Infection, Genetics and Evolution 27 (2014) 6–14

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Multi-drug resistant *Mycobacterium tuberculosis* complex genetic diversity and clues on recent transmission in Punjab, Pakistan

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ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form 14 June 2014
Accepted 17 June 2014
Available online 27 June 2014

Keywords:
Rifampin
Genotypic drug susceptibility testing
MDR-TB
Molecular epidemiology

ABSTRACT

Multi-Drug Resistant Tuberculosis (MDR-TB), *i.e.* bacilli resistant to rifampicin (RIF) and isoniazid (INH), is a major Public Health concern in Pakistan according to WHO estimates (3.5% and 32% of new and retreated cases, respectively). Previous Pakistanis reports identified a correlation between being MDR and belonging to Beijing or EAI lineages in one study, and belonging to "H4"-Ural Euro-American sublineage in another study. In addition, MDR-TB transmission was suspected in Karachi.

We tested MDR characteristics on a Punjab sample of 278 clinical isolates (without selection for Multi-Drug Resistance) including new and retreated cases collected from 2008 to 2012. All samples were characterized by a new, microbead-based method named "TB-SPRINT" (molecular diagnostic including spoligotype identification, and genetic resistance determinants to first-line anti-TB drugs RIF and INH). Isolates from 2011 to 2012 ($n = 100$) were further analyzed using 24-loci MIRU-VNTR.

We detected 8.7% MDR isolates ($CI_{95\%} = [5.0; 12.5]$), mainly among CAS lineage that predominates in this central-East region of Pakistan. Out of 20 MDR-TB cases, 12 different TB-SPRINT profiles were identified, limiting the suspicion of MDR-TB transmission. 24 MIRU-VNTR confirmed the unrelatedness of isolates with different TB-SPRINT profiles and discriminated 3 isolates with identical TB-SPRINT profiles.

In conclusion, our study did not confirm any of the correlations between Multi-Drug Resistance and lineage or sublineage in Punjab, Pakistan. MDR-TB isolates were diverse indicating that transmission is not pervasive. TB-SPRINT proved useful as a first step for detecting MDR-TB likely transmission events, before more extensive genotyping such as 15 or 24 MIRU-VNTR and thorough epidemiological investigation.

1. Introduction

Pakistan is a large and populated country (area: 796,095 km^2 and population: 179.2 million) where tuberculosis (TB) has been a public health concern for a long time. It resides westward to India, the country with highest estimated TB cases *i.e.* 2–2.4 million (WHO, 2013). With a current estimated incidence rate of 231/100,000 ($CI_{95\%} = [190; 276]$), and mortality burden of 62,000 ($CI_{95\%} = [27{,}000; 110{,}000]$), Pakistan has unfortunately moved three points higher from 8th to 5th rank in the list of 22 high TB burden countries of the world in 4 years (WHO, 2009, 2013). The situation is further compounded by drug resistance to the two first-line antibiotics, rifampin (RIF) and isoniazid (INH), which defines multidrug-resistant tuberculosis (MDR-TB). According to WHO, estimated rate of MDR-TB in Pakistan is 3.5% ($CI_{95\%} = [0.1; 12]$) in newly diagnosed patients and 32% in retreated cases ($CI_{95\%} = [7.5; 56]$) (WHO, 2013). Confidence intervals are, however, very large as thorough nationwide study is ongoing but has not yet been completed. These figures stress the need to trace the tuberculosis transmission dynamics and frequency of MDR in the country.

Standard genotyping for molecular epidemiology of *Mycobacterium tuberculosis* complex (MTBC) includes 15 or 24 MIRU-VNTR loci (Supply et al., 2001, 2006) and spoligotyping *i.e.* the detection of 43 spacers in the clustered regularly interspaced short palindromic repeats (CRISPRs) region (Lawson et al., 2012). MIRU-VNTR typing serves potential outbreak investigations, elucidation of

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http://dx.doi.org/10.1016/j.meegid.2014.06.017
1567-1348/

complex situations like mixed infections, laboratory cross contamination, and re-infection (Alonso-Rodriguez et al., 2008; Narayanan et al., 2010) while spoligotyping, much less discriminating locally, is mainly used as a first-line assay for global genetic diversity and phylogeographical purposes (Brudey et al., 2006).

The growing threat of MDR-TB and its effect on tuberculosis control measures has led to the development of robust multiplexed genotyping and resistance diagnostic tools like spoligoriftyping and TB-SPRINT (Gomgnimbou et al., 2013, 2012). These high throughput microbead-based assays have a number of advantages; they simultaneously provide: (1) spoligotype (genotype at CRISPR locus), (2) RIF and INH prospective genetic susceptibility of tuberculosis isolates with excellent sensitivity and specificity (Gomgnimbou et al., 2013).

The genetic diversity and transmission dynamics of MTBC in Pakistan have partly been reported before (Ali et al., 2007; Ayaz et al., 2012; Hasan et al., 2010, 2006; Tanveer et al., 2008). Tanveer et al. (2008) reported the genetic diversity of isolates collected countrywide between 2003 and 2005. They described the predominance of CAS1 clade especially in Punjab, in addition to the tendency for EAI and Beijing lineages to harbor MDR. Ayaz et al. (2012) identified in contrast Haarlem as associated with MDR and Hasan et al. (2010) found that extensively resistant isolates (XDR) were mostly CAS isolates in proportions similar to global diversity. Ali et al. (2007) focused on MIRU-VNTR diversity among CAS isolates and identified new MIRU-VNTR alleles as compared to those described in worldwide databases, suggesting a higher diversity of CAS isolates as compared to other countries.

