen.

In a survey of more than 4,000 patients culture positive for tuberculosis reported in the first quarter of 1991, multidrug resistance in *Mycobacterium tuberculosis* was reported to be more prevalent in New York City than in other geographic areas of the United States (1). Our laboratory performed in vitro susceptibility testing of 466 single isolates of *M. tuberculosis* obtained from 466 individual patients during a 1-month survey of all patients in New York City from whom samples were culture positive during April 1991 (4). Susceptibility testing by the agar proportion method revealed that 33% of these isolates were resistant to at least one antituberculosis drug, 26% were resistant to isoniazid (INH), 22% were resistant to rifampin (RIF), 8% were resistant to ethambutol (EMB), 1% were resistant to pyrazinamide (PZA), and 13% were resistant to streptomycin (SM). This survey was repeated during a 30-day period in 1994, when 367 isolates were submitted to our laboratory for evaluation of their drug susceptibility patterns. The prevalence of resistance to INH, RIF, EMB, and SM decreased in the time interval between the two surveys. The results of the repeated survey will be reported elsewhere (unpublished data).

The factors that most influence the emergence of drug-resistant strains include inappropriate treatment regimens and noncompliance on the part of patients in completing the prescribed courses of therapy (7). Scrutiny of treatment strategies has resulted in recommendations for inclusion of as many as three additional drugs to the standard three-drug regimen of INH, RIF, and PZA (13). Although SM is an injectable antibiotic and is considered to be less active than INH or RIF against *M. tuberculosis*, it is recommended as an alternative for initial therapy of tuberculosis in a four-drug regimen that also includes INH, RIF, and PZA (7).

As with other aminoglycosides, the mode of action of SM against mycobacterial species is through its binding to the 30S ribosomal subunit, which affects polypeptide synthesis and which ultimately leads to inhibition of translation (14). Alterations in the drug target via a reduced association of the 16S rRNA with the ribosomal S12 protein provide the basis of resistance to SM in *M. tuberculosis*. A point mutation at codon 43 of the *rpsL* gene encoding the S12 protein in several bacterial species including *M. tuberculosis* results in the substitution of an arginine for a lysine in the S12 protein (10). Mutations in regions surrounding nucleotides 530 or 912 in 16S rRNA also have been reported as mechanisms of SM resistance in *M. tuberculosis* (2, 3). As with *Escherichia coli*, substitutions in these regions or in the S12 protein sequence are associated with a decreased affinity of the ribosomes for SM. Other mechanisms of aminoglycoside resistance, including enzymatic modification and impermeability, have not been reported for *M. tuberculosis.*

Early detection of resistance and characterization of resistance mechanisms in *M. tuberculosis* may facilitate the choice of appropriate treatment strategies and ultimately the control of tuberculosis. We evaluated isolates from the survey repeated in 1994 for patterns of resistance that included SM, mechanisms of SM resistance, and potentially useful methods for early resistance detection.

MATERIALS AND METHODS

Bacteria and drug susceptibility testing. Cultures of *M. tuberculosis* on Löwenstein-Jensen agar slants were received from the laboratory of the New York City Department of Health. Antimicrobial susceptibility testing was performed by using the agar proportion method with Middlebrook 7H10 medium (8).

Nucleic acid procedures. Strain typing was performed in the laboratory of B. Kreiswirth at the New York City Public Health Research Institute by using a standardized restriction fragment length polymorphism (RFLP) method with a chemiluminescence-labeled amplification product of the insertion element IS*6110* as a genomic probe (12). Cellular extracts containing nucleic acid templates for amplification by PCR were prepared by vortexing cells in the presence of siliconized mini-glass beads in a Mickle cell disruptor as described previously (11). Amplification reaction mixtures contained 5 μl of lysate, 2.5 U of *Taq*

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TABLE 1. Mutations associated with SM resistance in 43 *M. tuberculosis* isolates from New York City

