Co-occurrence of amikacin-resistant and -susceptible *Mycobacterium tuberculosis* isolates in clinical samples from Beijing, China

Xiaobing Zhang1†, Bing Zhao2†, Hairong Huang3†, Yafang Zhu1, Junping Peng1, Guangming Dai3, Guanglu Jiang3, Liguo Liu1, Yanlin Zhao2 and Qi Jin1*

1MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100176, China; 2Chinese Center for Disease Control and Prevention, 155# Changbai Road, Changping District, Beijing 102206, China; 3Beijing Tuberculosis and Thoracic Tumor Research Institute, 97 Beimachang, Tongzhou Qu, Beijing 101149, China

*Corresponding author. Tel: +86-10-67877732; Fax: +86-10-67877736; E-mail: zdsys@vip.sina.com
†These three authors contributed equally to this work.

Received 10 August 2012; returned 4 October 2012; revised 6 February 2013; accepted 11 February 2013

**Objectives:** This study examined the phenomenon of heteroresistance in *Mycobacterium tuberculosis* clinical isolates obtained from retreated patients in Beijing, China between 2006 and 2011.

**Methods:** The iPLEX Gold assay platform was used to determine the prevalence of heteroresistance to injectable second-line drugs (amikacin, kanamycin and capreomycin) in resistant isolates.

**Results:** Heteroresistance was identified in 10.9% of 220 phenotypic amikacin-resistant isolates.

**Conclusions:** Heteroresistance was related mainly to the short duration and repeated use of amikacin and capreomycin during retreatment. These findings further our understanding of the evolution of resistance to injectable drugs used for tuberculosis treatment and help guide the rational use of injectable drugs during therapy.

**Keywords:** heteroresistance, MDR-TB, aminoglycoside antibiotics, molecular typing

**Introduction**

Despite recent efforts towards treatment and prevention, tuberculosis (TB) remains a major global health problem. An estimated 8.8 million incident cases and nearly 1.4 million deaths occurred from TB in 2010 and multidrug-resistant (MDR) TB accounted for 650,000 out of 12 million prevalent cases of TB worldwide. In China, MDR-TB strains were isolated from 5.7% of new cases and 26% of previously treated TB patients. The most effective first-line anti-TB drugs, isoniazid and rifampicin, could not be used to treat these patients. The injectable polypeptide agents (capreomycin) and aminoglycoside antibiotics (amikacin/kanamycin) represent one of four key drug classes used to treat MDR-TB infections. In China, patients with MDR-TB are treated with regimens containing various combinations of these anti-TB drugs, such as pyrazinamide, ethambutol, fluoroquinolones, aminoglycosides or capreomycin, thiacetazone and cycloserine or p-aminosalicylic acid. However, inadequate empirical treatment regimens have led to acquired resistance to injectable second-line drugs (and fluoroquinolones) and the emergence of extensively drug-resistant (XDR) TB. The prompt and precise determination of drug susceptibility profiles by drug susceptibility testing (DST) is important for avoiding the improper usage of anti-TB drugs and the development of drug resistance.

Heteroresistance, the simultaneous presence of drug-resistant and -susceptible populations in a sample, can lead to inconsistent DST results from clinical isolates. This situation is caused by either superinfection (multiple infecting strains) or mutation and the separation of a single strain into susceptible and resistant populations. The latter situation reflects an early stage in the adaptive evolution of drug resistance and is used as an indicator of the effectiveness of treatment.

Heteroresistance in clinical isolates is not directly detected by DST, although heteroresistance can be detected by molecular screening for known resistance mutations in *Mycobacterium tuberculosis* genes, such as mutations conferring resistance to rifampicin, isoniazid, ethambutol and fluoroquinolones. We used the iPLEX assay, a novel platform with high sensitivity for molecular genotyping, to determine the prevalence of
heteroresistance in amikacin-resistant isolates collected from 2006 to 2011 in Beijing, China. This report is the first systematic analysis of the occurrence of heteroresistance to injectable second-line drugs in clinical isolates collected in China and will help us understand the molecular basis of the progression of MDR- and XDR-TB in relation with aminoglycoside drugs.

