Detection of Kanamycin-Resistant Mycobacterium tuberculosis by Identifying Mutations in the 16S rRNA Gene

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Received 2 October 1997/Returned for modification 15 December 1997/Accepted 17 February 1998

In Mycobacterium smegmatis and a limited number of Mycobacterium tuberculosis strains, the involvement of alterations of the 16S rRNA gene (rrs) in resistance to kanamycin has been shown. To the extent which mutations in a specific region of the rrs gene and the kanamycin-resistant phenotype in clinically isolated M. tuberculosis strains were correlated, 43 kanamycin-resistant strains (MICs, ≥200 μg/ml), 71 kanamycin-susceptible strains, and 4 type strains were examined. The 300-bp DNA fragments carrying the rrs gene and the intervening sequence between the rrs gene and the kanamycin-resistant phenotype in clinically isolated M. tuberculosis isolates at positions 1400, 1401, and 1483 but not yet been established.

Kanamycin is one of the key second-line drugs for the treatment of tuberculosis. Patients who are suffering from tuberculosis caused by multidrug-resistant strains with resistance to the first-line antituberculosis drugs such as rifampin, isoniazid, ethambutol, streptomycin, or pyrazinamide have a poor prognosis (15). For such patients, kanamycin is one of the best choices for treatment. In bacteria, resistance to kanamycin is attributed to three mechanisms. One mechanism involves an aminoglycoside-modifying enzyme carried by transposons (22). The second mechanism is specific methylation of RNA. Modification of the rRNA at position 1405 or 1408 was responsible for kanamycin resistance (23). Caceres et al. (7) reported that the overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in Mycobacterium smegmatis. In addition, quinolone-resistant mutations were found on the gyrA gene, as has been seen in other bacteria (26). However, a method for the detection of kanamycin-resistant strains has not yet been established.

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In bacteria, resistance to kanamycin is attributed to three mechanisms. One mechanism involves an aminoglycoside-modifying enzyme carried by transposons (22). The second mechanism is specific methylation of RNA. Modification of the rRNA at position 1405 or 1408 was responsible for kanamycin resistance (5). The third mechanism involves nucleotide changes in the 3′ part of the 16S rRNA gene (rrs) (3, 9). The structural and functional organization of rRNA is highly conserved among bacteria, so it is reasonable to consider that the same mutation results in resistance to kanamycin in mycobacteria, as was seen in other bacteria. Recently, we have used a genetic conjugation system to show that the nucleotide substitution from A to G at position 1389 of the 16S rRNA gene in M. smegmatis was responsible for the kanamycin-resistant phe-
The susceptibility of the clinical isolates used in this study to first-line antituberculosis drugs

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>No. of strains with the following kanamycin susceptibility resistant to the drug(s):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>4</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>S</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
</tr>
<tr>
<td>R, I</td>
<td>3</td>
</tr>
<tr>
<td>R, S</td>
<td>2</td>
</tr>
<tr>
<td>R, E</td>
<td>0</td>
</tr>
<tr>
<td>I, S</td>
<td>1</td>
</tr>
<tr>
<td>I, E</td>
<td>8</td>
</tr>
<tr>
<td>S, E</td>
<td>2</td>
</tr>
<tr>
<td>R, I, S</td>
<td>8</td>
</tr>
<tr>
<td>R, I, E</td>
<td>7</td>
</tr>
<tr>
<td>R, S, E</td>
<td>8</td>
</tr>
<tr>
<td>I, S, E</td>
<td>6</td>
</tr>
<tr>
<td>R, I, S, E</td>
<td>14</td>
</tr>
</tbody>
</table>

*Abbreviations: R, rifampin; I, isoniazid; S, streptomycin; E, ethambutol.*

Notably, in the same study we showed with a limited number of strains that there exist some correlations between the mutation from A to G at position 1400 of the 16S rRNA gene, equivalent to position 1389 of *M. smegmatis*, and a kanamycin-resistant phenotype in *M. tuberculosis* (28).

