Journal of Medical Microbiology (2006), **55**, 599–603 DOI 10.1099/jmm.0.46261-0

Mutations in *mutT* genes of *Mycobacterium tuberculosis* isolates of Beijing genotype

Nicoletta Lari,[1] Laura Rindi,[1] Daniela Bonanni,[1] Enrico Tortoli[2] and Carlo Garzelli[1]

[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

Correspondence
Carlo Garzelli
garzelli@biomed.unipi.it

Received 25 July 2005
Accepted 9 January 2006

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 prevalence of drug resistance, but further studies are required to investigate the mutation rates of Beijing isolates in response to drug exposure.

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 (Ramaswamy & 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 (Rad *et al.*, 2003).

The aim of this investigation was to study the variation in *mutT* genes in clinical isolates of Beijing *M. tuberculosis* to evaluate the contribution, if any, of *mutT* gene mutations to drug resistance.

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

METHODS

Clinical isolates. A total of 30 *M. tuberculosis* isolates of Beijing genotype, obtained from 2002 to 2004 from separate patients with tuberculosis (TB) hospitalized in Tuscany, Italy, were studied. Nineteen isolates were from patients with primary TB with no records of previous anti-TB therapy, two isolates were from patients

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 IS*6110* 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 *gyr*A 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 proportion method. Drug resistance of Beijing isolates was compared with that of non-Beijing strains isolated in the same years and in the same geographic area as Beijing strains.

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). IS*6110*-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 TAAGTCCTGGCCGACGATGG and CAACTCGATGTGCCCCTTGG for *mutT4* gene, and GGCCATAAACGTCGGAAACTTG and CGCGTCCAGAAAACCATCGTAA 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 $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 0·1 % Triton X-100, 0·25 µM 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 sequencer; Pharmacia Biotech) using the Thermo Sequenase Cy5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech).

Table 1. Genotypes of non-Beijing strains

Genotypes were defined according to the SpolDB4 database (Brudey *et al.*, 2006). Abbreviations: CAS, central Asian; EAI, East African-Indian; LAM, Latino-American and Mediterranean. All non-Beijing isolates (resistant to multiple drugs or drug sensitive) displayed distinct IS*6110*-RFLP profiles, with the exception of the isolates of Bovis genotype showing the typical one-band pattern (data not shown).

Drug resistance	No. tested	Genotype	PGG
Multiple	1	Bovis	1
	5	LAM	2
	1	S	2
	3	Haarlem	2
	1	Haarlem	3
	2	T	2
	10	T	3
	1	Undefined	2
None	3	Bovis	1
	3	Africanum	1
	6	EAI	1
	4	CAS	1
	4	Haarlem	2
	3	LAM	2
	1	X	2
	2	T	3
	1	T	2
	1	Undefined	1

RESULTS AND DISCUSSION

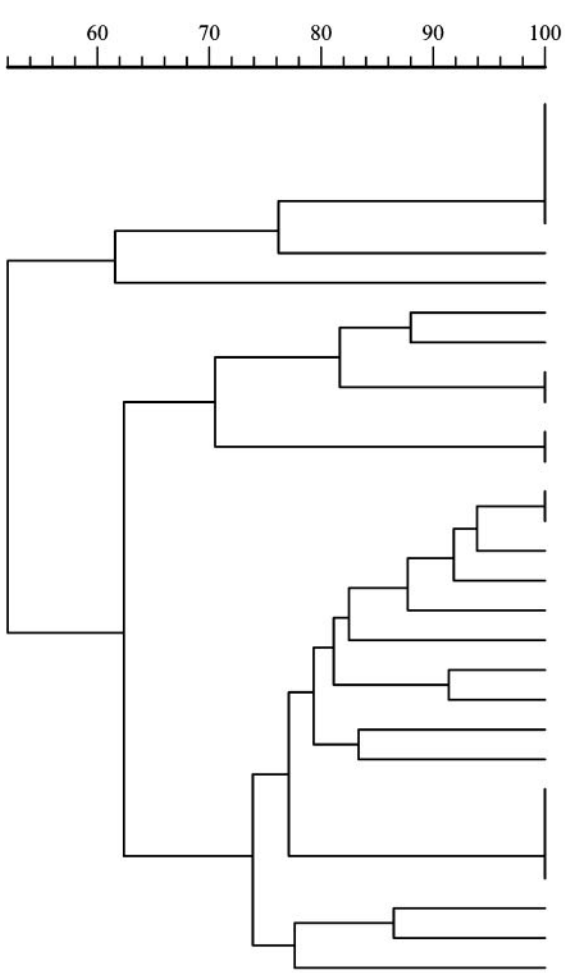
Molecular characteristics of Beijing isolates