Resistance to amikacin, kanamycin and capreomycin is associated with three mutations frequently detected in the 1400 region of the 16S rRNA gene (rrs), which is also referred to as the kanamycin resistance-determining region (KRDR), in *M. tuberculosis*.10,11 The presence of the A1401G substitution appears to be specific for the detection of amikacin and kanamycin resistance and is related to cross-resistance to amikacin and kanamycin (MICs ≥120 mg/mL). C1402T has also been observed in capreomycin- and amikacin-resistant isolates. In addition, the G1484T substitution has been found in capreomycin-resistant strains and isolates with cross-resistance to capreomycin, amikacin and kanamycin (MIC ≥160 mg/mL, amikacin MIC >64 mg/mL and kanamycin MIC >80 mg/mL).12,13 Thus, these three mutations indicate genotypic resistance to the injectable second-line drugs (amikacin, kanamycin and capreomycin).

**Materials and methods**

**Clinical isolates**

Two-hundred-and-twenty clinical isolates displaying phenotypic amikacin resistance were randomly selected from 6500 retreatment patients with pulmonary TB in Beijing Chest Hospital, China between 2006 and 2011. Beijing Chest Hospital is the largest specialized hospital for TB treatment in Beijing. Patients were from local and neighbouring cities in the north and central west region of China. More than 90% of these patients had finished directly observed therapy and had become relapse or failure cases. The selection of these isolates provided an opportunity to understand the relationship between injectable drug use and the evolution of drug resistance among retreated cases. This study was approved by the ethics review committee of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College.

**Antimicrobial susceptibility testing**

DST was carried out using the absolute concentration method on Löwenstein–Jensen slants. The extraction of genomic DNA was performed via disruption with beads and phenol–chloroform extraction.16 The genomic DNA of each isolate was used as a template and an ~350 bp segment (KRDR) of *rrs* was amplified using the primers *rrs.PCR.F123* and *rrs.PCR.R535*, as described previously.17 PCR mixtures (5 μL) contained 2 μL of the DNA template (10 ng/μL), primer mixture at a final concentration of 0.1 μM, 500 μM of each deoxynucleoside triphosphate, 4 mM MgCl₂, 0.2 U of DNA polymerase (Roche Molecular Systems Inc., Pleasanton, CA, USA) and 0.2 U of uracil-DNA glycosylase (ShineGene Molecular Biotechnology, Shanghai, China). Prior to PCR, an additional step at 45°C for 2 min was added to eliminate the carry-over contamination from previous PCRs.18 Primary PCR was performed as follows: denaturation at 95°C for 4 min; 45 cycles at 95°C for 30 s, 56.5°C for 30 s and 72°C for 1 min; and a final step at 72°C for 5 min. The PCR products were dephosphorylated with shrimp alkaline phosphatase according to the manufacturer’s protocol (Sequenom, Inc., San Diego, CA, USA). Sterile water was used as a negative control.

The iPLEX reaction mixture was added to the dephosphorylated primary PCR mixture and included 1 μL of each extend primer, 0.2 μL of terminator mixture, 0.2 μL of iPLEX Gold buffer and 0.041 μL of iPLEX enzyme (Sequenom, Inc.) in a final volume of 9 μL. PCR was performed according to standard procedures. After desalting by the addition of 6 mg of Clean Resin (Sequenom, Inc.) to each 384-well plate, we applied 10 μL of each iPLEX product to a 384-spot SpectroChip II (Sequenom, Inc.). Mass spectrometric analysis was performed and interpreted using MassARRAY Typer software version 4.0 (Sequenom, Inc.).

**Sensitivity of the iPLEX assay for detecting heteroresistance**

Genotypic analysis of the in vitro mixtures was performed to assess the sensitivity of this method for detecting heteroresistance. Two types of KRDR DNA fragments were used as components of the artificial heteroresistant samples, which were amplified from purified plasmids containing mutant and wild-type sequences (A1401G and WT1401, respectively). Three groups of samples with fixed total concentrations (1, 5 or 10 ng/μL) contained A1401G at seven ratios to the amount of WT1401: 1:10, 1:20, 1:100, 1:200, 1:1000, 1:2000 and 1:10000. These samples were subjected to the iPLEX assay.

**Mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing**

Molecular typing of the *M. tuberculosis* strains was performed by MIRU-VNTR genotyping analysis with 24 loci.19 The procedure relied on PCR amplification using primers for the flanking regions of the VNTRs and the determination of the amplicon sizes. The primers and PCR conditions were selected according to the data of Weniger et al.19 The size of the PCR products was analysed by electrophoresis using agarose gels.

**Verification of heteroresistant strains**

The KRDR region of the suspected heteroresistant isolates was amplified and cloned into the pGEM-T Easy system (Promega, Madison, WI, USA). Single clones for each isolate were cultured and purified plasmids were sequenced using T7 and SP6 commercial primers. KRDR genotypes from 12 clones of each isolate were analysed in parallel.

**Results and discussion**

Twenty-nine out of 220 amikacin-resistant isolates (13.2%) carried the wild-type *rrs* genotype. The other 191 isolates...
(86.8%) were genotypically resistant, exhibiting mutations in the 1400 region of rrs. The predominant mutation was the A1401G substitution. One isolate contained the G1484T substitution of rrs. No mutations were detected at position 1402. Twenty-four out of these 191 isolates (12.6%) were identified as heteroresistant and consisted of a mixture of wild-type and A1401G mutant strains.

MIRU-VNTR genotyping analysis with 24 loci revealed that these 24 heteroresistant isolates belonged to the Beijing family. There were 22 unique VNTR types carried by 22 isolates. The remaining two were identified as multiple strain infections, with two or three VNTR types in each isolate (Table 1). 24-loci VNTR genotyping had high discriminatory power for molecular genotyping of clinical isolates, even for the Beijing family strains prevalent in China. Consequently, the occurrence of amikacin heteroresistance may primarily be due to the segregation of a single strain under drug selection pressure.

According to the DST profile, our collection consisted of three groups of isolates. There were 45 non-MDR-TB isolates, 33 MDR-TB isolates and 142 XDR-TB isolates. Heteroresistance was detected in each of the three groups, but the percentage varied from 20% (9/45) among non-MDR-TB isolates to 15.2% (5/33) for MDR-TB isolates and 7.0% (10/142) for XDR-TB isolates (Table 2). Conversely, the 167 homoresistant isolates carrying only the A1401G or G1484T mutation included 31 non-MDR-TB isolates (18.6%), 21 MDR-TB isolates (12.5%) and 115 XDR-TB isolates (68.9%). Because the development of MDR-TB occurs via the accumulation of drug resistance mutations in these genes, the decreasing trend of heteroresistance as the overall degree of resistance increases (non-MDR > MDR > XDR) implies that this resistance is often likely emerging in the earlier stages of the development of XDR-TB drug resistance.

The A1401G mutation in rrs mediates amikacin and kanamycin resistance and this mutation also decreases the susceptibility of strains to capreomycin. Previous reports demonstrated that cross-resistant isolates that carried the A1401G mutation usually exhibited high-level resistance to amikacin (MIC > 64 mg/L) and kanamycin (MIC > 80 mg/L). In fact, cross-resistance to injectable drugs was observed among isolates with high-level resistance to these drugs. Among 220 isolates, 166 displayed cross-resistance to kanamycin and the other 54 were resistant to kanamycin and capreomycin simultaneously (Table 2). In total, 74.1% (163/220) and 81.5% (45/54) of the isolates with cross-resistance to kanamycin and kanamycin/capreomycin