To make sure that this observation would be generally applicable for the identification of clinically isolated *M. tuberculosis* strains with the kanamycin-resistant phenotype, we have amplified and sequenced the identical fragment reported previously (28) and compared the results.

In this paper, we discuss the use of sequencing of the *rrs* gene segment and PCR product-restriction fragment length polymorphism (PCR-RFLP) analysis as tools for the identification of kanamycin-resistant *M. tuberculosis* strains.

**MATERIALS AND METHODS**

**Bacterial strains.** Kanamycin-susceptible and -resistant clinical isolates of *M. tuberculosis* were kindly provided by the following institutions: Hiroshima University (Hiroshima, Japan), Osaka Prefectural Habikino Hospital (Osaka, Japan), Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (Tokyo, Japan), National Minami-Fukuoka Hospital (Fukuoka, Japan), National Fukuoka-Higashi Hospital (Fukuoka, Japan), National Sanyoso Hospital (Yamaguchi, Japan), National Ohmuta Hospital (Fukuoka, Japan), National Cuyhu Hospital (Aichi, Japan), Aihoku Hospital (Aichi, Japan), and National Sapporo-nanpo Hospital (Hokkaido, Japan). The type strains *M. tuberculosis* H37Rv, H37Ra, Aoyama B, and ATCC 35416 have been maintained on Ogawa egg medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan). Drug susceptibility testing was performed by the conventional method with Ogawa egg medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan). PCR was performed as follows: 94°C for 10 min, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, followed by 5 min at 72°C for completion. A PCR thermal cycler (MP, Takara Shuzo) was used throughout.

**PCR-based sequencing of PCR products.** PCR products were subjected to the purification step by agarose gel electrophoresis. The block containing the bands in which we were interested was sliced out of the agarose gel and frozen at −80°C for 10 min, followed by centrifugation at 15,000 × g for 5 min. The concentration of DNA fragments in the eluent obtained after the centrifugation was determined by comparing the density of the band with those of the HindIII-digested bacteriophage λ DNA fragments (Takara Shuzo) of known concentration. DNA sequences were obtained by a modified dideoxy procedure using 0.5 to 1 μg of template per sequencing reaction with an Autoseq DNA sequencing kit (Pharmacia Biotech) and an A.L.F. fluorescent auto DNA sequencer (Pharmacia Biotech). Every PCR product from both strands was sequenced.

**PCR-RFLP analysis.** The PCR products obtained by using primers TBr1250 and TBr38 were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, dissolved in TE buffer, and then digested with the restriction endonucleases TaiI, Tsp65I, BsrUI, and DdeI (New England Biolabs, Inc., Beverly, Mass.) according to the manufacturer’s procedure. Digested PCR products were separated by electrophoresis on a 4% agarose gel (NuSieve GTG agarose; FMC Bioproducts Inc., Rockland, Maine). The resulting bands were then visualized by staining with SYBR green nucleic acid gel stain (FMC Bioproducts).

