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EPIDEMIOLOGY

Comparison of the socio-demographic and clinical features of pulmonary TB patients infected with sub-lineages within the W-Beijing and non-Beijing *Mycobacterium tuberculosis*

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SUMMARY

Background: Highly lethal outbreaks of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis are increasing. *Mycobacterium tuberculosis* variant Beijing family and its members is regarded as a successful clone of *M. tuberculosis* that is associated with drug resistance in China. Understanding the genetic characteristics and molecular mechanism of drug resistant tuberculosis within Beijing family may help to clarify its origin and evolutionary history and the driving forces behind its emergence and current dissemination.

Methods: Totally of 1222 *Mycobacterium tuberculosis* isolates were recovered from patients in six counties of two provinces in eastern China within 2010/2012. Strain lineage and its major subgroups were studied respectively by using Spoligotyping and MIRU-VNTR. The 1st-line drug susceptibility was analyzed by proportional method and 2nd-line drug susceptibility was determined by the HAINs MTBDRsl test. The genetic characterization of drug resistance was analyzed by sequencing the previously reported genes and loci associated with drug resistance together with the multiple genotyping including MIRU-VNTR, Spoligotyping and LSP genotyping.

Results: Of the 1222 Mtb isolates, 298 (24.4%) were resistant to 1st-line drug and 73 (5.9%) were simultaneously resistant to INH and RIF namely MDR-TB. Respectively 23.8% of 1st-line drug resistant TB and 12.0% of the drug susceptible TB contained the mutation associated with 2nd-line drugs by HAINs test. The Spoligotyping of 1222 Mtb isolates revealed the 967 (79.1%) of the isolates belonged to the W-Beijing family. Within W-Beijing family, all 48 MDR-TB were observed in the isolates with simultaneous deletion of RD105 and RD207, with sub-lineage accounting for 88.7% of MDR-TB. Analysis of 24 MIRU-VNTR loci revealed that 88.2% (15/17) of MDR and extensively drug resistant (XDR) clustered isolates were sub-lineage 181.

Conclusions: Sublineage 181 might have the capacity to spread throughout the general community in rural China. This is the first report on the extensive association of sub-lineage 181 with MDR TB and possibly pre-XDR TB and XDR TB. It is important to monitor sublineage 181 to verify its heightened transmission and understand its importance in the global MDR–TB and XDR–TB epidemics.

1. Introduction

Tuberculosis (TB) is an emerging problem worldwide. The high mortality rates associated with multidrug resistant tuberculosis are particularly worrisome. Rural regions in China account for 80% of the country's land area and contain 60% of its population [1]. In rural areas, the prevalence and incidence of TB are much higher than in urban zones. In 2010, the estimated prevalence associated

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with TB in rural China were 569 cases per 100,000 population [1]. The situation worsens if the disease is caused by drug-resistant *Mycobacterium tuberculosis* (Mtb). Among new cases in some rural regions of China, the prevalence of resistance to at least one drug is 35–44%, and the prevalence of multidrug resistance (MDR; resistance to at least rifampin and isoniazid) among new cases could be as high as 22% [2].

In several Asian countries with high TB prevalence, a unique genotype of Mtb, known as the Beijing family genotype, has been found to be the dominant genotype [3–5]. During the last decade, the modern Beijing sublineage has been spreading throughout various geographic locations. This sublineage strain contains one copy of IS*6110* upstream of the NTF region [6]. Possible associations have been reported between epidemics caused by this genotype and its drug resistance [7,8] and high adaptability to the host intracellular environment [9]. Beijing family strains have spread widely throughout China. The modern Beijing sublineage has been found to comprise more than 80% of the Beijing genotype isolates in some parts of the country [10,11]. The genetic characteristics of the major sublineages, particularly those associated with drug-resistant Beijing family strains of TB in China, remain unknown. The aim of this study was to investigate the genetic diversity and molecular epidemiology of the major sublineages associated with M/XDR TB, and to understand the relationship between Mtb strains and the clinical characteristics of pulmonary tuberculosis (PTB) using current genetic methodology and cluster analysis.

2. Subjects and methods

2.1. Ethical considerations

This study was approved by the Fudan School of Public Health Institutional Review Board. Written informed consent was obtained from all patients who participated in the study.

