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Genetic diversity of multidrug-resistant tuberculosis in a resource-limited region of China

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SUMMARY

Objectives: To gain an insight into the genetic diversity of multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates in Chongqing Municipality, an MDR tuberculosis (MDR-TB) epidemic region of China.

Methods: In this study, a total of 208 *M. tuberculosis* isolates from smear-positive TB patients in Chongqing were genotyped by spoligotyping and mycobacterial interspersed repetitive unit–variable number tandem repeat typing (MIRU-VNTR). In addition, statistical analysis was performed to evaluate the distributions of drug susceptibility patterns and demographic data among different genotypes.

Results: Our results showed that 156 MDR *M. tuberculosis* strains (75.0%) belonged to the Beijing genotype, while the other 52 strains (25.0%) were non-Beijing genotype. The proportion of Beijing genotype in the re-treated patient group was significantly higher than that in the new patient group ($p = 0.013$), while drug resistance and demographic characteristics showed no statistically significant associations with Beijing genotype ($p > 0.05$). In addition, the 208 strains were clustered into 193 genotypes using a 10-locus VNTR set; the cumulative clustering rate was 12.98% and the HGDI was 0.9991.

Conclusions: Beijing genotype was the predominant genotype among the isolates from MDR-TB cases in Chongqing. The re-treated MDR-TB cases were more likely to be attributed to Beijing genotype infection. The 10-locus VNTR set demonstrated a good discrimination power for genotyping MDR *M. tuberculosis* isolates circulating in Chongqing Municipality.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), has been known as part of the human experience for many thousands of years.[1,2] The successful spread of TB worldwide reflects the effective mechanism of immune escape from the host immune response, favoring natural selection in *M. tuberculosis* strains.[1,3] Due to the application of effective anti-mycobacterial drugs, the prevalence of TB appeared to be under control.[4,5] Unfortunately, the emergence of drug-resistant TB, especially multidrug-resistant (MDR)-TB, defined as resistance to at least isoniazid and rifampin, has had a significant negative impact on TB control.[6]

The advance in molecular epidemiological tools has provided a reliable way to investigate molecular evolution over shorter and longer periods of time.[7–10] For *M. tuberculosis*, the application of several molecular markers harboring different molecular clocks has allowed us to access its complicated evolutionary history.[10] IS*6110*-restriction fragment length polymorphism (RFLP) DNA fingerprinting has been the genotyping technique used most widely for *M. tuberculosis*.[11] Despite providing high discriminatory power, this method is limited by its requirement for large quantities of high-quality DNA and cumbersome procedures. Recently, several PCR-based genotyping methods have been developed to compensate for the limitations of RFLP, including spoligotyping and mycobacterial interspersed repetitive unit–variable number tandem repeat typing (MIRU-VNTR), which has been considered as a good alternative method to RFLP.[12]

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Chongqing is the largest municipality located in southwestern China and has been classified as a hotspot for MDR-TB.[13] A recent epidemiological study demonstrated that the rates of primary and acquired MDR-TB were 3.8% and 26.9%, respectively.[13] Nevertheless, we still have no knowledge of the potential transmission profile of MDR-TB in this resource-limited region. In this study, in order to provide the basis for implementing control strategies for Chongqing, a total of 208 MDR *M. tuberculosis* isolates were genotyped by spoligotyping and MIRU-VNTR. The relationship between the molecular characteristics and drug susceptibility of phenotypes was also analyzed.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of the Chinese Center for Disease Control and Prevention. Patients were allowed to enter the study after agreeing to participate and signing an informed consent form.

2.2. Bacterial strains and culture conditions

From October 2011 to June 2013, a total of 208 MDR *M. tuberculosis* isolates, determined by conventional proportional drug susceptibility test,[6] were collected from 1976 smear-positive TB patients who were registered at local TB dispensaries in Chongqing. The concentrations of drugs in media were as follows: isoniazid 0.2 μg/ml, rifampin 40 μg/ml, ethambutol 2 μg/ml, streptomycin 4 μg/ml, ofloxacin 2 μg/ml, and kanamycin 30 μg/ml. A strain was declared resistant to a drug when the growth rate was >1% compared with the control. The MDR *M. tuberculosis* strains were defined as those resistant to both isoniazid and rifampin. All the isolates were subcultured on Lowenstein–Jensen medium.

