Molecular Characterization of Drug-Resistant Beijing Family Isolates of *Mycobacterium Tuberculosis* from Tianjin, China[1]

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Objective Tuberculosis remains a severe public health issue, and the Beijing family of *mycobacterium tuberculosis (M. tuberculosis)* is widespread in East Asia, especially in some areas in China, like Beijing and Tianjin. This study aimed at determining the mutation patterns of drug-resistant Beijing strains of *M. tuberculosis* isolated from Tianjin, China. **Methods** A total of 822 *M. tuberculosis* isolates were screened for drug resistance by an absolute concentration method and the genotype was identified by PCR. 169 drug-resistant isolates of the Beijing family were analyzed for the potential mutations in the *rpoB*, *katG*, *inhA* promoter region and in *rpsL*, *rrs* and *embB* genes, which are associated with resistance to rifampin (RFP), isoniazid (INH), streptomycin (SM) and ethambutol (EMB) respectively by PCR and DNA sequencing. **Results** Fifty-eight out of 63 RFP-resistant isolates were found to carry the mutations within the 81-bp RFP resistance determining region (RRDR) of the *rpoB* gene and the most frequent mutations occurred at codon 531 (44.4%), 526 (28.6%), and 516 (7.9%) respectively. 16 mutation patterns affecting 12 different codons around the RRDR of *rpoB* were found. Of 116 INH-resistant isolates, 56 (48.3%) had the mutation of *katG* 315 (AGC→ACC) (Ser→Thr), 3 (2.6%) carried S315N (AGC→AAC) and 27 (16.0%) had the mutation of *inhA*-15A→T. 84 out of 122 SM-resistant isolates (68.9%) displayed mutations at the codons 43 or 88 with AAG→AGG (Lys→Arg) of the *rpsL* gene and 22 (18.0%) with the mutations at positions 513A→C, 516C→T or 905 A→G in the *rrs* gene. Of 34 EMB-resistant isolates, 6 had mutation with M306V (ATG→GTG), 3 with M306I (ATG→ATT), 1 with M306I (ATG→ATA), 1 with D328Y (GAT→TAT), 1 with V348L (GTC→CTC), and 1 with G406S (GGC→AGC) in the *embB* gene. **Conclusion** These novel findings extended our understanding of resistance-related mutations in the Beijing strains of *M. tuberculosis* and may provide a scientific basis for development of new strategies for diagnosis and control of tuberculosis in China and other countries where Beijing strains are prevalent.

Key words: *Mycobacterium tuberculosis*; Mutation; Drug-resistance; Beijing family

INTRODUCTION

Tuberculosis (TB) is one of the most important infectious diseases in the world. The emergence and spread of drug-resistant strains of *M. tuberculosis*, especially multidrug-resistant (MDR) strains, defined as simultaneous resistant to at least rifampin (RFP) and isoniazid (INH), are serious threats to the control of tuberculosis and human health. An estimated 0.5 million cases of MDR-TB were found in 2007 and distributed in many countries. There were 112 000 new MDR-TB cases reported in China, which is secondary to India (131 000) in the world (www.who.int/tb/publications/global_report/2009/key_points/). The Beijing family of *M. tuberculosis* is globally widespread. It is particularly prevalent in East Asia and trends to be MDR[1-6]. Indeed, over 90% of *M. tuberculosis* strains from Beijing, China, are of this genotype[5].

Tianjin is a metropolitan of China with coverage of 11 919 square kilometres and a population of 12 million. There were more than 3 500 people diagnosed with TB during the past couple of years and many of them suffered from drug-resistant *M. tuberculosis* infection. However, little is known about what kind of drug-resistant *M. tuberculosis* spread in this area and what the molecular nature of these drug-resistant *M. tuberculosis* is. To address these issues, a total of 822 isolates collected from individual patients at the Tuberculosis Control Centers, Tianjin, China, from 2007 to 2008, were evaluated for the genotype and susceptivity of RFP, INH, streptomycin (SM) and ethambutol (EMB), which are commonly prescribed for the treatment of tuberculosis in China. And 169 drug-resistant *M.tuberculosis* Beijing strains were chosen to

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characterize the specific regions of the drug-resistant genes by PCR and sequencing. The loci studied were *rpoB* (RFP), *katG* (INH), *inhA* promoter region (INH), *rpsL* (SM), *rrs* (SM), and *embB* (EMB). This study aimed at characterizing the genes of drug-resistant Beijing family of *M. tuberculosis* in Tianjin, China, so as to provide a scientific basis for the diagnosis and control of tuberculosis in China and other countries where Beijing strains are prevalent.

