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Genotypic diversity of multidrug-, quinolone- and extensively drug-resistant *Mycobacterium tuberculosis* isolates in Thailand

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ABSTRACT

Drug-resistant tuberculosis (TB), which includes multidrug-resistant (MDR-TB), quinolone-resistant (QR-TB) and extensively drug-resistant tuberculosis (XDR-TB), is a serious threat to TB control. We aimed to characterize the genotypic diversity of drug-resistant TB clinical isolates collected in Thailand to establish whether the emergence of drug-resistant TB is attributable to transmitted resistance or acquired resistance. We constructed the first molecular phylogeny of MDR-TB ($n = 95$), QR-TB ($n = 69$) and XDR-TB ($n = 28$) in Thailand based on spoligotyping and proposed 24-locus multilocus variable-number of tandem repeat analysis (MLVA). Clustering analysis was performed using the unweighted pair group method with arithmetic mean. Spoligotyping identified the Beijing strain (SIT1) as the most predominant genotype ($n = 139$; 72.4%). The discriminatory power of 0.9235 Hunter–Gaston Discriminatory Index (HGDI) with the 15-locus variable-number tandem repeats of mycobacterial interspersed repetitive units typing was improved to a 0.9574 HGDI with proposed 24-locus MLVA, thereby resulting in the subdivision of a large cluster of Beijing strains (SIT1) into 17 subclusters. We identified the spread of drug-resistant TB clones caused by three different MLVA types in the Beijing strain (SIT1) and a specific clone of XDR-TB caused by a rare genotype, the Manu-ancestor strain (SIT523). Overall, 49.5% of all isolates were clustered. These findings suggest that a remarkable transmission of drug-resistant TB occurred in Thailand. The remaining 50% of drug-resistant TB isolates were unique genotypes, which may have arisen from the individual acquisition of drug resistance. Our results suggest that transmitted and acquired resistance have played an equal role in the emergence of drug-resistant TB. Further characterization of whole genome sequences of clonal strains could help to elucidate the mycobacterial genetic factors relevant for drug resistance, transmissibility and virulence.

1. Introduction

Despite improvements in tuberculosis (TB) treatment, with a success rate among all new TB cases reaching 86%, only 48% of multidrug-resistant tuberculosis (MDR-TB) patients are treated successfully (World Health Organization, 2014). The poor outcome of this treatment contributes to an increase in the mortality rate and leads to a greater risk of developing extensively drug-resistant tuberculosis (XDR-TB), which is considerably more difficult to treat. The World Health Organization classified the burden of MDR-TB and XDR-TB as a public health crisis. In Thailand, MDR-TB accounts for 2% and 19% of new TB and retreatment cases, respectively (World Health Organization, 2014). We analyzed data from routine second-line drug susceptibility testing of MDR-TB between 2003 and 2011. During this 8 years period, quinolone-resistant tuberculosis (QR-TB), which is one form of pre-XDR-TB, increased from 9% to 15% of MDR-TB isolates, while XDR-TB isolates, which are resistant to quinolone and a second-line injectable drug, comprised 4% of MDR-TB isolates (Chaiprasert et al., 2014). Elucidation of the factors driving the emergence of drug-resistant TB is required to optimize strategies for TB control.

Epidemiological investigation is the first-line approach in the identification of the emergence of an infectious disease. However, it does not provide sufficient information about the mechanisms

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of emergence. The emergence of drug-resistant TB is complicated by two potential mechanisms: (i) transmission of drug resistance from a drug-resistant strain (transmitted resistance) and (ii) acquired drug resistance by a drug-susceptible strain (acquired resistance) (Blower and Chou, 2004; Klopper et al., 2013). Molecular epidemiology has been used to clarify the cause of emerging drug-resistant TB in the community. The most notable application was the use of IS*6110*-based restriction fragment length polymorphism genotyping in the investigation of the emergence of MDR-TB in New York City from 1995 to 2001, which resulted in the discovery of the transmission of a specific strain of the W/Beijing genotype (Frieden et al., 1995; Munsiff et al., 2002).

A number of genotyping methods have been used to provide information concerning the genetic diversity among drug-resistant strains. Currently, multilocus variable-number of tandem repeat analysis (MLVA) is accepted as a standard polymerase chain reaction (PCR)-based method that is used in molecular epidemiology studies of TB, because it facilitates rapid analysis and provides digital data, which enable the comparison of data between laboratories (Mazars et al., 2001). MLVA data have been used in combination with classical epidemiologic data to provide detailed information on the relative proportion of acquired resistance versus transmitted resistance based on the genotypic diversity over time (de Beer et al., 2014; Marais et al., 2013; Shamputa et al., 2010).