No. of isolates (no. of strains[a])	SM MIC (μg/ml)	Resistance pattern[b]								Mutation		
		INH	RIF	RBT	EMB	THA	KN	PZA	CIP	*rpsL*	*rrs* (530 loop)	*rrs* (912 loop)
5 (4)	>500	−	−	−	−	−	−	−	−	+	−	−
8 (8)	10	−	−	−	−	−	−	−	−	−	−	−
2 (2)	10	+	−	−	−	−	−	−	−	−	−	−
1 (1)	>500	+	−	−	−	−	−	−	−	+	−	−
1 (1)	10	+	+	−	−	−	−	−	−	−	−	−
3 (3)	10	+	+	+	−	−	−	−	−	−	−	−
1 (1)	10	+	+	+	−	−	−	−	−	−	−	+
2 (2)	>500	+	+	+	−	−	−	−	−	+	−	−
2 (2)	>500	+	+	+	+	−	+	−	−	+	−	−
9 (2[c])	>500	+	+	+	+	+	+	−	−	+	−	−
2 (1)	>500	+	+	+	+	+	+	+	−	+	−	−
1 (1)	10	+	+	−	+	−	−	−	−	−	−	−
2 (2)	10	+	+	−	−	−	−	+	−	−	−	−
1 (1)	>500	+	+	−	+	+	−	−	−	+	−	−
1 (1)	>500	+	+	−	+	−	−	+	−	+	−	−
1 (1)	10	+	+	+	−	−	−	+	−	−	−	−
1 (1)	>500	+	+	+	+	−	+	−	+	+	−	−

[a] Number of strains as determined by RFLP typing.
[b] The drug concentrations tested by the agar proportion method were 500, 250, 100, 50, 25, 10, and 2 μg/ml for SM; 5, 1, and 0.2 μg/ml for INH; 1 μg/ml for RIF; 2 μg/ml for rifabutin (RBT); 5 μg/ml for EMB; 10 μg/ml for ethionamide (THA); 5 μg/ml for kanamycin (KN); 25 μg/ml for PZA; and 2 μg/ml for CIP.
[c] The RFLP patterns were identical for eight of nine isolates.

polymerase, 150 ng of DNA primers, and 0.2 mM (each) the four nucleotides in a final volume of 100 μl. Sense and antisense DNA primers for amplification and nucleic acid sequencing included ML51 and ML52 for the *rpsL* gene (306-bp product), TB53 and TB54 for the 530 loop region of *rrs* (238-bp product), and TB55 and TB56 for the 912 loop region of *rrs* (238-bp product) (6). Control PCR products possessing mutations in the 530 loop regions were kindly supplied by V. Kapur (University of Minnesota, Minneapolis). Conditions for thermocycling included 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The unincorporated nucleotides and primers were separated from amplified DNA by filtration through QIAquick PCR purification columns (Qiagen Inc., Chatsworth, Calif.). Sequencing reactions with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Division, Perkin-Elmer Corp., Foster City, Calif.) were performed with 6.5 μl of PCR-amplified DNA as the template and 3.2 pmol of either the forward or the reverse primer. The unincorporated dye terminators and primers were separated from the extension products by using Centri-sep spin columns (Princeton Separations Inc., Adelphia, N.J.). The products were dried in a vacuum centrifuge for 45 min at ambient temperature, resuspended in 6.5 μl of loading buffer (5:1; deionized formamide and 30 mg of blue dextran per ml–50 mM EDTA [pH 8.0]), heat denatured at 90°C for 2 min, and placed on ice. Immediately after denaturation, 5 μl of each sample was loaded onto a 6% acrylamide gel in an Applied Biosystems model 373A automated DNA sequencer. The gel was electrophoresed at a constant 30 W for 8 h. Sequence data were analyzed by using DNASIS, version 7.0, software (Hitachi Software Engineering America, Ltd., San Bruno, Calif.). PCR products were additionally evaluated for mutations by using a nonisotopic method for detecting a single-strand conformation polymorphism (SSCP) (5). Purified PCR products were denatured with methyl mercury hydroxide (final concentration, 20 mM) and were electrophoresed through Novex precast 4 to 20% gradient polyacrylamide gels (Novel Experimental Technology, San Diego, Calif.) in 1× TBE (Tris-borate-EDTA) for 2 h at 300 V and 13°C (buffer temperature). DNA bands were stained by placing the gels in 10 μM ethidium bromide for 15 min.

