Prevalence and molecular characteristics of drug-resistant *Mycobacterium tuberculosis* in Beijing, China: 2006 versus 2012

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Abstract

**Background:** As the epidemic of MDR-TB and XDR-TB becomes increasingly severe, it is important to determine the clinical characteristics and molecular epidemiology of MDR-TB and XDR-TB. Recently, many studies have shown that clinical features and molecular characteristics of drug-resistant strains vary in different geographical areas, however, further information is needed to assess the dynamic evolution of drug-resistant TB. Comparative studies between different time periods are necessary to elucidate the development of drug-resistant TB.

**Results:** A total of 255 and 537 strains were collected from Beijing Chest Hospital in 2006 and in 2012, respectively. Drug-resistance rates and mutations associated with resistance to first-line anti-tuberculosis (TB) drugs were compared. The overall rate of drug resistance among strains of TB in 2012 was 54.4 %, significantly higher than that in 2006 (34.9 %, \( P < 0.001 \)). Rates of resistance to each first-line drug (isoniazid, rifampicin, streptomycin and ethambutol) and to second-line drug ofloxacin increased significantly from 2006 to 2012. The overall MDR rate also increased significantly from 2006 (14.9 %) to 2012 (27.0 %). The rate of MDR increased significantly between these two time periods in previously treated cases (\( P = 0.023 \)) but not in new cases (\( P = 0.073 \)), and the rate of XDR was similar in new cases at the two time periods, but was marginally higher in 2012 in previously treated cases (\( P = 0.056 \)). Previous treatment was found to be a risk factor for drug-resistant TB, especially for MDR-TB. In addition, the proportion of drug resistant isolates in which *katG*, the *mabA*-inhA promoter, *oxyR-ahpC* intergenic region, *rpoB*, *rpsL*, and *embB* were mutated was similar in 2006 and 2012, however patterns of mutation in these loci were more diverse in 2012 compared to 2006.

**Conclusions:** Our data suggests that the prevalence of drug resistant TB remains high in Beijing, China, and that increasing rates of resistance in *M. tuberculosis* to all anti-TB drugs should be considered when choosing an optimal anti-TB regimen. Moreover, acquired multi-drug resistance may play a primary role in the MDR-TB epidemic in Beijing, China. Consequently, this highlights the importance of an earlier start to effective and supervised treatment in order to reduce the burden of retreatment.

**Keywords:** Tuberculosis, Drug-resistant, Molecular characteristics
Background
Although the incidence and mortality of tuberculosis (TB) have declined over the past decade, there were an estimated 9.0 million incident cases of TB and 1.5 million deaths in 2013 according to the World Health Organization [1]. Two major factors, including the lethal association of HIV with active TB disease, and the worldwide dissemination of multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains of Mycobacterium tuberculosis, contribute to the severe TB epidemic [1]. Globally, an estimated 3.5% of new cases and 20.5% of previously treated cases have MDR-TB. In 2013 there were an estimated 480,000 new cases of MDR-TB, and about 210,000 associated deaths [1]. China is one of the 27 MDR-TB high burden countries. According to the 2007 national survey of drug-resistant TB in China, 5.7% of new cases and 25.6% of previously treated cases were diagnosed with MDR-TB. Based on these survey results, it is estimated that there are 120,000 new cases of MDR-TB in China per year [2]. In addition, XDR-TB cases were reported by over 100 countries in 2013, and up to 9.0% of MDR-TB cases had XDR-TB in 2013 [1]. It is therefore urgent to control the epidemic of MDR-TB and XDR-TB.

In order to understand how drug-resistant TB develops and to find better ways to control MDR-TB and XDR-TB, it is essential to determine the clinical characteristics and molecular epidemiology of MDR-TB and XDR-TB. Recently, many studies have shown that clinical features and molecular characteristics of drug-resistant strains vary in different geographical areas [3, 4], such as, for example in different provinces in China [5–9]. These studies, however, have focused on the clinical and molecular characteristics of drug-resistant M. tuberculosis strains from a single time period, and are thus unable to assess the dynamic evolution of drug-resistant TB. Comparative studies between different time periods are thus necessary to elucidate the development of drug-resistant TB.

To better understand changes in the clinical and molecular characteristics of M. tuberculosis isolates in Beijing, China, we analyzed all strains collected from TB inpatients admitted to Beijing Chest Hospital (tertiary TB referral hospital) over two time periods separated by six years (2006 and 2012). Clinical information, drug-resistant phenotypes (including MDR and XDR phenotypes) and first-line drug-resistance-associated mutations were compared between these two time periods.

