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Research paper

Genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* isolates in Punjab, Pakistan

Zofia Bakuła[a], Hasnain Javed[b], Małgorzata Pleń[a], Nazia Jamil[b], Zarfishan Tahir[c], Tomasz Jagielski[a,*]

[a] Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Poland
[b] Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan
[c] Provincial TB Control Program, Lahore, Pakistan

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ABSTRACT

Pakistan ranks 5th among the world's highest tuberculosis (TB) burden countries and 6th among the countries with the highest prevalence of drug-resistant TB. However, insufficient data are available on the genetic structure of *M. tuberculosis* strains circulating in this country. The objective of this study was to explore the genetic diversity of multidrug-resistant *M. tuberculosis* isolates from Punjab, Pakistan with a combination of spoligotyping and 24-loci MIRU-VNTR typing.

Among a total of 127 MDR isolates studies, 53 spoligotypes were obtained, split into 14 clusters ($n = 88$, 69.3%, 2–29 isolates per cluster) and 39 (30.7%) unique patterns. At the phylogenetic level, the most prevalent sublineage was CAS1_DELHI ($n = 53$, 41.7%), mostly represented by ST 1942 ($n = 29$, 22.8%), followed by T1 ($n = 14$, 11%) and Beijing ($n = 10$, 7.8%). The remaining nine sublineages (CAS, MANU2, EAI5, T2, LAM10_CAM, H1, X1, H4 and CAS2) involved altogether 24 (18.9%) isolates. Twenty-six (20.5%) isolates could not be assigned to any specific clade. MIRU-VNTR typing identified 123 (96.8%), 97 (76.4%) and 65 (51.2%) unique types with a tolerance of 0, 1, and 2 locus differences between the patterns.

Upon combined spoligotyping and MIRU-VNTR typing analysis, 123 (96.8%), 108 (85%), and 91 (71.7%) unique types were identified if a tolerance of 0, 1, and 2 locus differences in the MIRU-VNTR patterns was assumed, respectively. Based on the clustering results, the transmission rate for MDR-TB cases under the study was calculated at 3.2%, 15%, and 28.3%.

Overall, three clades, namely CAS1_DELHI, T1, and Beijing accounted for the majority of MDR-TB cases in Pakistan. Up to a third of the cases were clustered upon combined spoligotyping and MIRU-VNTR typing, suggesting a moderate level of active transmission.

1. Introduction

Tuberculosis (TB) remains one of the deadliest diseases in the world, affecting approximately 10 million people every year (WHO, 2018). One of the reasons for the ongoing TB prevalence is the emergence of drug-resistant (DR) *Mycobacterium tuberculosis* strains, particularly those multi-drug resistant (MDR), defined as resistant to at least isoniazid (INH) and rifampicin (RIF), and extensively drug-resistant (XDR), defined as MDR with additional resistance to a fluoroquinolone (FQ) and a second-line injectable drug (SLID) (WHO, 2018).

Pakistan, populated by over 212 million people (Pakistan Bureau of Statistics; www.pbscensus.gov.pk) ranks 5th among the world's highest TB burden countries, with an estimated half million new TB cases per year. This number accounts for > 60% of the total TB burden in the WHO Eastern Mediterranean Region (WHO, 2017). Globally, Pakistan has the 6th and 4th highest prevalence of DR- and MDR-TB, respectively (WHO, 2017). The incidence of DR-TB in Pakistan has been aggravated by delays in diagnosis, unsupervised, inadequate drug regimens and poor patient adherence and follow-up (Braham et al., 2018). Furthermore, only very few large-scale, nation-wide TB epidemiological studies are available (Hasan et al., 2006; Tanveer et al., 2008; Hasan et al., 2010; Ali et al., 2014). This has been mainly attributed to the developing status of the country, coupled with low expenditures on TB research and surveillance.

* Corresponding author at: Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, I. Miecznikowa 1, 02-096 Warsaw, Poland.
E-mail addresses: zofiabakula@biol.uw.edu.pl (Z. Bakuła), m.plen@student.uw.edu.pl (M. Pleń), t.jagielski@biol.uw.edu.pl (T. Jagielski).

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Currently, the two most widely used methods in *M. tuberculosis* genotyping are spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive unit – variable-number tandem-repeat (MIRU-VNTR) typing. Both these methods are PCR-based and target repetitive loci in the *M. tuberculosis* genomes (Jagielski et al., 2016). Whereas spoligotyping, with a much less discriminatory potential, has been mainly used for phylogenetic and evolutionary purposes (Abadia et al., 2010; Lopes et al., 2013; Rasoahanitralisoa et al., 2017), MIRU-VNTR typing has been decisive for disclosing sources of infection, quantifying recent transmission and delineating transmission paths within different populations studied (Cowan et al., 2012; Jagielski et al., 2015; Hamblion et al., 2016; Teeter et al., 2017).