RESULTS

The 1994 New York City survey yielded a total of 367 viable cultures of *M. tuberculosis* which were tested in our laboratory between 22 July 1994 and 27 October 1994. In comparison with the 1991 survey, in which 13% of 466 isolates were SM resistant by the agar proportion susceptibility method, 45 isolates (12.3%) from the 1994 survey were found to be SM resistant. Twenty-four (56%) of these isolates were resistant to 500 μg of SM per ml (Table 1), and no susceptible subpopulations (on the basis of colony counts compared with drug-free controls colony counts) were detected for any of these isolates. Eight of 21 (38%) isolates with low-level SM resistance (MIC, 10 μg/ml) had susceptible subpopulations ranging from 10 to 50% of the colonies on drug-free control agar. Among the 43 SM-resistant isolates available for further testing, there were 13 patterns of susceptibility to the eight additional drugs tested. The fingerprint (RFLP) types within each of these patterns were unique except for common banding patterns observed for 2 of 13 isolates that were resistant only to SM and 10 of 11 isolates that were resistant to all drugs tested except ciprofloxacin (CIP) or CIP and PZA (Table 1).

Specific PCR products were obtained by using templates prepared from all 43 isolates. Amplimers for the *rpsL* gene fragment generated with primers ML51 and ML52 were 306 bp, those generated with TB53 and TB54 for the *rrs* (530 loop) were 238 bp, and those generated with TB55 and TB56 for the *rrs* (912 region) were 238 bp. The nucleotide sequence of the *rpsL* product agreed with previous findings (6) except for the findings at position 102, which was found to be a cytosine rather than a thymine in all isolates tested. This base change, however, is degenerate and does not alter the deduced amino acid sequence. All 24 isolates with high-level SM resistance (MIC, >500 μg/ml) possessed a common substitution of a guanine for an adenine at nucleotide 128 of the *rpsL* gene, resulting in a change from lysine (codon AAG) to arginine (codon AGG) in the S12 protein. This mutation was not present among any of the remaining isolates with low-level SM resistance. Mutations in the 530 loop of 16S rRNA were not found among any of the 43 isolates, but one isolate with low-level SM resistance had undergone a cytosine-to-guanine substitution at nucleotide 865 of the 16S rRNA sequence. This position corresponds to position 912 of the *E. coli* 16S rRNA sequence and is adjacent to a position previously associated with SM resistance in *M. tuberculosis* (A to G at position 866 [$A \rightarrow G_{866}$]) (2). Although for the isolate possessing the 912 loop mutation ~50% of its colonial growth was determined to be susceptible to SM by agar proportion testing, no peak corresponding to the wild-type nucleotide (adenine) was present on sequencing chromatograms. Genomic DNA from this strain was independently reamplified in duplicate, and the products were sequenced to confirm the mutation and to eliminate pos-

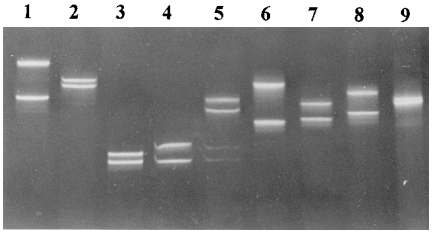

FIG. 1. SSCPs of wild-type (wt) and mutant PCR products representing genes involved in SM resistance in *M. tuberculosis*. Lanes: 1, $H_{37}Rv$ ($rpsL_{wt}$); 2, 94-3105 ($rpsL_{128}$); 3, $H_{37}Rv$ (rrs_{wt}) (912 loop region); 4, 94-2903 (rrs_{865}); 5, $H_{37}Rv$ (rrs_{wt}) (530 loop); 6, 83-3976 (rrs_{512}); 7, 791 (rrs_{516}); 8, 83-4325 (rrs_{491}); 9, 792 (rrs_{516}).

sible nucleotide misincorporations during the original amplification as an explanation for the presence of the substitution. The remaining 19 isolates, all with low-level SM resistance, possessed no mutations in any of the regions sequenced, and no dual peaks in 530 or 912 loop regions of the chromatograms were observed. None of the isolates had multiple mutations.