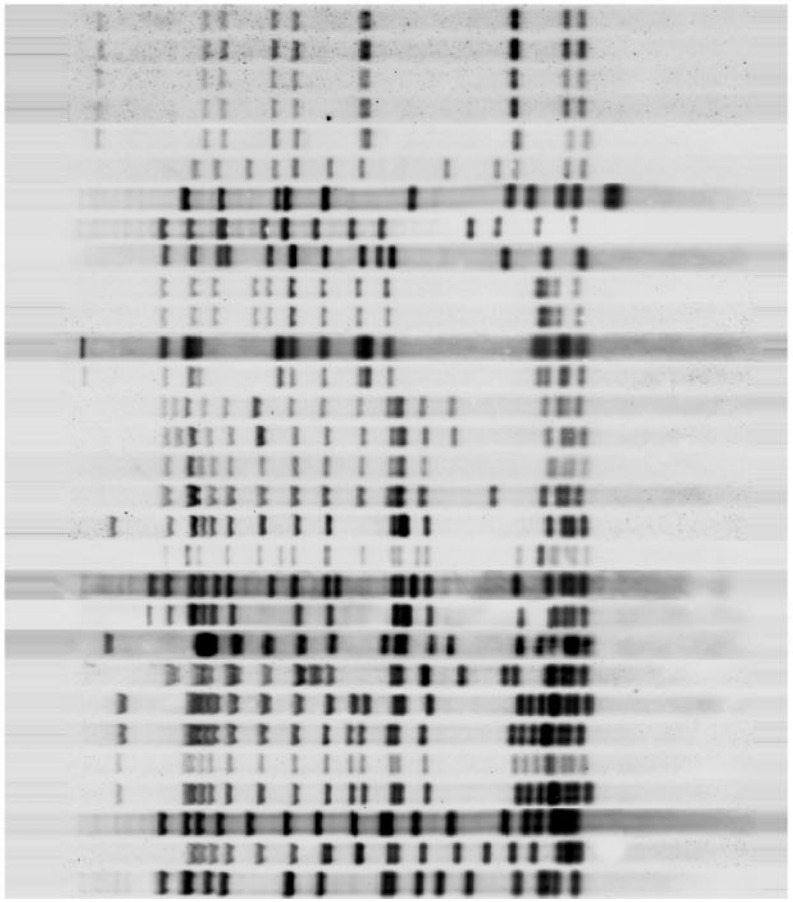
A total of 28 of the isolates assigned to the Beijing genotype showed the typical spoligotype pattern characterized by the deletion of spacers 1 to 34 in the direct repeat locus [octal number 000000000003771, share type (ST) 1]; the remaining 2 isolates showed deletions of spacers 1 to 36 and spacer 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 IS*6110*-RFLP analysis, the 30 Beijing isolates yielded a total of 20 distinct IS*6110* patters; 15 isolates (50 %) occurred in 5 distinct clusters with identical IS*6110* 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





Isolate code	*mutT4* codon 48	*mutT2* codon 58	Drug resistance
1131	wt	wt	0
1139	wt	wt	0
1149	wt	wt	0
1169	wt	wt	0
1142	wt	wt	0
1321	wt	wt	3
838	wt	wt	0
952	GGG	wt	1
763	GGG	wt	0
1172	GGG	wt	0
1191	GGG	wt	0
669	wt	wt	0
1120	wt	wt	0
1033	GGG	CGA	1
1284	GGG	CGA	1
1042	GGG	CGA	1
1184	GGG	CGA	0
1006	GGG	CGA	1
1146	GGG	CGA	0
884	GGG	CGA	0
946	GGG	CGA	0
974	GGG	CGA	0
1255	GGG	CGA	0
1156	GGG	CGA	0
1250	GGG	CGA	0
1269	GGG	CGA	0
1254	GGG	CGA	0
836	GGG	CGA	0
1041	GGG	CGA	0
804	GGG	wt	0