Results
Demographic and clinical characteristics of enrolled subjects
Our purpose in this study was to evaluate changes in the clinical characteristics and molecular epidemiology of drug-resistant TB and thus assess its dynamic evolution. We thus performed a comparative study of M. tuberculosis isolates collected at the Beijing Chest Hospital over two time periods separated by 6 years. The MTB strain bank at Beijing Chest Hospital was first established in 2005, and complete datasets were available for each year from 2006 to 2012 when we initiated this project. We reasoned that a 6-year window would reveal possible changes in the rate of occurrence of drug-resistant TB, so chose to analyze all isolates collected from inpatients in the Beijing Chest Hospital from 2006 to 2012. A total of 792 isolates were selected, including 255 and 537 isolates from 2006 to 2012, respectively. The mean age of patients in 2006 and 2012 was 48.8 ± 19.5 years and 51.4 ± 19.6 years, and the male to female ratio was 2.7 and 2.9, respectively.

As shown in Table 1, there were no significant differences in the clinical characteristics (including gender, age and treatment history) of each subgroup (any drug-resistant TB, MDR-TB and pan-susceptible TB cases) between cases in 2006 and 2012 (P > 0.05). Of note, however, the proportion of previously treated cases in 2012 (40.8%) was significantly higher than that in 2006 (31.0%, P = 0.008).

Drug-resistance patterns differ in 2006 and 2012
The overall rate of resistance in the MTB strains examined to any drug was 54.4% in 2012, significantly higher than that in 2006 (34.9%, P < 0.001), indicating the severe and worsening situation of drug-resistance in TB in China. Furthermore, the rate of resistance to any drug in 2012 was higher than that in 2006 in both new and previously treated cases (P < 0.05).

The proportion of drug-resistant M. tuberculosis isolates in 2006 was compared with that in 2012 (Table 2). The overall resistance level to each drug was significantly higher in 2012 (P < 0.05). Rates of resistance to rifampicin (RIF), streptomycin (STR), ethambutol (EMB) and ofloxacin (OFX) were significantly higher in new cases in 2012 (P < 0.05), while the proportion of isolates resistant to isoniazid (INH) were marginally higher (P = 0.054), and resistance to capreomycin (CAP) and amikacin (AMK) was not significantly changed (P > 0.05). The proportions of isolates resistant to INH, RIF, OFX and CAP in previously treated cases were significantly higher in 2012 (P < 0.05), but the proportions of isolates resistant to STR, EMB and AMK were not significantly changed (P > 0.05).

The overall rate of MDR-TB was 27.0% in 2012, significantly higher than that in 2006 (14.9%, P < 0.05). The proportion of MDR-TB in previously treated cases was significantly higher in 2012 compared to 2006 (P = 0.023), but there was no significant increase in new cases (P = 0.073). The overall pre-XDR rate was 13.6% in 2012, again, significantly higher than that in 2006 (6.7%, P = 0.004). However, the pre-XDR rate in 2012 did
not increase significantly in either new cases (P = 0.165) or previously treated cases (P = 0.090) compared with 2006. The rate of XDR-TB also increased significantly from 2006 (2.4 %) to 2012 (6.9 %). While the rate of XDR was similar in new cases during the two time periods, it was marginally higher in 2012 in previously treated cases (P = 0.056).

Factors associated with drug-resistant TB and MDR-TB
The risk factors associated with resistance to any drug and MDR-TB were analyzed based on pooled demographic data for all patients (Table 3). By univariate analysis, age and treatment history were significantly associated with resistance to any drug and MDR-TB (P < 0.05). Multivariate analysis confirmed that age and treatment history were independently associated with resistance to any drug and MDR-TB (P < 0.05).

Patients older than 64 years had a significantly lower risk of developing drug resistance relative to patients in the younger age group (<25 years), with an adjusted OR of 0.52 (95 % CI: 0.31–0.86, P = 0.002). This risk decreased significantly in MDR-TB cases, with an adjusted Table 1 General demographic characteristics of patients enrolled in 2006 and 2012