2.3. Extraction of genomic DNA

Genomic DNA was extracted from freshly cultured bacteria, as reported previously.[12] Following centrifugation at 13 000 rpm for 2 min, the bacterial cells were transferred to a microcentrifuge tube containing 500 μl Tris–ethylenediaminetetraacetic acid (TE) buffer. The supernatant was discarded and the pellet was resuspended in 500 μl TE buffer and heated in a 95 °C water bath for 1 h. The cellular debris was isolated by centrifugation at 13 000 rpm for 5 min and the DNA in the supernatant was used for PCR amplification reactions.

2.4. Genotyping

Spoligotyping was performed with a commercially available kit, in accordance with the manufacturer's instructions and a published report (Isogen Bioscience BV, Maarssen, Netherlands).[14] The original binary data were submitted to the SITVITWEB database to obtain the spoligotyping pattern.[15] Isolates were assigned shared spoligotype international types (SIT) by the SITVITWEB database. Beijing genotype strains were defined as those with the pattern that hybridized to at least three signals from the last nine spacer oligonucleotides (spacers 35 to 43) and the absence of hybridization to spacers 1 to 34.[16]

In addition, the MIRU-VNTR typing method based on a 10-locus VNTR set with high discriminatory power was carried out to determine the composition of strains isolated from patients, as described previously in the literature.[13] The composition of 20 μl PCR mixture was as follows: 10 μl 2 × PCR Mix, 2 μl of DNA template containing approximately 2 ng genomic DNA, 0.2 μM of each primer set. The PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min, and then 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The copy number at each locus was calculated according to the repeat and flank length. The Hunter–Gaston discriminatory index (HGDI) was used to evaluate the discriminatory power of the MIRU-VNTR loci, as reported previously.[17] The primers used in the study were all synthesized by Tsingke Company (Beijing, China).

2.5. Data analysis

BioNumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) was used to cluster the genotyping patterns, including spoligotyping and MIRU-VNTR. A minimum spanning tree was constructed by the UPMGA (unweighted pair group method with arithmetic mean) algorithm. We performed a Chi-square test to evaluate the associations among multiple categorical variables, and all calculations were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Differences with a *p*-value of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Spoligotyping

Among the 208 MDR *M. tuberculosis* strains, 156 (75.0%) belonged to Beijing genotype, while the other 52 (25.0%) were non-Beijing genotype. Strains classified into non-Beijing families included 17 strains from the T1 family (8.2%), four from the T2 family (1.9%), seven from the MANU2 family (3.4%), two from the H3 family (1.0%), one from the LAM9 family (0.5%), one from the S family (0.5%), and 20 of undefined genotypes (9.6%). Clustering analysis revealed that SIT1 was the largest lineage (69.8%, 143), belonging to the classical Beijing genotype. The second largest lineage was SIT53, assigned to the ill-defined T1 family, with 13 strains (6.3%) (Table 1).

Table 1
Distribution of spoligotypes shared by *Mycobacterium tuberculosis* in this study

Family	Clade[a]	SIT[b]	No. of isolates		
			New cases	Re-treated cases	Total (%)
Beijing	Beijing	1	65	78	143 (69.8)
		190	3	0	3 (1.4)
		260	0	1	1 (0.5)
		269	2	0	2 (1.0)
		621	0	7	7 (3.4)
Non-Beijing	T1	53	10	3	13 (6.3)
		913	0	1	1 (0.5)
		522	3	0	3 (1.4)
	T2	52	2	0	2 (1.0)
		1161	2	0	2 (1.0)
	MANU2	54	2	5	7 (3.4)
	LAM9	803	1	0	1 (0.5)
	H3	50	0	1	1 (0.5)
	H3	294	1	0	1 (0.5)
	S	1211	1	0	1 (0.5)
	Others	NA[c]	13	7	20 (9.6)
Total	105	103	208 (100.0)		

[a] Representing spoligotype families annotated in the SITVITWEB database.
[b] SIT from SITVITWEB database.
[c] NA represents the spoligotyping type that is not found in the SITVITWEB database.