**RESULTS**

**Nucleotide sequence analysis of 3’ part of 16S rRNA gene from kanamycin-resistant and -susceptible *M. tuberculosis*.** DNAs were extracted from a panel of kanamycin-resistant and -susceptible clinical isolates and type strains of *M. tuberculosis*. DNA fragments of 300 bp containing the 3’ one-fifth of the 16S rRNA gene and 38 bp of the intervening sequence between the 16S and the 23S rRNA genes were made by PCR and were used for PCR-based direct sequencing. When the chromosomal DNAs of these *M. tuberculosis* strains were analyzed by PCR by using the strategy described in Materials and Methods, the expected products with a length of 300 bp were obtained in all cases. To reduce artifacts resulting from the misincorporation of nucleotides by the Taq DNA polymerase and cloning errors, PCR-based direct sequencing was used. The PCR-amplified DNA fragments were separated by electrophoresis on 1% agarose gels and were cut out of the gels as blocks. Template DNA solutions were obtained in the supernatant after freezing the gel block and centrifuging it at 20,000 × g for 5 min. The nucleotide sequences of the regions in which we were interested were obtained by the dideoxy method by using PCR-amplified DNAs as templates. When comparing the nucleotide
sequences with those for the same region reported by us (24) and Kempsey et al. (16), all 71 clinically isolated kanamycin-susceptible M. tuberculosis strains and the 4 type strains (strains H37Rv, H37Ra, Aoyama B, and ATCC 35416) exhibited identical nucleotide sequences. In striking contrast, three different mutations were found among the 29 kanamycin-resistant M. tuberculosis strains. An A-to-G nucleotide substitution at position 1400, the most frequent mutation, was observed in 26 of the 43 (60.5%) kanamycin-resistant clinical isolates. The mutant genotype with a substitution from C to T at position 1401 was found only once (2.3%). As described above, most of the substitutions were single nucleotide substitutions. Double nucleotide substitutions (from C to A at position 1401 and from G to T at position 1483) were found in two isolates (4.7%). However, no mutations were identified in the specific region of the rrs genes of 14 kanamycin-resistant isolates (32.6%) examined in this study (Table 2).

**TABLE 2. Genotypes of rrs genes of kanamycin-resistant and -susceptible clinical isolates of M. tuberculosis**

<table>
<thead>
<tr>
<th>Kanamycin susceptibility</th>
<th>No. of strains tested</th>
<th>No. of strains with a mutation(s) at the following position(s):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(A → G)</td>
</tr>
<tr>
<td>Susceptible</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>Resistant</td>
<td>43</td>
<td>14</td>
</tr>
</tbody>
</table>

**FIG. 2.** Expected restriction digestion patterns of amplicons. The restriction digestion patterns of the DNA fragments in which we were interested, which were amplified by using primers TBrrs1250 and TBrrivs38 as described in the legend to Fig. 1, are presented schematically. (A) Alignment of nucleotide sequences of wild-type (wt) and mutant alleles from positions 1381 to 1420. The restriction endonuclease digestion sites of Tsp45I, TaqI, BsrUI, and DdeI are indicated below the sequences. (B) Alignment of nucleotide sequences of wild-type and mutant alleles from positions 1464 to 1503. The restriction endonuclease digestion site of DdeI is indicated below the sequences. (C) PCR-generated fragments of the wild-type sequence are digested by Tsp45I, whereas the fragments of strains with mutant alleles are not. (D) PCR-generated fragments of the wild-type sequence are digested by TaqI into three fragments, whereas the fragments of strains with mutant alleles are digested into two fragments. (E) PCR-generated fragments of the wild-type sequence and mutated sequence at position 1401 are resistant to digestion with BsrUI, whereas the fragments of strains with mutant alleles at position 1400 (from A to G) are digested into two fragments. (F) PCR-generated fragments of strains with the mutation at position 1483 (from G to T) are digested into four fragments, whereas the fragments of other strains are digested into three fragments.