| Characteristics         | Total   | Any drug resistant TB | MDR-TB | Pan-susceptible TB |
|-------------------------|---------|-----------------------|--------|-------------------|
|                         | 2006 (n = 255) | 2012 (n = 537) | P value | 2006 (n = 89) | 2012 (n = 292) | P value | 2006 (n = 166) | 2012 (n = 245) | P value |
| Gender                  |         |                      |        |                  |                   |        |                  |                   |        |
| Male                    | 186 (72.9) | 400 (74.5)  | 0.643  | 66 (74.2) | 223 (76.4) | 0.669  | 29 (76.3) | 102 (70.3) | 0.468  | 120 (72.3) | 177 (72.2) | 0.990 |
| Female                  | 69 (27.1)  | 137 (25.5)  |        | 23 (25.8) | 69 (23.6)  |        | 9 (23.7)  | 43 (29.7)  |        | 46 (27.7)  | 68 (27.8)  |        |
| Age group (years)       |         |                      |        |                  |                   |        |                  |                   |        |
| ≥25                     | 41 (16.1)  | 76 (14.2)   | 0.682  | 14 (15.7) | 46 (15.8)  | 0.728  | 6 (15.8)  | 32 (22.1) | 0.428  | 27 (16.4) | 30 (11.9)  | 0.271 |
| ≥44                     | 60 (23.5)  | 125 (23.3)  |        | 23 (25.8) | 82 (28.1)  |        | 11 (28.9) | 53 (36.6) |        | 37 (20.9) | 43 (17.5)  |        |
| ≥64                     | 92 (36.1)  | 185 (34.5)  |        | 38 (42.7) | 107 (36.6) |        | 18 (47.4) | 48 (33.1) |        | 54 (32.2) | 78 (32.5)  |        |
| >64                     | 62 (24.3)  | 151 (28.1)  |        | 14 (15.7) | 57 (19.5)  |        | 3 (7.9)   | 12 (8.3)  |        | 48 (30.5) | 94 (38.1)  |        |
| Treatment history       |         |                      |        |                  |                   |        |                  |                   |        |
| New cases               | 176 (69.0) | 318 (59.2)  | 0.008  | 37 (41.6) | 115 (39.4) | 0.712  | 5 (13.2)  | 21 (14.5) | 0.835  | 139 (83.7) | 203 (82.9) | 0.815 |
| Previously treated cases| 79 (31.0)  | 219 (40.8)  |        | 52 (58.4) | 177 (60.6) |        | 33 (86.8) | 124 (85.5)|        | 27 (16.3) | 42 (17.1)  |        |

Table 2 Comparison of drug susceptibility patterns between clinical M. tuberculosis isolates in 2006 and in 2012

| Susceptibility or resistance category | Total cases | New cases | Previously treated cases |
|--------------------------------------|-------------|-----------|--------------------------|
|                                      | 2006 (n = 255) | 2012 (n = 537) | P value |
| Any drug-resistance                  | 89 (34.9) | 292 (54.4) | <0.001  | 37 (21.0) | 115 (36.2) | 0.001  | 52 (65.8) | 177 (80.8) | 0.007 |
| All first-line drug resistance       | 78 (30.6) | 269 (50.1) | <0.001  | 30 (17.0) | 98 (30.8)  | 0.001  | 48 (60.8) | 171 (78.1) | 0.003 |
| INH                                  | 60 (23.5) | 209 (38.9) | 0.001  | 20 (11.4) | 57 (17.9)  | 0.054  | 40 (50.6) | 152 (69.4) | 0.003 |
| RIF                                  | 43 (16.9) | 164 (30.5) | <0.001  | 5 (2.8)   | 30 (9.4)   | 0.006  | 38 (48.1) | 134 (61.2) | 0.044 |
| STR                                  | 59 (23.1) | 189 (35.2) | <0.001  | 21 (11.9) | 63 (19.8)  | 0.026  | 38 (48.1) | 126 (57.5) | 0.149 |
| EMB                                  | 24 (9.4)  | 88 (16.4)  | 0.008  | 8 (4.5)   | 31 (9.7)   | 0.040  | 16 (20.3) | 57 (26.0)  | 0.306 |
| All MDR                              | 38 (14.9) | 145 (27.0) | <0.001  | 5 (2.8)   | 21 (6.6)   | 0.073  | 33 (41.8) | 124 (56.6) | 0.023 |
| INH + RIF                            | 4 (2.0)   | 25 (4.5)   | 0.007  | 1 (0.6)   | 4 (1.3)    | 0.463  | 3 (3.8)   | 21 (9.6)   | 0.105 |
| INH + RIF + STR/EMB                  | 34 (13.3) | 120 (22.3) | 0.003  | 4 (2.3)   | 17 (5.3)   | 0.105  | 30 (38.0) | 103 (47.0) | 0.165 |
| All second-line drug resistance      | 40 (15.7) | 172 (32.0) | <0.001  | 14 (8.0)  | 45 (14.2)  | 0.042  | 26 (32.9) | 127 (58.0) | <0.001 |
| OFX                                  | 37 (14.5) | 164 (30.5) | <0.001  | 13 (7.4)  | 42 (13.2)  | 0.049  | 24 (30.4) | 122 (55.7) | <0.001 |
| CAP                                  | 1 (0.4)   | 29 (5.4)   | 0.001  | 1 (0.6)   | 2 (0.6)    | 0.934  | 0         | 27 (12.3)  | 0.001 |
| AMK                                  | 9 (3.5)   | 42 (7.8)   | 0.021  | 1 (0.6)   | 7 (2.2)    | 0.168  | 8 (10.1)  | 35 (16.0)  | 0.204 |
| All pre-XDR                          | 17 (6.7)  | 73 (13.6)  | 0.004  | 2 (1.1)   | 10 (3.1)   | 0.165  | 15 (19.0) | 63 (28.8)  | 0.090 |
| All XDR                              | 6 (2.4)   | 36 (6.9)   | 0.008  | 1 (0.6)   | 4 (1.3)    | 0.463  | 5 (6.3)   | 32 (14.6)  | 0.056 |
Table 3 Factors associated with drug-resistant tuberculosis in all patients