Mutations in the *rpsL* and *rrs* PCR products were conveniently detected by using nonradioactive SSCP electrophoresis (Fig. 1). All *rpsL* mutants shared a common SSCP banding pattern (Fig. 1, lane 2), while all isolates with low-level or no resistance to SM presented the wild-type pattern (Fig. 1, lane 1). In addition to detection of the *rrs* (912 loop region) mutant, the SSCP procedure was capable of distinguishing four different single-nucleotide substitutions in the 530 loop of *rrs* (Fig. 1, lanes 6 to 9). Optimal conditions for detecting SSCP patterns specific for mutations in each of the three regions included electrophoresis of samples through 4 to 20% gradient acrylamide gels (1× TBE) for 2 h at 300 V and the use of a constant buffer temperature of 13°C during the run.

DISCUSSION

Decisions regarding the inclusion of specific antimicrobial agents in therapeutic regimens are influenced not only by the in vitro susceptibilities of the pathogens but also by the levels of resistance determined by MIC testing. This type of information is rarely available early in the disease process for slowly growing mycobacteria, such as *M. tuberculosis*, often making these decisions empirical. PCR-based methodologies which exploit abilities to detect specific nucleotide sequences present in low copy numbers may serve to address this dilemma if these sequences correlate with levels of resistance to specific drugs. In the present study, we have shown that the presence of *rpsL* sequences possessing mutations at nucleotide 128 (amino acid residue 43) correlate with in vitro phenotypic resistance to at least 500 μg of SM per ml. Our findings regarding this correlation differ from those presented in a previous report in which high MICs (≥160 μg/ml) were reported for two of six SM-resistant strains, and these were explained by *rrs* (912 loop region) mutations in the absence of *rpsL* mutations (6). The means of detecting the *rpsL* mutation is a convenient combination of PCR and then nonisotopic SSCP electrophoresis. Although we have not confirmed the suitability of this method for the direct detection of mutated *rpsL* sequences in clinical specimens (e.g., sputum), we have preliminary evidence that mutants present as 1% subpopulations may be detectable (data not shown). We were able to detect both *rpsL* and *rrs* genes using a single set of electrophoretic conditions. The nonradioactive SSCP procedure offers the additional advantages of eliminating the need for handling radioisotopes, casting and drying acrylamide gels, or performing autoradiography. In contrast, the cold SSCP method offers a high-throughput capability for screening point mutations in larger collections of isolates with precast gels.

Since only 1 of 19 isolates with low-level SM resistance (MICs, 10 μg/ml) and none of the highly resistant isolates possessed mutations in the *rrs* regions, it is apparent that the 530 loop and 912 loop regions of mycobacterial 16S rRNA play only minor roles in explaining SM resistance within this group of organisms. This conclusion differs from that of a previous study of 38 SM-resistant isolates in which 5 of 23 multidrug-resistant isolates and 4 of 15 isolates resistant only to SM possessed 530 loop 16S rRNA mutations and mutations in *rpsL* accounted for resistance in 20 of the 38 isolates. No mechanism of resistance was reported for nine isolates (3). Another study reported a possible interplay of mutations in both regions to explain SM resistance in a single strain (9). These findings underscore the need for future studies of SM resistance in *M. tuberculosis* which focus on mechanisms of low-level resistance. Levels of aminoglycoside resistance that are considered low relative to those mediated by altered ribosomal binding by the drug in certain other bacterial genera are often conferred by drug-modifying enzymes or by reductions in drug uptake. One or both of these alternative mechanisms may play a role in low-level SM resistance in *M. tuberculosis.*

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