Table 1. General description of heteroresistant isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Type of treatment</th>
<th>DST profile</th>
<th>MIRU-VNTR type</th>
<th>Duration of anti-TB treatment</th>
<th>Injectable drugs used</th>
<th>Ratio of wild-type to mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>retreated</td>
<td>22423362534442173353924</td>
<td>3 years</td>
<td>AMK</td>
<td>7:5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>retreated</td>
<td>22423352644442173353834</td>
<td>8 months</td>
<td>AMK</td>
<td>10:2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>retreated</td>
<td>25402335244442173153823</td>
<td>12 years</td>
<td>AMK</td>
<td>4:8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>retreated</td>
<td>25423352664442173153823</td>
<td>10 years</td>
<td>AMK/KAN/CAP</td>
<td>3:9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>2242332324321514356822</td>
<td>—</td>
<td>AMK/CAP</td>
<td>11:1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>retreated</td>
<td>24523332544442173353624</td>
<td>10 years</td>
<td>AMK</td>
<td>9:3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>retreated</td>
<td>244233323213421514356822</td>
<td>6 years</td>
<td>AMK</td>
<td>5:7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>retreated</td>
<td>24423352644442173353823</td>
<td>2 years</td>
<td>AMK</td>
<td>6:6</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>retreated</td>
<td>234233526444421513737393</td>
<td>6 months</td>
<td>none</td>
<td>5:7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>224233526644421733537372</td>
<td>—</td>
<td>—</td>
<td>3:9</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>NA</td>
<td>244233526444421513737372</td>
<td>—</td>
<td>—</td>
<td>4:8</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>retreated</td>
<td>24423352544442163353824</td>
<td>4 years</td>
<td>AMK/CAP</td>
<td>6:6</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>retreated</td>
<td>3442444452544442173353823</td>
<td>4 years</td>
<td>AMK/CAP</td>
<td>4:8</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>retreated</td>
<td>24423352644442173153624</td>
<td>4 years</td>
<td>AMK/CAP</td>
<td>7:5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>retreated</td>
<td>24403352544442173353824</td>
<td>13 years</td>
<td>AMK/CAP</td>
<td>5:7</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>retreated</td>
<td>24423362544442163153823</td>
<td>5 years</td>
<td>AMK/CAP</td>
<td>4:8</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>retreated</td>
<td>22423352664442173343822</td>
<td>17 years</td>
<td>AMK</td>
<td>9:3</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>NA</td>
<td>22423352664442173353823</td>
<td>—</td>
<td>—</td>
<td>3:9</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>retreated</td>
<td>23423336(5,3)2644425171353623</td>
<td>3 years</td>
<td>AMK</td>
<td>2:10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>retreated</td>
<td>24324342534442151353925</td>
<td>21 years</td>
<td>AMK/CAP</td>
<td>10:2</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>retreated</td>
<td>24403352544442173353823</td>
<td>13 years</td>
<td>AMK/CAP</td>
<td>8:4</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>retreated</td>
<td>2442233523444441513353824</td>
<td>5 years</td>
<td>AMK/CAP</td>
<td>9:3</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>retreated</td>
<td>233233342444427153353634</td>
<td>5 years</td>
<td>AMK/CAP</td>
<td>8:4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>retreated</td>
<td>234233342444425163354744</td>
<td>1 year</td>
<td>AMK</td>
<td>9:3</td>
<td></td>
</tr>
</tbody>
</table>

AMK, amikacin; CAP, capreomycin; KAN, kanamycin.

a’NA’ was used for outpatients for whom the isolates were not identified. Therefore, no description of the duration or treatment or usage of drugs is provided.

bThe treatment regimens included amikacin, kanamycin and/or capreomycin.

cThe ratio of the two rrs genotypes as detected by the pGEM-T Easy system.
were homoresistant isolates, respectively. Furthermore, 73.1% (122/167) and 6.6% (11/167) of these isolates displayed high-level resistance to amikacin/kanamycin (MIC ≥ 100 mg/L for both amikacin and kanamycin) and amikacin/kanamycin/capreomycin (MIC ≥ 100 mg/L for amikacin, kanamycin and capreomycin), respectively. In contrast, heteroresistant isolates were present at a lower frequency among cross-resistant isolates. The corresponding frequencies were 11.4% (19/166) and 9.3% (5/54) for isolates with cross-resistance to kanamycin and kanamycin/capreomycin, respectively. High-level cross-resistance to amikacin and kanamycin was observed in 11/24 (45.8%) heteroresistant isolates. No heteroresistant isolates exhibited high-level resistance to amikacin, kanamycin and capreomycin.