MATERIALS AND METHODS

Patients and Bacterial Isolates: A total of 822 *M. tuberculosis* were isolated from individual patients (562 male and 260 female aged from 13 to 87 with a mean of 42±19) and obtained from the Tuberculosis Control Centers, Tianjin, China, from 2007 to 2008. A total of 703 isolates were collected from new diagnostic cases and 119 from previously treated patients who had being treated with the first-line anti-TB drugs for 4 to 23 months before their sputa were obtained or once infected with TB 3 to 48 years ago. Cultured on Lowenstein-Jensen plates, individual isolates were characterized with 2-thiophene carboxylic acid (TCH) and paranitrobenzoic acid (PNB) selective media. Their profiles of drug susceptibility were evaluated by using the absolute concentration method with Lowenstein-Jensen plates of INH of 1 mg/L and 10 mg/L, SM of 10 mg/L and 100 mg/L, RFP of 50 mg/L and 250 mg/L, and EMB of 5 mg/L l and 50 mg/L. Drug resistance was defined as the isolate grew more than 1+ in drug-contained plates when grew well in drug-free plates.

DNA Isolation and PCR: The drug-sensitive and resistant isolates and control strain of *Mycobacterium* H37Rv were harvested and killed by heating at 80 ℃ for 90 min. The genomic DNA was extracted from individual isolates with the cetyltrimethylammonium bromide (CTAB) method. After qualification and quantification, the genomic DNA was used as the template and the contained *rpoB* (RFP), *katG* and *inhA* regulator sequence (INH), *rpsL* and *rrs* (SM), and *embB* (EMB) genes were amplified by PCR with a 50 μL mixture of 1 μL chromosomal DNA, 0.2 mmol/L dNTP, 400 nmol/L each primer, and 1.25 U of Pyrobest DNA polymerase (TaKaRa Biotechnology, Dalian, China). The PCR reactions were denatured at 94 ℃ for 5 min and subjected to 35 cycles of 94 ℃ for 1 min, 54-62 ℃ for 1 min, and 72 ℃ for 1 min, followed by a final extension at 72 ℃ for 10 min. The sequences of specific primers, the sizes of amplicons and the annealing temperatures were presented in Table 1.

DNA sequencing Analysis: Partial PCR products were characterized by DNA sequencing with the specific primers on an ABI Prism 3730 automated DNA sequencer (ABI Prism) by the Shanghai Sunsoon Bio-Technology Company, Shanghai, China. The resulting DNA sequences were analyzed with Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nih.gov/BLAST). The specific mutations in protein sequences of individual isolates were identified.

Genotyping: To identify a strain belonging to the Beijing evolutionary lineage, PCR was preformed with 4 pairs of primers reported by Warren *et al.*[7], and the PCR products were visualized by agarose gel

TABLE 1

Primers Used in the Study to Amplify and Sequence the Different Loci, Annealing Temperatures and Amplicon Sizes

Gene (Accession No.)	Primer	Sequence	Annealing Temp (℃)	Amplicon size (bp)
*rpoB**(BX842574)	Forward	CAG ACG TTG ATC AAC ATC CG	54	305
	Reverse	TAC GGC GTT TCG ATG AAC		
*katG***(X68081)	Forward	GCG GCG GTC GAC ATT	62	273
	Reverse	CTC GAG GAA ACT GTT GTC CC		
*inhA****(BX842576)	Forward	GCA GCC ACG TTA CGC TCG TGG	60	149
	Reverse	CGA TCC CCC GGT TTC CTC CGG		
rpsL#(BX842574)	Forward	GGC CGA CAA ACA GAA CGT	58	622
	Reverse	GTT CAC CAA CTG GGT GAC		
rrs(BX842576)#*	Forward	GAT GAC GGC CTT CGG GTT GT	60	504
	Reverse	AGG CCA CAA GGG AAC GCC TA		
embB(BX842584)#**	Forward	TGA TAT TCG GCT TCC TGC TC	60	417
	Reverse	ACC GCT CGA TCA GCA CAT AG		

Note. *-[42]. **-[43]. ***-[21]. #-[44]. #*-[34]. #**-[45].

electrophoresis.

Statistical Analysis： The difference between groups was analyzed through the chi-square or Fisher's exact test by using SPSS for windows 16.0. A value of $P<0.05$ was considered statistically significant.