Fig. 1. IS*6110* fingerprints, *mutT4* and *mutT2* mutations, and drug resistance of 30 clinical isolates of *M. tuberculosis* of Beijing genotype. IS*6110*-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

Drug*	Genotype			
	Beijing		Non-Beijing	
	Total tested	No. (%) of drug resistant isolates†	Total tested	No. (%) of drug resistant isolates†
INH	30	6 (20·0)	473	44 (9·3)
RIF	30	0 (0)	473	12 (2·5)
ETH	30	1 (3·3)	464	11 (2·4)
PZA	30	1 (3·3)	445	19 (4·3)
Multiple drugs	–	1 (3·3)	–	11 (2·3)

*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.

ACKNOWLEDGEMENTS

This work was financially supported by MIUR (PRIN-2004) and, partly, by the Italian Istituto Superiore di Sanità (National Research Programme on AIDS-2003, ISS grant no. 50F.18).

REFERENCES

Anh, D. D., Borgdorff, M. W., Van, L. N., Lan, N. T., van Gorkom, T., Kremer, K. & van Soolingen, D. (2000). *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. *Emerg Infect Dis* **6**, 302–305.

Bifani, P. J., Mathema, B., Kurepina, N. E. & Kreiswirth, B. N. (2002). Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends Microbiol* **10**, 45–52.

Brudey, K., Driscoll, J. R., Rigouts, L. & 63 other authors (2006). *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol* **6**, (in press).

Filliol, I., Driscoll, J. R., van Soolingen, D. & 44 other authors (2003). Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. *J Clin Microbiol* **41**, 1963–1970.

Glynn, J. R., Whiteley, J., Bifani, P. J., Kremer, K. & van Soolingen, D. (2002). Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. *Emerg Infect Dis* **8**, 843–849.

Horst, J. P., Wu, T. H. & Marinus, M. G. (1999). *Escherichia coli* mutator genes. *Trends Microbiol* **7**, 29–36.

Kamerbeek, J., Schouls, L., Kolk, A. & 8 other authors (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* **35**, 907–914.

Lari, N., Rindi, L., Bonanni, D., Tortoli, E. & Garzelli, C. (2004). Beijing/W *Mycobacterium tuberculosis* in Italy. *Emerg Infect Dis* **10**, 958–959.

Lari, N., Rindi, L., Sola, C., Bonanni, D., Rastogi, N., Tortoli, E. & Garzelli, C. (2005). Genetic diversity, determined on the basis of *katG*463 and *gyrA*95 polymorphisms, spoligotyping, and IS*6110* typing, of *Mycobacterium tuberculosis* complex isolates from Italy. *J Clin Microbiol* **43**, 1617–1624.

Mizrahi, V. & Andersen, S. J. (1998). DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? *Mol Microbiol* **29**, 1331–1339.

Mokrousov, I., Otten, T., Vyazovaya, A. & 7 other authors (2003). PCR-based methodology for detecting multidrug-resistant strains of *Mycobacterium tuberculosis* Beijing family circulating in Russia. *Eur J Clin Microbiol Infect Dis* **22**, 342–348.

Rad, M. E., Bifani, P., Martin, C. & 8 other authors (2003). Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerg Infect Dis* **9**, 838–845.

Ramaswamy, S. & Musser, J. M. (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* **79**, 3–29.

Rindi, L., Lari, N., Bonanni, D. & Garzelli, C. (2004). Detection of *Mycobacterium tuberculosis* principal genetic groups by a duplex real-time PCR targeting the *katG* and *gyrA* genes. *J Microbiol Methods* **59**, 283–287.

Sreevatsan, S., Pan, X., Stockbauer, K., Connell, N., Kreiswirth, B., Whittam, T. & Musser, J. (1997). Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci U S A* **94**, 9869–9874.

van Embden, J. D. A., Cave, M. D., Crowford, J. T. & 8 other authors (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* **31**, 406–409.