International Journal of Antimicrobial Agents 45 (2015) 137–143

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Molecular characterisation of extensively drug-resistant *Mycobacterium tuberculosis* isolates in China

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ARTICLE INFO

Article history:
Received 30 May 2014
Accepted 28 September 2014

Keywords:
Extensively drug-resistant tuberculosis
Molecular characterisation
Genotype
Resistance-conferring mutations

ABSTRACT

The emergence of extensively drug-resistant tuberculosis (XDR-TB) in China is a great threat to TB control. To determine the molecular characterisation of XDR-TB isolates from China and the correlations between specific drug resistance-associated mutations and different genotype strains, 58 XDR-TB isolates were sequenced in eight drug loci, including *katG*, *inhA*, *oxyR–ahpC* intergenic region, *rpoB*, *eis*, *rrs*, *gyrA* and *gyrB*, and were genotyped using spoligotyping and analysis of the noise transfer function region. Compared with the phenotypic data, the sensitivities and specificities for DNA sequencing were 87.9% and 100.0% for isoniazid (INH), 91.4% and 98.3% for rifampicin (RIF), 60.4% and 100.0% for kanamycin (KAN) and 81.0% and 100.0% for ofloxacin (OFX), respectively. A combination of eight drug loci predicted XDR-TB phenotypes with 53.4% sensitivity (31/58 isolates) and 100.0% specificity. The most frequent mutations among these XDR-TB isolates were *katG*315 and *inhA*-15 (for INH), 531, 526 and 516 in *rpoB* (for RIF), *rrs*1401 and *eis*–10 (for KAN) and 94, 90 and 91 in *gyrA* (for OFX). Also, among these XDR-TB isolates, 44 (75.9%) were identified as Beijing genotype strain, of which 31 (70.5%) belonged to the modern Beijing sublineage. *inhA*-8, *rpoB*526 and *rpoB*531 mutations demonstrated significant statistical associations with ancient and modern Beijing family sublineage ($P < 0.05$). However, Beijing and non-Beijing genotypes showed no association with specific resistance-conferring mutations. These results will be helpful in designing new molecular biology-based techniques to diagnose XDR-TB in China.

1. Introduction

Extensively drug-resistant tuberculosis (XDR-TB) has recently emerged as a significant global public health threat [1]. Multidrug-resistant tuberculosis (MDR-TB) is resistant to at least the first-line antituberculous agents isoniazid (INH) and rifampicin (RIF). Treatment of MDR-TB requires use of costly, toxic and less effective second-line drugs for ≥18 months because the bacilli are resistant to the first-line drugs used for routine TB treatment. XDR-TB is a type of MDR-TB with additional resistance to any fluoroquinolone and at least one of the three second-line injectable drugs, namely capreomycin (CAP), kanamycin (KAN) and amikacin. Treatment options for XDR-TB are very limited and the prognosis is extremely poor [2]. Up to February 2011, a total of 69 countries, including China, reported at least one case of XDR-TB [3].

XDR-TB usually results from inadequate antibiotic treatment or from direct person-to-person transmission of drug-resistant strains. Rapid and accurate detection of drug resistance could allow for prompt and adequate adjustments to treatment and could minimise transmission of drug-resistant strains. Determining the molecular characterisation of drug resistance will be helpful for establishing rapid molecular diagnostic methods. However, geographic variations in the location, type and frequency of resistance-conferring mutations are frequently observed [4,5].

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http://dx.doi.org/10.1016/j.ijantimicag.2014.09.018
0924-8579/

Moreover, as the most globally predominant genotype, Beijing family strains are associated with drug resistance and resistance-conferring mutations in some regions.

China is one of the countries with the high levels of drug-resistant TB, and the epidemic of XDR-TB also remains a serious problem of TB control. According to the national baseline survey on TB in 2007–2008 [6], the observed prevalence of XDR-TB among all new and previously treated pulmonary TB patients was 0.68%, so it was estimated that there were 8200 incident cases of confirmed XDR-TB. Although China has a high annual risk for XDR-TB, little information has been obtained regarding the genotypes and drug resistance-associated mutations of XDR-TB in most Chinese regions [7,8]. This is a great obstacle for developing rapid molecular diagnostic methods for XDR-TB isolates. The present study was undertaken to characterise mutations prevalent in 58 XDR-TB clinical isolates from 19 provinces of China with respect to various target loci. Eight drug loci were examined for INH (*katG*, *inhA* and the *oxyR–ahpC* intergenic region), RIF (*rpoB*), KAN (*eis* and *rrs*) and ofloxacin (OFX) (*gyrA* and *gyrB*), which are commonly prescribed as TB treatments in China. Furthermore, to gain an insight into the association of specific mutations conferring drug resistance with different genotype strains, spoligotyping and noise transfer function (NTF) region analysis was performed.