In general, 15-locus variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) and 24-locus MIRU-VNTR are standard methods used in routine epidemiologic investigations and phylogenetic analysis, respectively (Supply et al., 2006). However, these techniques have the disadvantage of low discriminatory power between the Beijing family of *Mycobacterium tuberculosis* strains (Allix-Béguec et al., 2014; Gurjav et al., 2014), which is the most prevalent genotype in Thailand (Faksri et al., 2011; Park et al., 2000; Prodinger et al., 2001; Srilohasin et al., 2014). Hypervariable loci have been proposed to improve the efficiency of subtyping Beijing clusters. In this study, we selected nine additional loci, which are described as highly polymorphic loci with allelic diversity among Beijing stains above 0.5 in previous reports (Allix-Béguec et al., 2014; Iwamoto et al., 2007; Murase et al., 2008).

Currently, genotyping of *M. tuberculosis* is not routinely performed in Thailand. Therefore, information about emerging drug-resistant TB is based predominantly on classical epidemiologic data without corresponding data informed by genetics (Jiraphongsa et al., 2011; Lapphra et al., 2013; Punnotok et al., 2000). In this study, we aimed to characterize the genotypic diversity of drug-resistant TB clinical isolates (including MDR-TB, QR-TB and XDR-TB) collected in Thailand to establish whether the ongoing problem of drug-resistant TB is caused by the transmission of predominant clones or the emergence of acquired resistance. Spoligotyping, a simple and rapid method for detecting polymorphisms within the direct repeat locus, was used as a first-line genotyping tool. Furthermore, a proposed 24-locus MLVA was used for further subtyping of drug-resistant *M. tuberculosis* isolates. The discriminatory power of proposed 24-locus MLVA was evaluated in comparison with the standard 15-locus MIRU-VNTR to determine the effect of the nine additional loci on the subtyping of Beijing strains.

2. Materials and method

2.1. Mycobacteria and drug susceptibility testing

Two hundred and ten *M. tuberculosis* isolates from 196 TB patients were retrieved from stock cultures of clinical isolates deposited at the Drug-Resistant Tuberculosis Research Fund, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University from December 2003 to March 2013. The mycobacterial collection consisted of isolates from thirty provinces in all six geographical regions of Thailand. All isolates were obtained from decontaminated sputum cultured on Lowenstein–Jensen medium, followed by drug susceptibility testing using the proportion method on Middlebrook 7H10 agar quadrant plates. In total, 98 MDR-TB isolates, 82 QR-TB isolates and 30 XDR-TB isolates were successfully retrieved. The stock cultures were stored in Middlebrook 7H9 media with 15% glycerol at −20 °C. The study protocol was approved by the Ethical and Scientific Committees of the Faculty of Medicine Siriraj Hospital, Mahidol University (EC No. Si 029/2557).

2.2. Genotyping of *M. tuberculosis*

2.2.1. Spoligotyping

Genomic DNA was obtained by extraction using the cetyltrimethyl-ammonium bromide-sodium chloride method, as previously described (van Soolingen et al., 1991). The whole sequence of the direct repeat (DR) region, including all spacers between the DR loci, was amplified by simplex PCR. The PCR amplicons were then used to perform spoligotyping with a commercially available kit (Ocimum Biosolutions, India) as previously described (Kamerbeek et al., 1997). The binary spoligotype pattern was converted into a 15-digit octal code, which was later used to query the SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) proprietary database of the Pasteur Institute of Guadeloupe for classification into clade and spoligotype international type (SIT) (Demay et al., 2012).

2.2.2. Multilocus variable-number of tandem repeat analysis

A set of standard 15-locus MIRU-VNTR (Supply et al., 2006), a consensus 4-locus set of hypervariable loci (1982, 3232, 3820 and 4120) (Allix-Béguec et al., 2014), a promising 4-locus set for subtyping Beijing family (2074, 2372, 3155 and 3336) (Murase et al., 2008) and a highly polymorphic locus (2163a) (Iwamoto et al., 2007) were amplified by multiplex PCR or a simplex PCR using 5′ fluorescently labeled primers as shown in Supplementary Table S1. The multiplex PCR and simplex PCR master mixes were prepared as summarized in Supplementary Tables S2 and S3, respectively. PCR reactions were performed under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 7 min. The size of the amplified PCR products was determined by capillary electrophoresis on a 3130 genetic analyzer or a 3500 genetic analyzer (Applied Biosystems, USA). The copy number of each locus was automatically estimated using GeneMapper® software v.5 (Applied Biosystems, USA). When an ambiguous peak or no peak was observed, the sample was subjected to re-analysis by simplex PCR with unlabelled primers. In this case, the size of the PCR amplicon was determined by 2% agarose gel electrophoresis or capillary electrophoresis (SV1210 Microchip electrophoresis system, Hitachi High-Technologies Corporation, Japan), and the copy number of each locus was calculated manually.

2.3. Discriminatory power of genotypic methods and clustering analysis

The allelic diversity of each locus and the discriminatory power of the different genotypic methods were evaluated based on the *h*-value (Selander et al., 1986) and Hunter–Gaston Discriminatory Index (HGDI) (Hunter and Gaston, 1988), respectively. For clustering analysis, the unweighted pair group method with arithmetic mean (UPGMA) was used to construct a rooted tree based on the