**DISCUSSION**

In an earlier study, we showed that kanamycin resistance in M. smegmatis is due to some alterations in a 30S ribosome subunit (31). Recently, the precise positions of the mutations in M. smegmatis were identified by use of the conjugation system (28) (Fig. 4). The base substitution at position 1389 (from A to G), which is equivalent to position 1400 of M. tuberculosis, was found in the high-level kanamycin-resistant mutants. Kanamycin-resistant M. smegmatis had other mutations, from T to A at position 1387 (equivalent to position 1398 of M. tuberculosis) and from G to T at position 1473 (equivalent to position 1483 of M. tuberculosis). The mutation at position 1473 in M. smegmatis was characterized as the key mutation for viomycin and capreomycin resistance as well as kanamycin resistance (28). In Escherichia coli, mutations at positions 1408, 1409, and 1491 (equivalent to positions 1400, 1401, and 1483 of M. tuberculosis, respectively) caused resistance to kanamycin, paromomycin, and other aminoglycoside antibiotics (9, 20).
In this study of *M. tuberculosis*, mutated bases were observed in the DNAs of 29 strains of kanamycin-resistant clinical isolates. Twenty-six of 29 strains carried the base substitution from A to G at position 1400 (equivalent to position 1389 of *M. smegmatis* and position 1408 of *E. coli*), which appeared to be responsible for kanamycin resistance. In addition to this mutation, two clinical isolates possessed the mutation from C to A at position 1401 (equivalent to position 1390 of *M. smegmatis* and position 1409 of *E. coli*), as well as the mutation from G to T at position 1483 (equivalent to positions 1473 and 1491 of *M. smegmatis* and *E. coli*, respectively). The mutation at position 1473 in *M. smegmatis* was characterized as the key mutation for viomycin, capreomycin, and kanamycin resistance. In *E. coli*, an equivalent mutation appeared to be responsible for resistance to aminoglycoside antibiotics. So, the mutation at position 1401, together with the mutation at position 1483 in *M. tuberculosis* found in this study, may be responsible for kanamycin resistance. Interestingly, nucleotide 1401 is thought to hydrogen bond with nucleotide 1483. So, the conversion from a C to an A at position 1401 and a G to a T at position 1484 weakens the strength of hydrogen bonding (Fig. 3). The same kind of nucleotide substitution which weakens the strength of hydrogen bonding was found at position 1401 (from C to T). This mutation was not seen in kanamycin-resistant *M. smegmatis* but was seen in *E. coli*. By analogy, it appears possible that this mutation may be responsible for the kanamycin resistance in the *M. tuberculosis* strains that we studied.

In the kanamycin-resistant *M. tuberculosis* strains analyzed in this study, mutations in the *rrs* gene fragment were found in 67.4% (29 of 43) of the strains. This percentage is lower than that for mutations in the specific region of the *rpoB* gene (more than 95%) in rifampin-resistant *M. tuberculosis* (21, 25, 27, 29). Similar observations were made for both streptomycin- and isoniazid-resistant *M. tuberculosis* strains. Drug resistance cannot be explained by mutations in one gene region in these cases. At least three enzymes are involved in resistance to isoniazid in *M. tuberculosis* (2, 4, 30, 32), and at least two genes are responsible for streptomycin resistance (12, 15, 21). So, causes of resistance other than the mutations in the region studied in this experiment should be considered. One candidate gene that may be involved in kanamycin resistance is elongation factor G. Aminoglycoside antibiotics have been reported to bind to the A site of the 30S ribosome and inhibit translocation (11, 18). Elongation factor G is known to be involved in translocation. Fusidic acid-resistant mutants of *Salmonella typhimurium* with mutations in elongation factor G.

![FIG. 3. Analysis of the PCR products by PCR-RFLP analysis. The DNA fragments in which we were interested were amplified by using primers TBrrs1250 and TBrrivs38, as described in the legend to Fig. 1, digested with restriction endonucleases, and analyzed by electrophoresis on a 4% agarose gel. (A) PCR-generated fragments were digested with Tsp45I. (B) PCR-generated fragments were digested with TaqI. (C) PCR-generated fragments were digested with BsrUI. (D) PCR-generated fragments were digested with DdeI. Lane M, DNA marker; lane 1, PCR product from a kanamycin-susceptible isolate; lane 2, PCR product from a kanamycin-resistant isolate with the wild-type sequence; lane 3, PCR product from a kanamycin-resistant isolate with a mutation at position 1401 (C to T); lane 4, PCR product from a kanamycin-resistant isolate with a mutation at position 1400 (A to G); lane 5, PCR product from a kanamycin-resistant isolate with mutations at position 1401 (C to A) and position 1483 (G to T). Numbers on the left indicate the lengths (in base pairs) of the DNA marker (pUC118 HindIII digests).](http://jcm.asm.org/)