2. Materials and methods

2.1. Mycobacterium tuberculosis isolates

In total, 58 XDR-TB isolates comprised all of the identified XDR-TB isolates collected from 2005–2009 stored in the *M. tuberculosis* bank of the National Reference Laboratory of Tuberculosis (Beijing, China). These isolates were recovered from patients with confirmed pulmonary TB in institutes for TB control and cure as well as TB hospitals distributed in 19 of 31 provinces in mainland China. The numbers isolated from each province were as follows: Fujian, 6; Guangdong, 4; Guangxi, 2; Hunan, 4; Hubei, 2; Jiangxi, 2; Chongqing, 1; Anhui, 4; Zhejiang, 6; Shanghai, 4; Gansu, 2; Xizang (Tibet), 1; Henan, 2; Hebe, 2; Beijing, 2; Shanxi, 4; Shaanxi, 2; Liaoning, 5; and Heilongjiang, 3. The same number of *M. tuberculosis* isolates identified as pan-susceptible were selected as negative controls using a random number table matched by region and isolation time. Only one isolate was included for each patient in this study. *M. tuberculosis* H37Rv (ATCC 27294) was used as a reference strain.

2.2. Drug susceptibility testing (DST)

DST was performed at the National Reference Laboratory of Tuberculosis using a conventional proportion method on Löwenstein–Jensen medium according to the World Health Organization (WHO) guideline in 2008 with the following critical concentrations: 0.2 μg/mL for INH; 40 μg/mL for RIF; 30 μg/mL for KAN; and 2.0 μg/mL for OFX. *M. tuberculosis* H37Rv (ATCC 27294) was used as a control for each batch of DST.

2.3. DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from freshly cultured bacteria using a conventional cetyltrimethylammonium bromide (CTAB) method [9]. The primers used in this study and their nucleotide positions in the drug loci are listed in Table 1. Each PCR mixture was prepared in a volume of 30 μL containing 15 μL of 2× *Taq* Master Mix (Takara, Ōtsu, Japan), 1 μL of the forward and reverse 5 μM primers, 12 μL of distilled H_2O and 1 μL of genomic DNA. The PCR programme consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, and a final extension step of 72 °C for 5 min. PCR products were sent for sequencing using the same primers as in the PCR amplification. All sequence data were aligned with the corresponding sequences of the reference *M. tuberculosis* H37Rv strain (GenBank accession no. NC_000962) using BioEdit v.7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.4. Spoligotyping and data analysis

Spoligotyping was performed using 43 covalently bound oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 as previously described by Kamerbeek et al. [10]. The results in binary format were entered in an Excel spreadsheet (Microsoft Corp., Redmond, WA) and were compared with the spoligotyping database SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp).

2.5. IS6110 in the noise transfer function region

All Beijing family strains identified by spoligotyping were then amplified by PCR to detect the presence or absence of IS*6110* in the NTF region [11] using the following primers: F-6110, 5′-CCAGATATCGGGTGTGTCGAC-3′; and R-6110, 5′-TGCCGTTGTCGAAATCTAAACCC-3′. Modern Beijing strains inserted in the NTF region yielded amplified products of ca. 1500 bp, whilst ancient Beijing strains without insertion yielded ca. 300 bp PCR products.

2.6. Statistical analysis

SPSS for Windows v.16.0 (SPSS Inc., Chicago, IL) was used to perform the χ^2 test analysis. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Drug susceptibility testing and DNA sequencing

A total of 116 clinical isolates, including 58 XDR-TB isolates and 58 pan-sensitive isolates, were analysed. Mutations conferring resistance to anti-TB drugs identified in the 58 XDR-TB isolates are summarised in Table 2. The agreement of DST and DNA sequencing varied for each drug and locus combination. When discrepant results occurred between both methods, repeat testing was performed. If the repeated result conflicted with the original data, a third round of testing was conducted, with the final value representing two out of the three cycles.

3.2. Isoniazid and katG, inhA and the oxyR–ahpC intergenic region

Among the 58 XDR isolates, 27 (46.6%) had only *katG* mutations, 9 (15.5%) had only *inhA* mutations and 4 (6.9%) had only *oxyR–ahpC* intergenic region mutations, whilst 11 (19.0%) harboured double mutations in these three fragments. As anticipated, the most commonly changed sites were *katG*315 (36 isolates; 62.1%) and *inhA* promoter 15 base mutation (13 isolates; 22.4%). By contrast, none of the 58 pan-susceptible isolates possessed a mutation in these three target fragments. Compared with the phenotypic data, detection of mutations for the combination of *katG*, *inhA* and the *oxyR–ahpC* intergenic region exhibited a sensitivity of 87.9% and a specificity of 100.0%. This assay was better than those obtained using the data for either gene alone (Table 3).