In this study, spoligotyping and 24-loci MIRU-VNTR typing were applied to characterize the epidemiological landscape of MDR-TB in Punjab, Pakistan.

2. Materials and methods

2.1. Isolates

The study sample was selected out of a collection of 3056 *M. tuberculosis* isolates. The isolates represented as many TB patients admitted to different tertiary care hospitals between January 2013 and June 2015 and originated from seven major cities of the Punjab province of Pakistan. Only isolates defined as MDR upon 1st-line drug susceptibility testing were included for the study. Thereby, the final study sample of 127 isolates was achieved.

The isolates were recovered from sputa of 65 (51.2%) males and 62 (48.8%) females (age range, 15 to 80 years; mean age, 32.6 ± 13.2 years; 35.8 ± 12.9 for males and 29.2 ± 12.8 for females).

Primary isolation, culturing, and species identification were performed with standard mycobacteriological methods in Provincial TB Reference Laboratory, Institute of Public Health, Lahore, as described earlier (Javed et al., 2018).

This study was carried out in accordance with the recommendations of ethical policy of the University of Punjab.

2.2. Drug susceptibility testing

Conventional drug susceptibility testing (DST) was performed using the standard 1% proportion method on the Löwenstein-Jensen (L-J) medium, following the WHO recommendations (WHO, 2008). The *M. tuberculosis* H37Rv reference strain was used as a quality control.

The critical concentrations for specific drugs were as follows: INH, 0.2 mg/L; RIF, 40 mg/L; EMB, 2 mg/L; STR, 4 mg/L; KAN, 30 mg/L; AMK, 30 mg/L; CAP, 40 mg/L; and OFX, 4 mg/L (WHO, 2008).

XDR-TB was defined as MDR-TB with additional resistance to OFX and one of the SLIDs. The pre-XDR phenotype was defined as MDR with either resistance to ofloxacin (OFX) or any of the SLIDs (i.e. AMK, KAN or CAP) (Banerjee et al., 2008; World Health Organization, 2018).

2.3. DNA extraction

Isolation and purification of chromosomal *M. tuberculosis* genomic DNA was done using the cetyl-trimethyl ammonium bromide (CTAB) method, as described elsewhere (van Embden et al., 1993). The purified DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) and quantified with the NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, USA). The DNA samples were diluted to the required concentration (10 ng/μL) and stored at −20 °C until used.

2.4. Spoligotyping

Forty-three spacer spoligotyping was performed with the Spoligotyping Kit according to the manufacturer's instructions (Ocimum Biosolutions, Hyderabad, India) with a PCR Master Mix (TopTaq, Qiagen, Hilden, Germany) and 20 ng (2 μL) of the template DNA per sample.

The *M. tuberculosis* H37Rv and *M. bovis* BCG reference strains were used as quality controls in each run. Spoligotype shared types (ST) and phylogenetic clades (sublineages and families) were assigned according to the SITVIT WEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) housed by the Institut Pasteur de Guadeloupe (Demay et al., 2012). Spoligotypes not assigned to any clades with the SITVIT WEB database, were compared with the MIRU-VNTR*plus* database (Allix-Béguec et al., 2008).

Furthermore, isolates were analyzed with the newly released SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/) (Couvin et al., 2018).

2.5. MIRU-VNTR typing

MIRU-VNTR analysis was carried out at 24 loci, with primers and amplification conditions essentially as described previously (Supply et al., 2006). For PCR set-up, reagents from the PCR Master Mix (TopTaq, Qiagen, Hilden, Germany) were used.

Twenty-four-digit MIRU-VNTR codes were analyzed by MIRU-VNTR*plus* software (Allix-Béguec et al., 2008).

The allelic diversity of each MIRU-VNTR locus was calculated with the Hunter and Gaston discriminatory index (HGDI), using the following formula:

$$DI = 1 - \left[\frac{1}{N(N-1)}\right]\sum nj(nj-1),$$

where N is the total number of isolates, and nj is the number of isolates representing each type (Hunter and Gaston, 1988). Each locus was designated as being: (i) highly discriminatory (HGDI > 0.6), (ii) moderately discriminatory (0.3 ≤ HGDI ≤ 0.6) or (iii) poorly discriminatory (HGDI < 0.3) (Sola et al., 2003).

The genetic diversity index (GDI) for each typing method was calculated as a quotient of the total number of genetic patterns to the total number of isolates.

2.6. Dendrogram and minimum spanning tree construction

A dendrogram was constructed based on combined spoligotyping and MIRU-VNTR typing data, by using MIRU-VNTR*plus* software and UPGMA algorithm (Allix-Béguec et al., 2008).

A minimum spanning tree (MST) was drawn based on the spoligotyping data by using MIRU-VNTR*plus* software (Allix-Béguec et al., 2008). The maximum difference allowed within a MST complex was a change at two probes.

