Mutations in mutT genes of Mycobacterium tuberculosis isolates of Beijing genotype

Nicoletta Lari,1 Laura Rindi,1 Daniela Bonanni,1 Enrico Tortoli2 and Carlo Garzelli1

1 Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, Università di Pisa, I-56127 Pisa, Italy
2 Centro Regionale di Riferimento per i Micobatteri, Laboratorio di Microbiologia e Virologia, Ospedale Careggi, I-50134 Firenze, Italy

INTRODUCTION

Molecular typing of Mycobacterium tuberculosis strains isolated in several countries in recent years has revealed that a family of strains known as ‘Beijing’ (or ‘Beijing/W’ or ‘W-Beijing’) is widespread around the world (Bifani et al., 2002; Filliol et al., 2003; Glynn et al., 2002). M. tuberculosis strains of Beijing genotype are mostly prevalent in Asia, but recent data suggest that they have spread to eastern Europe and Indo-China, being significantly more prevalent among younger patients than older patients in Vietnam (Anh et al., 2000). There is concern that the Beijing family may have a predilection for drug resistance, especially multidrug resistance (Glynn et al., 2002). In fact, the Beijing genotype of M. tuberculosis, with a higher prevalence of drug-resistance mutations than non-Beijing strains, has been identified in 40–50% of the clinical isolates studied in Russia during the last decade (Mokrousov et al., 2003).

In M. tuberculosis, resistance to anti-mycobacterial drugs is exclusively due to genomic mutations in specific genes (Ramawamy & Musser, 1998). With other bacteria, mutated phenotypes commonly result from defects in DNA repair (Horst et al., 1999), so it has been suggested that Beijing strains may have defective DNA repair systems, which would confer a mutator phenotype allowing an increased mutation rate, thus leading to a selective advantage during exposure to anti-mycobacterial drugs.

An in silico analysis has shown that most mismatch-repair systems commonly found in Escherichia coli (Mizrahi & Andersen, 1998) are missing in M. tuberculosis, and only a number of putative genes encoding DNA repair enzymes, such as mutT, ogt, mutM and mutY, have been detected in the M. tuberculosis genome (Rad et al., 2003). Analysis of strains representing different branches of the Beijing genotype has shown that the Beijing strains display unique missense alterations in putative mut genes, including two of the mutT type (the ORF Rv3908, designated mutT4 and mutT2) and ogt. These polymorphisms were found to be characteristic and unique to the Beijing phylogenetic lineage. The mutator phenotype was found to increase the prevalence of drug resistance, but further studies are required to investigate the mutation rates of Beijing isolates in response to drug exposure.

Abbreviations: PGG, principal genotypic group; TB, tuberculosis.

Missense alterations in genes mutT4 and mutT2, which encode DNA repair enzymes, were sequenced from 30 clinical isolates of Mycobacterium tuberculosis of Beijing genotype, mostly from patients with primary tuberculosis, to evaluate their contribution to anti-mycobacterial drug resistance. The mutation Arg to Gly at codon position 48 (CGG to GGG) of mutT4 was found in 21 isolates; of these, 16 isolates also harboured the mutation Gly to Arg at position 58 (GGA to CGA) of mutT2. No statistically significant association was found between mutT4 and mutT2 mutations, and drug resistance. Furthermore, no mutations in mutT4 or mutT2 were found in any of 24 isolates resistant to multiple drugs, nor in 28 anti-mycobacterial drug-susceptible isolates of different genotypes. These data confirm that the polymorphism of mutT genes is characteristic and unique to the Beijing phylogenetic lineage. The mutator phenotype does not appear to increase the prevalence of drug resistance, but further studies are required to investigate the mutation rates of Beijing isolates in response to drug exposure.
previously treated with anti-TB drugs; previous TB and/or drug treatment was unknown for the remaining nine patients. A total of 24 non-Beijing strains resistant to multiple drugs and 28 fully susceptible non-Beijing strains isolated from 1993 to 2003, and during 2002 and 2003, in the same geographic area as the Beijing strains, were selected from our collection and used as controls. Assignment of isolates to the different genotypes was performed on the basis of the spoligotyping assay. All isolates were subjected to IS6110 RFLP typing and assigned to one of the three principal genotypic groups (PGGs) delineated by Sreevatsan et al. (1997) on the basis of the polymorphisms at codon 463 of the katG gene and codon 95 of gyrA gene (see below). The genotypes of the control isolates are reported in Table 1. Susceptibility of the isolates to isoniazid, rifampicin, ethambutol and pyrazinamide was determined by the radiometric BACTEC 460 TB system (Becton Dickinson) according to the protocol.

**Molecular typing assays.** Spoligotype analysis of isolates was performed as described by Kamerbeek et al. (1997), and the spoligotypes were compared to those contained in the SpolDB4 database (Brudey et al., 2006). IS6110-RFLP analysis of isolates was performed according to the standardized method described by van Embden et al. (1993). Polymorphisms at codon 463 of the katG gene and at codon 95 of gyrA gene of the isolates were evaluated by a real-time PCR assay, as previously reported (Rindi et al., 2004).