In addition, the phenotypic determination of heteroresistance by the proportion method clarified the different levels of drug resistance between heteroresistant and homoresistant isolates. The level of drug resistance was determined by the proportion of resistant bacteria to the total number of bacteria in an isolate. Here, the average proportions of resistant bacteria for strains were calculated among three groups of isolates (wild-type, heteroresistant and homoresistant), which were identified by iPLEX analysis. For amikacin drug susceptibility testing, these average proportions were 0.10%, 45.35% and 72.65%, respectively, with an obvious difference observed between the second and third groups (t-test, P<0.001), as shown in Figure 1. A similar trend was displayed for detecting resistance to kanamycin among these isolates (the corresponding percentages were 0.20%, 54.0% and 74.33%, respectively). Nearly half of the heteroresistant isolates had resistant bacteria proportions of <40%, whereas all of the homoresistant isolates had proportions >40%. These data revealed the lower level of resistance in heteroresistant isolates, which was mainly due to the presence of susceptible bacteria. Thus, inconsistent DST results may be related to changes in the composition of susceptible and resistant bacteria in clinical isolates with low-level resistance to certain drugs. This result also indicated that heteroresistance to aminoglycoside antibiotics in clinical isolates occurred in the intermediate stage of drug resistance evolution.

In our collection, heteroresistance was detected in 10.9% (24/220) of phenotypic amikacin-resistant isolates and 12.6% (24/191) of genotypic amikacin-resistant isolates. This percentage of heteroresistance is slightly higher than the 8.6% of genotypic amikacin-resistant isolates reported in South Africa. Previous studies have demonstrated that heteroresistance is more likely to appear in geographic areas with high TB incidence and in chronic patients due to multiple infections. Here, 83.3% (20/24) of the heteroresistant isolates identified in this study were obtained from relapse or failure cases and most of them had received anti-TB treatment for >3 years (Table 1). These patients had experienced multiple treatments with amikacin or capreomycin for short periods of 1–3 months. Treatment non-compliance or serious side effects represented the two major causes of short-duration treatment. In contrast, this history of drug usage occurred only in ~20% of isolates with single sub-populations of mutant genotype M. tuberculosis strains. More than 70% of A1401G homoresistant isolates were obtained from patients who received treatment with regimens containing amikacin or capreomycin for a duration of 6 months.

The prevalence of heteroresistance has been underestimated by the methods used in previous studies. Increased evidence of this phenomenon has been observed among clinical isolates and specimens by using novel methods, such as real-time PCR screening systems. In this study, the iPLEX assay was applied to identify amikacin resistance genotypes of the rrs gene. The sensitivity of this method was assessed by detecting a series of artificial mixtures (WT1401 and A1401G, the wild-type and mutant genotypes of KRDR, respectively). A ratio of 1:200 indicated that one resistant clone would be detected among 200 clones at a fragment concentration of 1 ng/μL. In our findings, the ratios of these two genotypes in clinical isolates were in the range of 2:10 to 1:1, as determined using the pGEM-T
Easy clone system (as shown in Table 1). Thus, the sensitivity of this method was sufficient for identifying the occurrence of heteroresistance in clinical isolates and providing an unambiguous result, as shown in Figure 2.

The iPLEX assay is a novel platform with high sensitivity for molecular genotyping and includes a primer extension process designed to detect the different alleles at the single nucleotide level. In contrast to other molecular methods, it allows multiplex detection of molecular genotypes for many genes within the same reaction. This characteristic enables the acquisition of information about heteroresistance to five to seven major anti-TB drugs at the same time. Further study of subpopulations of drug-resistant <i>M. tuberculosis</i> strains would help describe the evolution of drug resistance when multiple drugs are used.

In China, amikacin and capreomycin are frequently used for MDR-TB treatment. According to recent data, amikacin-resistant <i>M. tuberculosis</i> strains were present in 7% of new cases, 21% of retreatment cases and 48% of MDR-TB patients in Henan province. Analysis of the history of clinical use of injectable drugs (kanamycin, amikacin and capreomycin), DST profiles and molecular genotypes would further elucidate the clinical conditions that lead to XDR-TB.

**References**