The present study uncovering 278 isolates investigates recent TB-diversity in Punjab, making use for the first time of 24 MIRU-VNTR loci on 2011 and 2012 isolates in Pakistan. We show that CAS lineage predominates in Punjab, exhibiting allele diversity in the range of what could be expected according to worldwide studies, and that MDR-TB transmission is not highly prevalent in Pakistan.

2. Methods

2.1. Mycobacterial isolates

The isolates included in this study form a convenient sample of 278 *M. tuberculosis* culture isolates out of a recent global analysis of TB diversity in Pakistan (Yasmin, unpublished work). These isolates were collected by 3 different local hospitals of Punjab from different patients (one sample/patient). Species identification was performed phenotypically by local hospitals and was subsequently confirmed by successful genotyping analysis as presented in this study, targeting *M. tuberculosis* complex loci such as CRISPR. All samples represent unbiased collections of circulating isolates as the unavailability of treatment history and reliable phenotypic drug susceptibility data impeded any selection. One hundred ninety-nine isolates ($n = 199$) were obtained from Rawalpindi, a city located in the suburbs of Islamabad, so that we refer to this sample as "Islamabad pole" ($n = 115$ in 2008, $n = 19$ in 2010, $n = 65$ in 2011), and 79 from Lahore–Faisalabad pole (72 from Lahore, $n = 43$ in 2009, $n = 9$ in 2011, and $n = 20$ in 2012; 7 from Faisalabad, $n = 1$ in 2010, $n = 6$ in 2011).

Cetyl-trimethyl-ammonium-bromide (CTAB) DNA (van Soolingen et al., 1991) or crude thermolysates DNAs (three cycles of freeze and thaw followed by centrifugation and removal of cell pellets) were prepared in our central laboratory at Faisalabad. All the DNA samples were stored at −20 °C and shipped under dry ice to the French laboratory for further characterization.

2.2. Genotyping methods

High-throughput TB-SPRINT was performed on all samples on a Luminex 200® flow cytometry device (Luminex Corp, Austin, TX) as previously described (Gomgnimbou et al., 2013, 2012). This technique provides, simultaneously or separately according to experimenter choice, spoligotype profile along with the mutations most commonly conferring rifampin (*rpoB* Hot spot region coverage) and isoniazid resistance (*katG*, *inhA*). Here, both approaches (separate and combined analyses) were applied, the partial analyses being lately complemented as sufficient funding could be obtained. Standardized 24 loci MIRU-VNTR analysis was performed on 100 isolates and succeeded for 95 isolates with sufficient DNA quality and quantity (Faisalabad, $n = 6$; Lahore, $n = 25$; Rawalpindi $n = 64$). Amplicon's length was analyzed on agarose gels according to in-house developed duplex format (G. Refregier, unpublished data) that obtained excellent performance in international proficiency testing studies (de Beer et al., 2012). Numerical data were combined in a Microsoft Excel spreadsheet file and uploaded to Bionumerics software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). The genetic diversity was laid out as a minimum spanning tree (MST) using default settings in Bionumerics. The Shared International Types (SIT) corresponding to spoligotype patterns were identified using the International SITVITWEB database available at http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/. Assignation to clades was performed by MIRU-VNTR*plus* using best-match assignation tool (Weniger et al., 2012) taking into account all genotyping information available: spoligotype patterns and/or MIRU-VNTR profile. The output was occasionally translated by a specialist to conform to SITVITWEB denominations.

2.3. Statistical analyses

Allelic diversity of each MIRU-VNTR locus was calculated using Hunter and Gaston Discriminatory Index (HGDI) (Hunter and Gaston, 1988) and loci were designated as highly (HGDI > 0.6), moderately (HGDI 0.3–0.6) and poorly discriminatory (HGDI < 0.3). Homogeneity tests were performed using Chi^2 test and correlation analyses using Pearson correlation test in R (www.r-project.org).

3. Results

3.1. Genetic diversity of MTBC in Punjab as assessed by spoligotype and MIRU-VNTR profiles

Out of 278 isolates screened by TB-SPRINT (Islamabad pole-Rawalpindi-, $n = 199$; Lahore–Faisalabad pole, $n = 79$, Fig. 1), 217 (78%) gave interpretable result for 43-spacers spoligotype patterns. The relatively high number of failed analyses ($n = 61$) was mainly due to loss of DNA quality during sample storage for 2008 isolates from Rawalpindi ($n = 59$ out of 115 samples from this period). As this pole was largely sampled, 140 isolates were successfully typed which was sufficient for genetic diversity assessment. When excluding this subset of samples with poorer DNA quality and quantity, sensitivity of spoligotyping was above 98% ($n = 161$ out of 163). Altogether, 69 different spoligotypes were identified, only 47 (68%) of which were already described in the most recent database SITVITWEB (Demay et al., 2012). Out of 100 isolates from 2011 to 2012 from which sufficient DNA could be retrieved, 95 isolates were successfully genotyped at 24 MIRU-VNTR classical loci (Supply et al., 2006) (Faisalabad + Lahore $n = 31$, Rawalpindi $n = 64$).

Lineage assignation was performed by MIRU-VNTR*plus* best-match labeling using 24 MIRU-VNTR and/or spoligotype depending on availability, and using human expert interpretation of spoligotype signatures. We were able to assign a lineage to 221 isolates with either spoligotype pattern and/or 24 MIRU-VNTR pattern (Islamabad pole, $n = 144$; Lahore–Faisalabad pole, $n = 77$, Supplementary file S1). Global population structure of MTBC in Punjab consisted