**FIG. 4.** Locations of mutations in the *rrs* genes that cause aminoglycoside resistance in three bacterial species. A portion including the bases that were reported to be responsible for aminoglycoside resistance are reproduced from the secondary structure proposed by Moazed and Noller (20). Arrows indicate the bases responsible for resistance in *E. coli* (9), *M. tuberculosis* (this study), and *M. smegmatis* (28).
exhibited the kanamycin-resistant phenotype (14). Considering all of these facts, searching for mutations in the elongation factor G gene of kanamycin-resistant *M. tuberculosis* strains may lead to another cause of kanamycin resistance. The existence of genetic elements carrying aminoglycoside-modifying enzymes such as Tn5 in *E. coli* (22) have not yet been disproven. Moreover, there may exist specific methylases that can modify the rRNA at position 1405 or 1408, as described for *Streptomyces lividans* (5). In addition, permeability barriers (13) are also candidates for the cause of kanamycin resistance in *M. tuberculosis*.

Finally, we have tried to develop a rapid and simple method for the detection of kanamycin-resistant *M. tuberculosis* by applying PCR-RFLP techniques. The wild-type sequence of the *rrs* gene turned out to contain two restriction sites near the position where mutations associated with kanamycin resistance were found. It was suggested from the sequence of the amplon that all PCR-generated DNA fragments with mutations at either position 1400 or position 1401 were resistant to *Tsp*45I, whereas PCR-generated DNA fragments of the wild-type sequence were digested into two fragments of 175 and 125 bp (Fig. 2A and C). *Tai*I was supposed to digest the wild-type amplicon into three fragments with lengths of 171, 98, and 31 bp, whereas the PCR product carrying the mutant sequence will be cut into only two fragments of 202 and 98 bp (Fig. 2A and D). The mutant allele with the base conversion from A to G at position 1400 has the *BatUI* restriction sequence (CG ^ CG; spanning from positions 1400 to 1403) (Fig. 2A). The PCR-generated DNA fragment with this mutant sequence was supposed to be digested into two fragments with lengths of 178 and 122 bp, respectively, whereas the PCR-generated DNA fragment with the wild-type sequence or some other mutant sequence was supposed to be resistant to this enzyme (Fig. 2E). In addition, the mutation at position 1483 (from G to T) converts the PCR product into one that is digestible by the restriction endonuclease *Dde*I (recognition sequence, C ^ TRAG) (Fig. 2B) into four fragments with lengths of 196, 48, 38, and 14 bp (Fig. 2F). The lengths of the DNA fragments obtained by restriction digestion exhibited good agreement, with one exception, with the lengths suggested by the nucleotide sequence of amplicon when analyzed by agarose gel electrophoresis (Fig. 3); the exception was a 14-bp *Dde*I fragment which was too short to be detected with the agarose gel used in this study. All of the mutant genotypes found in this study can be identified by digesting the PCR products with at least three restriction endonucleases. However, the displacement of restriction fragments in *Tai*I and *Dde*I digestion are very small, and much care should be taken in running the assays and interpreting the results.

It is not clear that the results obtained in this study with clinically isolated strains only from Japan are applicable to strains from other countries. For strains resistant to other drugs, such as rifampin and streptomycin, however, the genes with mutations responsible for resistance in Japanese isolates were the same as the genes with mutations responsible for resistance in isolates from other countries (15, 25, 27). From this finding, the results obtained in this study may be applicable to strains from all over the world. In addition, further studies are necessary to answer the question of whether the results obtained in this study are applicable to nontuberculous mycobacterial strains.

**ACKNOWLEDGMENTS**

We thank N. Hirota, N. Miyazaki, H. Fukunaga, K. Ishibashi, S. Yamori, S. Kawahara, S. Sumi, and all members of the Department of Clinical Laboratory in Habikino Hospital for gifts of clinical mycobacterial isolates.

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