RESULTS

Among the 822 isolates, 753 belonged to the Beijing family. Furthermore, 169 out of 753 Beijing isolates were resistant to at least one drug tested while 12 out of 69 non-Beijing isolates were resistant to at least one drug. There was no statistical significance in terms of drug-resistance rates between Beijing and non-Beijing family strains. Analysis of 169 drug-resistant Beijing strains from individual patients (with 119 new diagnostic cases and 50 previously treated cases and 117 male and 52 female aged from 15 to 87 with a mean of 42±19) revealed that there were 63 RFP-resistant, 116 INH-resistant, 122 SM-resistant, 34 EMB-resistant and 53 MDR-TB isolates. Of the 169 drug resistant isolates, 69, 48, 38, and 14 were resistant to one, two, three, and four drugs respectively.

In order to understand the molecular mechanism underlying the drug resistance of these Beijing family isolates, the *katG*, *inhA promoter*, *rpoB*, *rpsL*, *rrs* and *embB* genes of 169 drug-resistant isolates together with 16, 12, 7, and 9 isolates that were sensitive to INH, RFP, SM, and EMB respectively were amplified by PCR and analyzed by sequencing. The results were shown in Table 2. Most drug sensitive isolates displayed the same sequences as those in control except that 7 SM-sensitive isolates carried a silent mutation of K121K (AAA→AAG) in the *rpsL* gene.

TABLE 2

Genotypic Characteristics of Drug-resistant Isolates

Drug*-gene (NO.)	Polymorphism	NO.	Percent
RFP-*rpoB*(63)	531TCG-TTG(Ser-Leu)	28	44.44
	526CAC-GAC(His-Asp)	9	14.29
	526CAC-TAC(His-Tyr)	5	7.94
	526CAC-CGC(His-Arg)	2	3.17
	526CAC-CTC(His-Arg)	1	1.59
	526CAC-AAC(His-Asn)	1	1.59
	516GAC-GTC(Asp-Val)	2	3.17
	516GAC-TAC(Asp-Tyr)	1	1.59
	511CTG-CCG(Leu-Pro)，516GAC-GGC(Asp-Gly）	1	1.59
	512AGC-GGG(Ser-Gly)，516GAC-GGC(Asp-Gly)	1	1.59
	533CTG-CCG(Leu-Pro)	1	1.59
	533CTG-CCG(Leu-Pro), 503AAG-ACG(Lys-Thr)	1	1.59
	513CAA-AAA(Gln-Lys)	2	3.17
	522TCG-TTG(Ser-Leu)	2	3.17
	BX842574.1, AATT deletions in positions 76828-76831, T deletion in position 76834****	1	1.59
	WT**	5	7.94
INH-*katG*(116)	315AGC-ACC（Ser-Thr）	56	48.28
	315AGC-AAC（Ser-Asn）	3	2.59
	295CAG-CCG（Gln-Pro）	1	0.86
	WT**	56	48.28
INH-*inhA* promoter(116)	-15C-T	27	23.28
	WT**	89	76.72
SM-*rpsL*(122)	43AAG-AGG(Lys-Arg),121AAA-AAG(Lys-Lys)	66	54.10
	88AAG-AGG(Lys-Arg),121AAA-AAG(Lys-Lys)	18	14.75
	121AAA-AAG(Lys-Lys)	38	31.15
SM-*rrs*(122)	513A-C	17	13.93
	516C-T	4	3.28
	905A-G	1	0.82
	WT**	100	81.97
EMB-*embB*(34)	306ATG-GTG(Met-Val)	6	17.65
	306ATG-ATT(Met-Ile)	3	8.82
	306ATG-ATA(Met-Ile)	1	2.94
	328GAT-TAT(Asp-Tyr)	1	2.94
	348GTC-CTC(Val-Leu)	1	2.94
	406GGC-AGC(Gly-Ser)	1	2.94
	WT**	21	58.82

Note. *RFP，rifampin; INH, isoniazid; SM, streptomicin; EMB, ethambutol; ** WT, wild type; ***Affeced the codons 513 514, and 515 of *rpoB*.

58 out of 63 RFP-resistant isolates displayed various mutations in the *rpoB* gene including 81-bp RRDR (codons from 507 to 533). One of them combined with the mutation outside of the RRDR (Shown in Table 2). The prevalent point mutations were at codon 531 (28 out of 63, 44.4%) for S531L, 526 (18 out of 63, 28.6%) for H526D, H526Y, H526N, or H526L, 516 (5 out of 63, 7.9%) for D516V, D516G, or D516Y. In total, 16 mutation patterns were found in 12 different codons in the RRDR and out of the RRDR of the *rpoB*.