| Factors                  | Resistance to any drug (n = 381) | MDR-TB (n = 183) | Pan-susceptible TB (n = 411) | MDR-TB vs Pan-susceptible TB | Resistance to any drug vs Pan-susceptible TB |
|--------------------------|----------------------------------|------------------|-----------------------------|------------------------------|----------------------------------------------|
|                          |                                  | Odds ratio (95% CI) | P value | Adjusted odds ratio (95% CI) | P value | Odds ratio (95% CI) | P value | Adjusted odds ratio (95% CI) | P value |
| **Gender**               |                                  |                   |         |                             |         |                   |         |                             |         |
| Male                     | 289                              | 131              | 297    | Reference                   | Reference |                       |         | 0.83 (0.60–1.14)             | 0.250   |
| Female                   | 92                               | 52               | 114    | 1.03 (0.70–1.52)            | 0.865    | 0.83 (0.60–1.14)     | 0.250   |
| **Age group (years)**    |                                  |                   |         |                             |         |                   |         |                             |         |
| ~25                      | 60                               | 38               | 57     | Reference                   | Reference |                       |         | 1.25 (0.78–1.99)             | 0.734   |
| ~44                      | 105                              | 64               | 80     | 1.20 (0.71–2.03)            | 0.892    | 1.05 (0.51–2.19)     | 0.718   | 1.04 (0.68–1.61)             | 0.969   |
| ~64                      | 145                              | 66               | 132    | 0.75 (0.45–1.24)            | 0.155    | 0.60 (0.30–1.21)     | 0.033   | 0.99 (0.61–1.60)             | 0.610   |
| >64                      | 71                               | 15               | 142    | 0.16 (0.08–0.31)            | <0.001   | 0.12 (0.05–0.27)     | <0.001  | 0.48 (0.30–0.75)             | 0.011   |
| **Treatment history**    |                                  |                   |         |                             |         |                   |         |                             |         |
| New cases                | 152                              | 26               | 342    | Reference                   | Reference |                       |         | 1.04 (0.68–1.61)             | 0.969   |
| Previously treated cases | 229                              | 157              | 69     | 29.93 (18.35–48.81)         | <0.001   | 32.64 (19.40–54.92)  | <0.001  | 7.47 (5.37–10.39)             | <0.001  |

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OR of 0.12 (95% CI: 0.05–0.27, P < 0.001). Previously treated cases were associated with a higher risk of developing drug resistance, with an adjusted OR of 7.11 (95% CI: 5.09–9.92, P < 0.001), and the risk increased significantly in MDR-TB cases, with an adjusted OR of 32.64 (95% CI: 19.40–54.92, P < 0.001).

Drug resistance associated mutations in 2006 and 2012

Using a combination of mutations in katG, the mabA-inhA promoter and the oxyR-ahpC intergenic region, DNA sequencing was able to identify 86.7% (52/60) and 85.6% (179/209) of INH-resistant isolates collected in 2006 and 2012, respectively. Similarly, 88.4% (38/43) and 92.1% (151/164) of RIF-resistant isolates were detected based on mutations in the RRDR region of rpoB, 79.7% (47/59) and 76.2% (144/189) of STR-resistant isolates were detected based on mutations in rpsL, and 54.2% (13/24) and 62.5% (55/88) of EMB-resistant isolates were detected based on mutations in embB. In addition, 8.2% (19/231) and 11.4% (51/449) of EMB-susceptible isolates in the 2006 and 2012 groups were also found to harbor mutations in the embB gene. Other target mutations were not found in INH, RIF and STR susceptible isolates in the 2006 and 2012 collections.