2.2. Study settings

The study was performed in six rural counties from two provinces in China including JI, GU and GA in Jiangsu Province and CA, TE and XI in Shandong Province, which together comprise 1,700,000 people residing in rural areas. According to Direct Observed Treatment, Short course (DOTS) implemented in China, all TB patients were referred to local TB health facilities for diagnosis and treatment during the study period. Microbiological diagnostics included examination of three sputum specimens by smear microscopy and two specimens by cultivation. All patients diagnosed with TB should be registered using standardized categories and treated according to recommendations of the World Health Organization and the International Union against Tuberculosis and Lung Diseases [12,13].

2.3. Study design

Study subjects were all patients with PTB who were registered in local TB dispensaries of the study areas from 2010 to 2012. Within the study period, a total of 1222 Mtb isolates were recovered from patients consecutively diagnosed with PTB. Sputum samples were collected in specially designed plastic boxes and sent to the national TB reference laboratory in Shandong Province for culture and drug susceptibility testing (DST) to first-line drugs (FLD). The isolates were cultivated on Löwenstein–Jensen medium and forwarded to School of Public Health at Fudan University for molecular analysis. Data regarding demographic characteristics (patient age, sex, and geographic origin) and findings of smear microscopy were obtained from well-designed questionnaires and medical records.

2.4. Identification of strains and DST

Identification of the isolates was performed using standard microbiological tests (niacin accumulation and nitrate reduction). DST to FLD was carried out using the proportion method in egg-based medium. The drug-susceptibility definitions used have been reported elsewhere [14]. The Genotype®MTBDRsl assay (Hain Lifescience Gmbh, Nehren, Germany) was then performed to detect resistance to second-line drugs (SLD).

Detection of mutations in the Mtb isolates was subsequently carried out [15]. Previously reported drug resistance-related mutations have been detected by DNA sequencing, including the following nine genes: *rpoB* (RIF), *katG* and *inhA* (INH), *embB* (EMB), *gyrA* (fluoroquinolones; FQs), *rrs* (KAN, CAP, and AMK), *eis* (KAN) and *tlyA* (CAP). The previously reported drug resistance-determining regions were amplified using locus-specific primers [5]. Sequence data generated by the 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) were reviewed for confidence levels using a sequence scanner, and chromatograms were analyzed for the presence or absence of mutations by comparison with published sequences of H37Rv using the SeqMan alignment application of the DNAStar Lasergene (version 8.0) software. All mutations were confirmed by sequencing the reverse strand, except when they were abundant, in which case a subset of isolates were retested for each mutation.

2.5. Strain family and sublineage determination

Spoligotyping was performed using membranes and equipment provided with a commercially available spoligotyping kit (Isogen Bioscience BV, Maarssen, Netherlands), according to the manufacturer's instructions [16]. Results were compared with the SITVIT2 proprietary database of Institut Pasteur de la Guadeloupe, which is an updated version of previously released SpolDB4 [17] and SITVITWEB [18] databases.

The IS*6110* NTF region was analyzed according to previous studies [6,19]. Three IS*6110* loci (IS21-1, IS21-5 and IS21-19), which have been shown to be present between ancestral and modern sublineages [20], were amplified by polymerase chain reaction (PCR) to detect their presence or absence. Primers and details of the assay have been described previously [20].

The LSP of each strain was investigated, as described by Caws et al. [21]. LSPs of Beijing genotype strains were determined based on the deletion of five regions of difference (RDs) found in Mtb (RD105, RD142, RD150, RD181, and RD207), using PCR as described by Gagneux et al. [22]. Strains with only an RD105 deletion were designated the 105 sublineage. Strains with RD105 and RD207 deletions were designated the 207 sublineage. Strains with RD105, RD181, and RD207 deletions were designated the 181 sublineage. Strains with RD105, RD142, RD181, and RD207 deletions were designated the 142 sublineage. Strains with RD105, RD150, RD181, and RD207 deletions were designated the 150 sublineage.

LSPs of non-Beijing genotype strains were determined as follows: a specific 7-bp deletion in the Mtb pks gene was investigated using the primers described by Caws et al. [21] Strains with the 7-bp deletion were designated the Euro-American lineage. For strains that did not have the 7-bp deletion, deletion of RD750 was investigated using the primers described by Gagneux et al. [22] Strains with an RD750 deletion were designated the East African lineage. For strains not having the RD750 deletion, the Mtb specific deletion (TbD1) was investigated using the primers described by Brosch

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