**mutT genes mutations.** Mutations in mutT genes were searched for by nucleotide sequencing using oligonucleotide primers pairs designed to amplify a 398 bp fragment of the mutT4 gene and a 675 bp fragment of the mutT2 gene, both containing the mutation sites previously reported by Rad et al. (2003). The primers pairs were TAAGTCTTGCGGAGCATGGA and CAACCTGATGGCCTGCCTGG for mutT4 gene, and GGCCTATACGTCGGAACCTGG and CGCGTCAAGAAAACCATCGTAA for mutT2. PCR was performed in 0.5 ml micro-centrifuge reaction tubes in a final volume of 50 μl, containing 50 mM Tris/HCl (pH 9.0), 1.5 mM MgCl2, 15 mM (NH4)2SO4, 0.1% Triton X-100, 0.25 mM primers, 200 μM dNTP, 1 U DyNAzyme EXT DNA polymerase and 30 ng DNA; after an initial denaturation step of 94°C for 3 min, the amplification was performed with a PCR Express thermal cycler (Hybaid), set for 1 min at 94°C, 1 min at primer annealing temperatures (64°C for mutT4 and 65°C for mutT2), 2 min at 72°C for 30 cycles, followed by one final 4 min extension cycle at 72°C. Direct sequencing of PCR products was carried out with a semi-automated apparatus (ALFexpress DNA sequencing; Pharmacia Biotech) using the Thermo Sequenase Cy5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech).

**RESULTS AND DISCUSSION**

**Molecular characteristics of Beijing isolates**

A total of 28 of the isolates assigned to the Beijing genotype showed the typical spoligotype pattern characterized by the deletion of spacers 1 to 34 in the direct repeat locus [locus number 000000000003771, share type (ST) 1]; the remaining 2 isolates showed deletions of spacers 1 to 36 and spacer 40 (isolate no. 838, octal number 000000000000731, ST 406), and deletions of spacers 1 to 34 and spacers 38 to 42 (isolate no. 946, octal number 000000000003401, ST 940). All Beijing isolates belonged to PGG 1. By IS6110-RFLP analysis, the 30 Beijing isolates yielded a total of 20 distinct IS6110 patterns; 15 isolates (50%) occurred in 5 distinct clusters with identical IS6110 fingerprints, of these, 3 clusters contained 2 isolates, and 2 clusters contained 4 and 5 isolates (Fig. 1).

**Drug resistance and mutT mutations**

As shown in Table 2, 6 Beijing isolates (20.0%) were resistant to isoniazid; such frequency, however, was not statistically different from that of isoniazid resistance (9.3%) detected in 473 non-Beijing strains isolated in the same years and in the same geographic area. Resistance to rifampicin was not detected in any Beijing isolate; only one isolate displayed multiple resistances to isoniazid, ethambutol and pyrazinamide.

As shown in Fig. 1, nucleotide sequencing of mutT genes of Beijing isolates showed a base substitution at codon 48 of mutT4, consisting of a change of wild-type codon CGG to GGG resulting in the amino acid substitution of Arg by Gly, in 21 isolates; of these, 16 isolates also harboured a mutation at codon 58 of mutT2, a change of the wild-type codon GGA to CGA, resulting in an amino acid substitution of Gly by Arg. Resistance to one drug (isoniazid) was detected in 5 of the 21 isolates mutated in mutT4 (4 isolates also displayed the mutT2 mutation); 1 of the 9 isolates with wild-type mutT genes displayed multiple resistances. The association of
Fig. 1. IS6110 fingerprints, mutT4 and mutT2 mutations, and drug resistance of 30 clinical isolates of M. tuberculosis of Beijing genotype. IS6110-RFLP patterns were compared and the dendrogram was constructed by using the UPGMA clustering method and the Dice coefficient by the Gelcompar 4.1 software package (Applied Maths). Isolate codes, wild-type (wt) or mutated codons of mutT4 and mutT2 genes, and drug resistance, expressed as number of drugs to which the isolate is resistant, are shown on the right of each RFLP panel.
**Table 2.** Pattern of drug resistance of *M. tuberculosis* isolates of Beijing genotype compared to isolates of non-Beijing genotype

<table>
<thead>
<tr>
<th>Drug*</th>
<th>Beijing</th>
<th></th>
<th>Non-Beijing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total tested</td>
<td>No. (%) of drug resistant isolates†</td>
<td>Total tested</td>
</tr>
<tr>
<td>INH</td>
<td>30</td>
<td>6 (20-0)</td>
<td>473</td>
</tr>
<tr>
<td>RIF</td>
<td>30</td>
<td>0 (0)</td>
<td>473</td>
</tr>
<tr>
<td>ETH</td>
<td>30</td>
<td>1 (3-3)</td>
<td>464</td>
</tr>
<tr>
<td>PZA</td>
<td>30</td>
<td>1 (3-3)</td>
<td>445</td>
</tr>
<tr>
<td>Multiple drugs</td>
<td>–</td>
<td>1 (3-3)</td>
<td>–</td>
</tr>
</tbody>
</table>

*ETH, ethambutol; INH, isoniazid; PZA, pyrazinamide; RIF, rifampicin.
†Determined by use of the radiometric BACTEC 460 TB system.

mutT4 and mutT2 mutations with drug resistance was not statistically significant (*P*=0.637 by Fisher’s exact test).

No mutation in mutT4 or mutT2 was found in any of 24 non-Beijing isolates resistant to multiple drugs, nor in any of 28 drug-susceptible non-Beijing strains.

**Conclusions**

This investigation, although carried out in a local setting where Beijing strains represent only a small proportion of the *M. tuberculosis* complex isolates (Lari et al., 2004, 2005), confirms that the polymorphism of the putative genes conferring a mutator phenotype is characteristic and unique to the Beijing phylogenetic lineage, as reported by Rad et al. (2003). Although the missense alterations detected in mutT genes do not appear to increase prevalence of resistance in the Beijing isolates of the present study, which are mostly primary isolates, it cannot be ruled out that the mutator phenotype might increase the rate of drug resistance mutations when strains are exposed to the selective pressure of anti-TB therapy. On this subject, further studies directly investigating the response of Beijing strains to anti-TB drugs are required.

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**REFERENCES**