The frequencies of common mutations in INH, RIF, STR or EMB resistant isolates were similar in 2006 and in 2012: katG315 (71.7% vs 58.4%, P = 0.062), mabA-inhA –15 (15.0% vs 19.6%, P = 0.418); rpoB531 (55.8% vs 63.4%, P = 0.361), rpoB526 (16.3% vs 17.1%, P = 0.902); rpsL43 (64.4% vs 64.0%, P = 0.975); embB306 (33.3% vs 35.2%, P = 0.863). Data on common mutation patterns in these drug-resistant isolates are shown in Table 4. In addition, the embB306 mutation was detected in 4.5% (11/231) and 6.5% (29/449) of EMB-susceptible isolates in the 2006 and 2012 collections, respectively. Moreover, patterns of mutation in the loci examined were more diverse in 2012 compared to 2006.

Discussion

In this hospital-based study, the rate of resistance to any drug and the MDR rate of M. tuberculosis isolates was found to increase significantly from 2006 to 2012, reflecting the serious drug-resistant TB epidemic in China. Overall, the percentage of previously treated cases in 2012 was higher than that in 2006, suggesting that treatment of previously treated cases is still a big challenge in controlling the TB epidemic in China. Furthermore, the proportion of previously treated cases among the MDR-TB cases increased to 40.8% in 2012 compared to that in 2006 (31.0%). This implies that acquired multi-drug resistance may play an increasing role in the MDR-TB epidemic in China.

Among the new TB cases in this study, rates of resistance to each first-line drug in the 2006 isolate collection were consistent with those found in the National survey of drug-resistant TB conducted in 2007 [2]. However, rates of resistance to each first-line drug were significantly increased in 2012. The rate of resistance to the second-line drug OFX also increased from 7.4% in 2006 to 13.2% in 2012 among new TB cases. The increasing rate of OFX resistance rate should thus be considered when choosing an optimal anti-TB regimen. As the new cases in this study had not received therapy or were in treatment for less than 1 month, the rate of drug resistance among new cases should reflect the transmission of drug-resistant TB.

Among previously treated cases examined in our study, rates of resistance of M. tuberculosis isolates to INH, RIF, OFX and CAP were also higher in the 2012 collection compared with the 2006 collection. Overall rates of drug-resistance to each first-line drug in 2006 were much higher than that in the National survey [2]. This difference might be attributed to differences in sampling methods and the subjects targeted. For the national survey, isolates were selected from across 10 provinces of China using a cluster-randomized sampling method [2]. In contrast, in this study, patients were recruited from one tertiary TB referral hospital, i.e., most subjects were hospitalized TB patients with relatively serious symptoms. Overall, the increasing rate of drug-resistance in previously treated cases underlines the importance of standard treatment and the necessity of optimizing the treatment regimen according to the results of drug susceptibility testing.

