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High diversity of multidrug-resistant *Mycobacterium tuberculosis* Central Asian Strain isolates in Nepal

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ABSTRACT

Objectives: Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) poses a major public health problem in Nepal. Although it has been reported as one of the dominant genotypes of MTB in Nepal, little information on the Central Asian Strain (CAS) family is available, especially isolates related to multidrug resistance (MDR) cases. This study aimed to elucidate the genetic and epidemiological characteristics of MDR CAS isolates in Nepal.

Methods: A total of 145 MDR CAS isolates collected in Nepal from 2008 to 2013 were characterized by spoligotyping, mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis, and drug resistance-associated gene sequencing.

Results: Spoligotyping analysis showed CAS1_Delhi SIT26 as predominant (60/145, 41.4%). However, by combining spoligotyping and MIRU-VNTR typing, it was possible to successfully discriminate all 145 isolates into 116 different types including 18 clusters with 47 isolates (clustering rate 32.4%). About a half of these clustered isolates shared the same genetic and geographical characteristics with other isolates in each cluster, and some of them shared rare point mutations in *rpoB* that are thought to be associated with rifampicin resistance.

Conclusions: Although the data obtained show little evidence that large outbreaks of MDR-TB caused by the CAS family have occurred in Nepal, they strongly suggest several MDR-MTB transmission cases.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains a major public health problem globally. Although TB is a preventable and curable disease, the World Health Organization (WHO) estimated that 10.4 million new cases occurred around the world in 2015 alone, and that 1.4 million of these cases resulted in death (WHO, 2016). The majority of deaths were reported in developing countries, with more than half occurring in Asia (58%).

Nepal is one of the Asian countries that experiences a large number of TB cases every year. For example, in 2015, Nepal reported a TB mortality rate of 21%, and estimates of TB prevalence and incidence were 215 and 156, respectively, per 100 000 inhabitants (WHO, 2016). Furthermore, despite a TB control program run by the government, the number of TB cases in Nepal has not decreased over the last decade. The reason behind this phenomenon remains unknown; thus, comprehensive studies on MTB transmission in Nepal are needed.

Genotyping of MTB isolates has proven to be a powerful tool for investigating suspected outbreaks, the source of transmission, the transmission chain, and circulating strains (Crawford, 2003).

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Spoligotyping (Kamerbeek et al., 1997), mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR) analysis (Supply et al., 2006), and IS*6110* restriction fragment length polymorphism (RFLP) typing (Beggs et al., 2000) are commonly used techniques for genotyping. However, IS*6110* RFLP typing is a time-consuming technique and the comparison of results between laboratories is difficult (Varma-Basil et al., 2011). As a result, spoligotyping and MIRU-VNTR have more often been used in recent molecular epidemiological studies. Moreover, both of these genotyping methods are reliable, discriminative, and technically feasible for comparisons between laboratories (Mazars et al., 2001).

MTB consists of four major lineages based on specific genetic markers and geographical areas: lineage 1 (Indo-Oceanic lineage), lineage 2 (East-Asian Lineage, includes Beijing family), lineage 3 (East African Indian, includes Delhi/CAS family), and lineage 4 (Euro-American lineage) (Comas et al., 2009; Filliol et al., 2003; Gagneux et al., 2006). Previous studies on the genotype of MTB isolates in Nepalese patients reported the lineage 3 Central Asian Strain (CAS) family as the dominant family in Nepal (Malla et al., 2012). Other studies have also reported the CAS family as dominant (Ali et al., 2007; Hasan et al., 2006; Singh et al., 2007; Singh et al., 2015) and to be a contributor to multidrug resistance (MDR) in TB in South Asian countries (Yasmin et al., 2014). For instance, in India, Stavrum et al. (2009) associated the CAS family with multidrug-resistant tuberculosis (MDR-TB), and in Pakistan it was linked to the increasing prevalence of MDR-TB and emergence of extensively drug-resistant (XDR) TB (Hasan et al., 2010). It is believed, therefore, that the emergence of MDR-MTB in the CAS family poses a serious threat to the success of TB control programs in the region. Likewise, in Nepal, MDR-MTB is considered to be one of the major emerging threats to the success of TB control. For instance, in 2010, a study conducted in Nepal showed an MDR-MTB prevalence of 11.7% in re-treated cases (NTPN, 2010), but the latest national anti-TB drug resistance survey conducted between 2011 and 2012 showed that MDR-MTB prevalence increased to 15.4% in re-treated cases (NTPN, 2014). Nonetheless, a study is yet to be conducted in Nepal to determine the genetic characteristics of MDR in MTB of the CAS family.