Of 116 INH-resistant isolates, 60 isolates (51.7%) carried mutations in the *katG* gene while

other 27 isolates (23.3%) carried mutation at position -15 C→T in the *inhA* promoter region.

Analysis of 122 SM-resistant isolates revealed that all of the isolates carried a silent mutation at codon 121 (AAA→AAG, K→K) in the *rpsL* locus. Furthermore, 84 out of 122 isolates had additional mutations at codon 43 or 88 (AAG→AGG, K→R) in the *rpsL* gene while 22 of the 122 isolates had additional mutations in the *rrs* gene.

Characterization of 34 EMB-resistant isolates indicated that 13 isolates (38.2%) carried mutations while 10 isolates (29.4%) happened at codon 306 in the *embB* gene.

Of the 53 MDR-isolates, 44 carried mutations in the *rpoB* and *katG* genes or the *inhA* promoter region, 8 isolates carried mutation in *rpoB* but not in *katG* or *inhA* promoter region, only 1 harbored no mutations in any of the above genes.

Thirty (17.8%) of the 169 analyzed *M. tuberculosis* isolates did not exhibit any mutations in the core regions of *rpoB*, *inhA* promoter region, *katG*, *rpsL*, *rrs*, or *embB.*

DISCUSSION

We studied 822 isolates from individual patients with TB and it was found that 91.6% of the isolates (753) belonged to the Beijing family. which was similar to the result in a previous report from Beijing, China[5]. Such rate was higher than that in other near regions[4, 8-10]. These novel data demonstrated that Beijing family of *M. tuberculosis was* highly prevalent in Tianjin, China.

Analysis of the RFP-resistant isolates indicated that 92.1% of the RFP-resistant isolates had mutations in the 81-bp RRDR of the *rpoB* gene which encodes for the β-subunit of the RNA polymerase[11-13]; 78.3% (36/46) of RFP-resistant *M. tuberculosis* isolates with mutations of *rpoB* 531 and 526, which are responsible for high level of RFP-resistance, displayed resistant to RFP at 250 mg/L. The mutation 531TTG in *rpoB* is the most common one as also observed in other areas[11, 13-14]. Furthermore, 7.9% of the RFP-resistant isolates carried the mutation at position 516 which was higher than the 4% in other areas of China[11-12], but was lower than the rates in other countries[9, 14-15]. Two isolates, which were MDR and resistant to RFP at 250 mg/L, had the mutation at position 522 that was seldom detected in other regions in Asia[16-18] and it was suggested that the mutation in this codon was associated with the high level of RFP resistance. Mutations in the codons 511 and 512 were also accompanied by 516[16-18]. One MDR isolate from a previously treated case was resistant to high levels of INH and RFP and displayed a novel mutation pattern with deletion in the *rpoB* gene, mutations at the codons 513, 514, and 515, but with wild type of the *katG* and *inhA* promoter. Furthermore, one new mutation K503T was found outside of the RRDR together with codon 533 and this isolate was also from a previously treated patient showing high level of INH and RFP resistance. These diversities of mutations in the *rpoB* gene suggested that RFP resistance in the Beijing Family strains of *M. tuberculosis* from Tianjin may be more complicated than that from other areas, calling for more attention when diagnosis of drug-resistant *M. tuberculosis* infection is made.

The percentages of *katG* 315 and *inhA* promoter mutations in INH-resistant isolates ranged from 46% to 100%[5, 9, 19-20] and from 2.0% to 35%[5, 20-24], respectively. Beijing family isolates from other countries usually carry a higher percentage of the *katG* S315T shift[4, 9, 25]and lower frequency of mutations in the *inhA* promoter region compared to non-Beijing family isolates[4]. This study showed that only 50.8% of the INH-resistant Beijing family isolates carried mutations at codon 315 of the *katG* gene, lower than that reported[4, 9, 25], but the mutation rate (23.3%) in the *inhA* promoter was higher than that in Korea[4]. A previous study has found that the mutation of S315T in the *katG* gene was associated with high resistance to INH[26] while the mutations in the *inhA* promoter are generally related to intermediate-level (MIC from 0.1 to 0.4 mg/L) resistance[27]. However, findings from our study showed that only 15 out of 56 INH-resistant isolates that carried the mutation of *katG* S315T were highly resistant to INH, while 14 out of 27 isolates that carried the wild type of the *katG* gene and with the mutation at position -15 C→T in the *inhA* promoter showed high resistance to INH at 10 mg/L. These unique characteristics of the isolates from Tianjin were different from that reported[26-27]. Notably, 50 out of 56 INH-resistant isolates with the mutation of S315T in the *katG* gene were also resistant to other drugs, which was similar to the findinig in a previous report[26]. Our study also showed that a novel mutation of *katG* D295K (CAG-CCG) in a high level INH-resistant isolate from a previously treated case was different from the rare mutation (CAG-AAG)[28]. However, no deletion of the *katG* in our isolates was detected. Interestingly, 29 out of 116 INH-resistant isolates, including 11 highly resistant to INH, did not display any mutation either in the *katG* gene or in the *inhA* promoter. Their INH resistance may be mediated by the changes in other chromosome loci (*oxyR-ahpC*, *kasA*, *ndh*), and the efflux pumps and/or other mechanisms remained to be investigated[19, 29-30].