The MDR and XDR rates in the 2006 isolates were similar to those found in the National survey [2] among new cases, but were much higher for previously treated cases. This may also be due to differences in the patients targeted as discussed above. Moreover, the rates of MDR and XDR MTB isolates had increased in 2012 compared to 2006 both among new cases and previously treated cases, although the difference was not significant for new cases. This data suggests that acquired MDR might outweigh primary resistance in the MDR epidemic in China. This finding is different from that of Gao et al. [10] who found that it is recent transmission of M. tuberculosis including transmission of MDR strains, that contributes most highly to the TB epidemic in China. This difference in findings may be due to variation in regions sampled: Gao et al. collected strains from five counties within five different provinces [10], while the study population examined here came from one hospital in Beijing. Previous studies have shown that drug-resistance rates vary in different provinces [11]. Pre-XDR rates increased significantly from 2006 to 2012. Since pre-XDR is an important step in the development of drug resistance from MDR to XDR, this finding presents an additional alarming indication of the worsening situation of XDR-TB in China.
| Drug | Locus | Mutated position | 2006 | 2012 | Total |
|------|-------|------------------|------|------|-------|
|      |       | Mutated patterns | Relative frequency % (No. of mutant isolates/No. of drug-resistant isolates) | Mutated patterns | Relative frequency % (No. of mutant isolates/No. of drug-resistant isolates) | Mutated patterns | Relative frequency % (No. of mutant isolates/No. of drug-resistant isolates) |
| INH | katG  | Codon 315        | AGC→ACC 68.3 (41/60) | AGC→ACC 66.5 (118/209) | AGC→ACC 59.1 (159/269) |
|     |       |                  | AGC→ACA 1.7 (1/60) | AGC→ACA 0.5 (1/209) | AGC→ACA 0.7 (2/269) |
|     |       |                  | AGC→AAC 1.7 (1/60) | AGC→AAC 1.0 (2/209) | AGC→AAC 1.1 (3/269) |
|     |       |                  | AGC→CGC 0.5 (1/209) | AGC→CGC 0.5 (1/269) | AGC→CGC 0.3 (1/269) |
|     |       | Other mutations  | – | – | – |
|     | mabA-inhA promoter | –15 | –15 C→T 15.0 (9/60) | –15 C→T 19.6 (41/209) | –15 C→T 18.6 (50/269) |
|     |       |                  | –6.7 (4/60) | –3.3 (7/209) | –4.1 (11/269) |
|     | oxyF-ahpC intergenic region | –10 | –10 C→T 0 | –10 C→T 2.4 (5/209) | –10 C→T 1.9 (5/269) |
| RIF | rpoB  | Codon 526        | CAC→TAC 9.3 (4/43) | CAC→TAC 7.3 (12/164) | CAC→TAC 7.7 (16/207) |
|     |       |                  | CAC→GAC 4.7 (2/43) | CAC→GAC 2.4 (4/164) | CAC→GAC 2.9 (6/207) |
|     |       |                  | CAC→AAC 2.3 (1/43) | CAC→AAC 1.2 (2/164) | CAC→AAC 1.4 (3/207) |
|     |       | Other mutations  | – | 16.3 (7/43) | – |
| STR | rpsL  | Codon 306        | ATG→GTG 25.0 (6/24) | ATG→GTG 19.3 (17/88) | ATG→GTG 29.5 (23/112) |
|     |       |                  | ATG→ATA 8.3 (2/24) | ATG→ATA 11.4 (10/88) | ATG→ATA 10.7 (12/112) |
|     |       | Other mutations  | – | 15.3 (9/59) | – |
| EMB | embB  | Codon 43         | AAG→AGG 59.3 (35/59) | AAG→AGG 63.5 (120/189) | AAG→AGG 62.5 (155/248) |
|     |       |                  | AAG→AAC 5.1 (3/59) | AAG→AAC 0.5 (1/189) | AAG→AAC 1.2 (3/248) |
|     |       | Other mutations  | – | – | 12.9 (32/248) |
| Mutation          | Frequency (Count/Total) |
|-------------------|-------------------------|
| ATG→ATC           | 1.1 (1/88)              |
| ATG→CTG           | 1.1 (1/88)              |
| Other mutations   | 20.8 (5/24)             |

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Previous treatment is a well-known risk factor for drug-resistant TB and MDR-TB, and the prevalence of MDR-TB can be up to 10 times higher after unsuccessful treatment [12]. In this sense, results obtained here are consistent with previous observations: the risk of suffering MDR-TB among previously treated cases was 32.64 times higher than that in new cases. The implementation of DOTS is thus still very important for effectively controlling drug-resistant TB and MDR-TB, especially with respect to supervising patients to complete the treatment. We also found that people older than 64 years of age had a lower risk of drug-resistant TB and MDR-TB. This is consistent with the conclusion of a systematic review of European studies which concluded that MDR-TB cases are more likely to occur in patients younger than 65 years of age [13]. The higher risk of getting MDR-TB in people under 65 years may be attributed to the use of RIF for anti-TB treatment from around 1965. TB cases in older patients are usually considered as relapse cases, and the infecting strains may be more ancient, and carry a lower risk of becoming resistant to RIF.

Between 2006 and 2012, there was more or less no difference in the molecular detection rate for first line drug-resistance in our study. It should be noted that mutations in embB were also found in EMB-susceptible isolates [14]. Here, embB306 mutations were found in 4.5 % and 6.5 % of EMB-susceptible isolates in 2006 and 2012, respectively. This percentage is lower than previously reported in Russia (31.2 %) [14] and Singapore (20.0 %) [15], and may be due to different percentages of multi-drug resistance among EMB-susceptible isolates. Hazbon et al. collected 807 M. tuberculosis isolates from Colombia, Mexico, New York and Texas, and found that the association between embB306 mutations and resistance to increasing numbers of anti-TB drugs was significant in each region, suggesting the role of embB306 mutations in broad drug resistance [16]. The frequency of the embB306 mutation in one setting may thus be influenced by the percentage of multi-drug resistant isolates. Accordingly, the embB306 mutation does not appear to be a reliable marker for predicting EMB resistance in Beijing, China.