The main purpose of the present study was to conduct a genetic analysis of MDR in MTB of the CAS family and to understand its molecular epidemiological features and transmission dynamics in Nepalese TB patients.

Materials and methods

Sample collection and drug susceptibility testing

A total of 601 MDR-MTB isolates collected from April 2008 to March 2013 by the German Nepal Tuberculosis Project (GENETUP) were used, of which 145 MDR-MTB CAS family isolates were purposively selected. Isolates were collected from a decentralized National Tuberculosis Program (NTP) network of 11 MDR-TB treatment centers and 66 sub-treatment centers. The location of treatment centers and the number of samples collected from each center are shown in Figure S1 in the **Supplementary Material** in the online version, at http://dx.doi.org/10.1016/j.ijid.2017.06.010. All samples were obtained from different individuals. Epidemiological features of patients were also collected from hospital medical records. A phenotypic drug susceptibility test (DST) was performed using the proportional method on Löwenstein–Jensen (LJ) medium with standard critical concentrations for isoniazid (INH) (0.2 μg/ml), rifampicin (RIF) (40 μg/ml), streptomycin (STR) (4 μg/ml), and ethambutol (EMB) (2 μg/ml) (WHO, 2009).

DNA extraction

Mycobacterial colonies on positive LJ cultures were suspended in 300 μl of distilled water and heated for 20 min at 95 °C. Heated samples were sonicated in an ultrasonic water bath apparatus (Elma Hans Schmidbauer GmbH & Co. KG, Germany) for 15 min and centrifuged for 5 min at 10 000 × *g*. Next, the bacterial DNA-containing supernatant was retrieved and used for further molecular analysis.

Spoligotyping

All isolates were analyzed by spoligotyping, as described by Kamerbeek et al. (1997). Briefly, the direct repeat (DR) region was amplified with a pair of primers, and the resulting PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were immobilized on the membrane. Spoligotyping data were analyzed using the SITVIT database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) to determine the spoligotype international type (SIT) (Demay et al., 2012).

MIRU-VNTR typing

MIRU-VNTR typing was performed by amplifying 24 loci, including 12 MIRU loci (MIRU2, MIRU4, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU26, MIRU27, MIRU31, MIRU39, and MIRU40), four exact tandem repeat (ETR) loci (ETR-A, ETR-B, ETR-C, and ETR-F), four Queens University Belfast (QUB) loci (QUB11a, QUB11b, QUB26, and QUB4156), and four VNTR loci (VNTR424, VNTR1955, VNTR2401, and VNTR3690), as described by Supply et al. (2006).

Sequencing of drug resistance-associated genes

Isolates clustered by a combined analysis of spoligotyping and MIRU-VNTR typing were analyzed further by sequencing of the drug resistance-associated genes, i.e. rifampicin resistance determining region (RRDR) in *rpoB* for RIF resistance and *katG* coding and *inhA* promoter regions for INH resistance, as described previously (Poudel et al., 2012).

Data management and analysis

Demographic data including age, sex, and treatment history for TB were analyzed using IBM SPSS Statistics version 19.0 (IBM Corp., Armonk, NY, USA) and PRISM version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Individual and cumulative Hunter Gaston Discriminatory Indices (HGDI) were calculated to determine the discriminatory power of each MIRU-VNTR locus and overall loci (Hunter and Gaston, 1988). The discriminatory power of each locus was considered high (HGDI > 0.6), moderate ($0.3 \leq$ HGDI ≤ 0.6), or poor (HGDI < 0.3), as suggested by Sola et al. (2003). A cluster was defined as two or more isolates sharing an identical spoligotype and MIRU-VNTR pattern, and the clustering rate was calculated using the formula 'number of clustered isolates'/'total number of isolates' (Glynn et al., 1999). A phylogenetic tree was constructed by unweighted pair group method with arithmetic mean (UPGMA) using an online MIRU-VNTRplus Web-based application (http://www.miru-vntrplus.org) (Weniger et al., 2010).

Results

Demographic information

Age, sex, and treatment history information of patients from whom 145 CAS and 456 non-CAS MDR isolates were obtained were