The *rpsL* and *rrs* genes encode the ribosomal protein S12 and the 16S rRNA, respectively and their changes are responsible for the SM resistance. Our findings showed that 68.9% SM-resistant isolates carried mutations in the *rpsL* gene，a rate higher than that in previous reports[6, 31-33], while 18.0% of the SM-resistant isolates carried mutations in *rrs*, which is in accordance with previous studies[6, 31-33]. However, only 54.1% of the SM-resistant Beijing family strains of *M. tuberculosis* isolates carried the mutation of K43R in *rpsL* which was lower than that in other regions[6, 9]. We surprisingly found in our study a high rate of the *rpsL* K88R (14.8%) mutation, which has not been reported so far in other areas[6, 9, 33] except in Japan[32]. Notably, the mutation 513 A→C was detected to be the most common but no 516 C→T in *rrs* of Beijing family isolates were found in Russia[9]. we observed the same result that 68 out of 84 isolates carried the mutations (K43R and K88R) in the *rpsL* gene and showed a high level of resistance to SM (100 mg/L), supporting the notion that the mutations of K43R and K88R in *rpsL* may be responsible for the high level of the SM resistance[31-35]. Interestingly, our isolates, regardless of the SM resistance status, had a silent mutation (K121K) in *rpsL*, demonstrating that the K121K mutation is not associated with the SM resistance[20]. Finally, 16 out of 122 SM-resistant isolates displayed no mutation at these regions tested and their SM-resistance may be mediated by the functional changes in efflux pumps, such as Tap[36], and DrrABC[37] or by other mechanisms.

Few data is available on the mutation rates of Beijing family of EMB-resistant isolates. We found that 29.4% of the isolates carried the mutations in the *embB* gene, which was lower than the rate of the overall EMB-resistant isolates from other areas[1, 6, 16, 38-39]. Furthermore, 28 out of the 34 EMB-resistant isolates were of multiple-drug resistance (MPR) and 13 of them carried mutations in *embB*, while none of mono-EMB resistant isolates were observed. This finding suggested that mutations in the *embB* gene may be more easily induced in MPR isolates[40-41]. We also found a new mutation of *embB* V348L (GTC→CTC) carried by a patient previously treated. Notably, 70.6% of EMB-resistant isolates did not carry any mutation in the amplified fragment of the *embB* gene, indicating that other mechanisms also contributed to the development of EMB-resistance in these isolates from Tianjin. We are interested in further examining whether genetic changes in the *embC* gene[39] or the functional changes in efflux pumps such as DrrABC[37], are responsible for the development of EMB resistance in *M. tuberculosis* isolates.

Interestingly, 30 out of 169 drug-resistant *M. tuberculosis* isolates displayed no single mutations in our study and 10 of them were resistant to multiple drugs. Possibly, other genetic variants may be involved in the drug resistance or the mutations may be present outside of the sequences. Therefore, further studies to determine the molecular mechanisms underlying the drug resistance are warranted.

In summary, 169 drug-resistant Beijing family isolates of *M. tuberculosis* were identified in our study that were resistant at least to one first-line anti-TB drugs in Tianjin, China. Most drug-resistant *M. tuberculosis* isolates carried the common genetic mutations, but some of them displayed unique mutations, which may provide a scientific basis for design of new diagnostic tools of drug-resistant *M. tuberculosis* in Tianjin, China. Potentially, findings from our study may contribute to better understanding of the spread of Beijing family of *M. tuberculosis* in population.

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