Mutation rates at common loci in specific genes associated with drug-resistance were similar in 2006 and 2012. It has previously been shown that antibiotic resistance associated mutations can impair bacterial fitness [17, 18]. However, acquisition of compensatory mutations in drug resistant strains can restore their ability to survive. It is possible that the reason why the mutation rate at common loci in the drug-resistant isolates in our study was unchanged may be that mutations in these common loci are associated with other compensatory mutations that lead to lower fitness costs.

Our study, however, has some limitations. The isolates examined were collected from only one TB referral hospital in Beijing. Patients admitted to this hospital tend to be severe cases or to have received therapy in other hospitals but with poor effect. Thus the incidence of drug-resistant TB may be overestimated, and may not reflect the average level of the whole country. Well-designed studies with a wide coverage of different regions in China should thus be conducted in the future.

Conclusions

Results from this study indicate that the prevalence of drug resistant TB remains high in Beijing, China, and suggest that increasing rates of resistance in M. tuberculosis to all anti-TB drugs should be considered when choosing optimal anti-TB regimens for treatment. The rate of MDR and XDR in M. tuberculosis isolates was higher in 2012 compared to 2006, especially in isolates from previously-treated cases, suggesting that acquired multi-drug resistance may increasingly be playing a primary role in the MDR-TB epidemic in China. These findings highlight the importance of an earlier start on effective and supervised treatment in order to reduce the burden of retreatment.

Methods

M. tuberculosis isolates and drug susceptibility testing (DST)

A total of 255 and 537 M. tuberculosis isolates collected in 2006 and 2012, respectively, were obtained from Beijing Bio-Bank of clinical resources on Tuberculosis at Beijing Chest Hospital. All isolates were recovered from inpatients diagnosed with pulmonary TB. If several isolates had been recovered from the same patient at different time points, only the earliest isolate was included in this analysis. Clinical investigations were conducted in accordance with the principles expressed in the Declaration of Helsinki, and this study was approved by the Ethics Committee of Beijing Chest Hospital. Written informed consent was not obtained from patients as the data were analyzed anonymously.

DST was performed using the proportion method on Löwenstein-Jensen medium, according to WHO guidelines, with the following concentrations of anti-TB drugs: INH - 0.2 μg/ml, RIF - 50 μg/ml, STR - 10 μg/ml, EMB - 5.0 μg/ml, OFX - 2.0 μg/ml, levofloxacin (LFX) - 2.0 μg/ml, CAP - 40 μg/ml, AMK - 30 μg/ml. Strains were deamed to be resistant to a specific drug when the growth rate was ≥1 % that of the control. Both OFX and LFX susceptibility testing were performed, but as results showed that all LFX-resistant isolates were also resistant to OFX, LFX-resistance data were not included in the analysis.
Data collection and definitions
Demographic and clinical information on enrolled patients, including gender, age, address and TB treatment history, were obtained from inpatients’ medical records. New cases were TB patients who had never been treated with anti-TB drugs or that had been treated for less than 1 month. Previously treated cases were TB patients who had been treated with anti-TB drugs for 1 month or longer. MDR-TB was defined as resistance to at least INH and RIF [19]. Although kanamycin resistance is included in the WHO definition of pre-XDR and XDR, this drug is rarely used to treat TB at the Beijing Chest Hospital. In this study, XDR-TB was therefore defined as resistance to INH and RIF plus OFX and at least one injectable second-line drug (CAP or AMK). Pre-XDR TB was defined as resistance to INH and RIF plus either OFX or a second-line injectable drug (CAP or AMK), but not both.

Detection of drug resistance associated gene mutations
Loci associated with hot-spots of drug-resistance to first line anti-TB drugs (INH, RIF, STR and EMB), including katG, the mabA-inhA promoter, oxyR-ahpC intergenic region, rpoB RRDR (RIF-resistance-determining region), rpsL and embB were sequenced in this study. Genomic DNA was extracted from freshly cultured M. tuberculosis using a conventional cetyltrimethylammonium bromide (CTAB) method [20]. All the primers for amplification of target nucleotide positions and DNA sequencing are listed in Table 5. For each target gene, the volume of PCR mixture was 25 μL, containing 12 μL of 2× Taq Master Mix, 1 μL of forward and reverse primers (10 μM), 10 μL of distilled H2O and 1 μL of genomic DNA. For katG, rpoB RRDR, embB, and rpsL, the PCR program comprised an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 62 °C for 45 s and 72 °C for 35 s, and a final step of 72 °C for 4 min. For the mabA-inhA promoter and oxyR-ahpC intergenic region, the PCR program comprised an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, and a final step of 72 °C for 4 min. All PCR products were sent for sequencing using primers which were the same as those used for PCR amplification. Sequencing data was aligned with the corresponding sequences of the M. tuberculosis H37Rv reference strain using BLASTn optimized for megablast on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis
Pearson chi-square tests or Fisher exact tests were used to compare drug-resistance rates between isolates collected in 2006 and in 2012. Univariate analysis of categorical variables was performed with the Pearson chi-square test or Fisher exact test as appropriate. Univariate and multivariate logistic regression analyses were used to analyze drug-resistance-associated risk factors. Variables with a P value less than 0.05 in the univariate analysis were analysed further by multivariable logistic regression analysis. A two-sided P value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS statistics 19.0.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Beijing Chest Hospital. Written informed consent was not obtained from patients as the data were analyzed anonymously.

Availability of data and materials
All of the data are complete in this study, no supplementary data are attached.

Table 5 Primers for PCR amplification and DNA sequencing

| Gene            | Amplified Region (bp) | Orientation | Oligonucleotide sequence (5′→3′) | Tm (°C) |
|-----------------|-----------------------|-------------|----------------------------------|---------|
| katG            | 580 to 1257           | Forward     | GTAGAGGTTGTCTATTGGGGCAAG         | 59.8    |
|                 |                       | Reverse     | GTCTCGGTGGATCAGCTTGTA           | 59.8    |
| mabA-inhA promoter | −436 to 182 (mabA) | Forward     | ATGCCGCTCTTCCCAGACTT            | 58.0    |
|                 |                       | Reverse     | TCACATTCCGACGCCAAACAG           | 60.0    |
| oxyR-ahpC intergenic region | 285 (oxyR) to 312 (ahpC) | Forward | CCCCTATGCAATGCACAACAA            | 60.0    |
|                 |                       | Reverse     | TTGGAGGTGCTGTCTGCTG            | 60.0    |
| rpoB RRDR       | 916 to 1572           | Forward     | GGTCGCTATAAGGTCAACAAG          | 61.0    |
|                 |                       | Reverse     | GTACACGATCTGCTGCTAACC          | 62.1    |
| embB            | 847 to 1581           | Forward     | GATGATTGGGTGCGGCTGGTA          | 62.0    |
|                 |                       | Reverse     | GTAGTGAATACGGCTTGCTGGA         | 62.9    |
| rpsL            | 254 to 877            | Forward     | GAATCCAGTGGTGGGCAAGCTAT        | 58.8    |
|                 |                       | Reverse     | CTCAACGCCACCATACAAAT           | 55.9    |
Abbreviations
AMK: amikacin; CAP: capreomycin; CTAB: cetyltrimethylammonium bromide; DST: drug susceptibility testing; EMB: ethambutol; INH: isoniazid; LFX: levofloxacin; MDR: multidrug-resistant; OFX: ofloxacin; Rif: rifampicin; RRDR: rifampicin-resistance-determining region; STR: streptomycin; TB: tuberculosis; XDR: extremely drug-resistant.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
QQY and ADS conceived this study, and WWJ and HRH participated in its design. QJL, FX and QJL carried out the experiments, and QJL, LS and YJL participated in data analysis. QQY drafted the manuscript and WWJ and ADS revised the manuscript. All authors read and approved the final manuscript before submission.

Acknowledgements
The authors thank colleagues in the Department of Clinical Epidemiology and the Evidence Based Medicine Center in Beijing Children’s Hospital for statistical advice. All the isolates used in this study were obtained from the “Beijing Bio-Bank of clinical resources on Tuberculosis” (D131100005313012), of the National Clinical Lab on Tuberculosis, Beijing Chest Hospital. We are grateful to Dr. Joy Fleming and Dr. Igor Mokrousov for critical reading and scientific editing of the final revised manuscript.

Funding
This work was supported by the grants of National Natural Science Foundation of China (81271889, 81450057 and 81541062) and the Infectious Diseases Special Project, Minister of China (2012ZX10003002009).

Received: 4 January 2016 Accepted: 29 April 2016
Published online: 12 May 2016

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