2.7. Cluster definition

A spoligotyping cluster was defined as two or more isolates sharing identical spoligotypes (identical ST designation in the SITVIT database). A MIRU-VNTR cluster was defined as two or more isolates sharing identical 24-loci VNTR typing profile. However, since stability of the analyzed loci was also taken into consideration, more relaxed cluster definitions were also assumed, allowing for one or two repeat/locus differences in the spoligotyping/MIRU-VNTR patterns (Li et al., 2018).

The final clustering was based on a combination of spoligotyping and MIRU-VNTR typing results. Accordingly, a cluster was defined as two or more isolates with identical spoligotypes and 24-loci VNTR typing profiles (with a tolerance of 0, 1 or 2 locus differences between the MIRU-VNTR patterns).

Clustering rate (CR) was defined as a percentage of clustered isolates among all isolates genotyped.

2.8. Statistical analysis

Data were analyzed with the $\chi 2$ test using Excel spreadsheets (Microsoft, Redmond, USA). A P value of 0.05 or less was considered statically significant.

3. Results

3.1. Drug susceptibility testing

Complete DST patterns of isolates under the study are provided in Supplementary Table 1. Three-fourths (n = 93; 73.2%) of the isolates met the definition of pre-XDR-TB. The most common drug resistance profile was INH + RIF + OFX + STR + EMB (n = 53; 41.7%), followed by INH + RIF + OFX + EMB (n = 21; 16.5%). Almost every tenth (n = 16; 12.6%) isolate was categorized as XDR. Most (n = 10; 62.5%) of such isolates were MDR with an extra resistance to OFX, STR, KAN, AMK, CAP, and EMB.

3.2. Spoligotyping

Out of 127 isolates spoligotyped, 53 distinct patterns were observed (GDI = 0.42) (Table 1, Fig. 1). Approximately one-third (n = 39; 30.7%) of the isolates were represented by a unique pattern, whereas the others (n = 88; 69.3%) were split into 14 clusters (2–29 isolates per cluster).

When a single or double spoligotyping probe variation was tolerated between any two patterns, 113 (CR = 89%) and 123 (CR = 96.8%) isolates were clustered, respectively.

Upon comparison with the SITVIT database, 26 (20.5%) of the isolates could not be assigned to any shared type (ST) described in the spoligotype database (Table 1). Half (n = 52; 51.5%) of the remaining 101 isolates, were assigned to three major STs i.e. ST 1942 (n = 29; 22.8%), ST 26 (n = 12; 9.4%) and ST 53 (n = 11; 8.6%). Phylogenetically, all isolates were classified into eight main lineages and 12 sub-lineages (Table 1). The most abundant families were CAS, T, and Beijing, accounting for 62 (48.8%), 16 (12.6%) and 10 (7.8%) of the isolates, respectively. The remaining five lineages (MANU, EAI, LAM, H, and X) involved 13 (10.2%) isolates.

The spoligotype-derived MST was composed of six major branches (Fig. 2). The largest Complex 1 (n = 67) essentially contained isolates from the CAS family (n = 58) or orphan types (n = 9). The Complex 2 (n = 24) included, apart from the orphan types (n = 3), isolates from the T (n = 16), MANU (n = 4), and X (n = 1) families. The Complex 3 (n = 7) included isolates from the CAS family (n = 3) and orphan types (n = 4) whereas the Complex 5 (n = 2) of Haarlem family (n = 1) and orphan type (n = 1). The other two branches included isolates of the Beijing (Complex 4, n = 10) or orphan type (Complex 6, n = 2). There were 15 singletons of the EAI (n = 3), H (n = 2), LAM (n = 2), CAS (n = 1) families, and and orphan types (n = 7). The genotype ST 1942 (CAS) had a central position in the MST.

Twenty-six (20.5%) isolates, that could not be assigned to any specific lineage in the SITVIT database, were further analyzed with the MIRU-VNTR*plus* database. For 17 (65.4%) isolates, the nearest possible lineage was Dehli/CAS subfamily, while the remaining isolates were identified as representing the EAI (n = 2; 7.7%), Uganda I (n = 2; 7.7%), URAL (n = 2; 7.7%), Cameroon (n = 1; 3.8%), Haarlem (n = 1; 3.8%), and S (n = 1; 3.8%) families (Supplementary Table 2).

The spoligotypes were further analyzed with the newly released SITVIT2 database and 13 (10.2%) different designations were noted when compared with SITVIT (Table 1). Whereas five orphan types were assigned to either CAS (n = 2), Manu1 (n = 2) or Manu2 (n = 1) clades, eight isolates were reassigned (CAS to Cas1-Delhi, n = 3; T2 to T1, n = 2; CAS to Cas1-Kili, n = 1; EAI5 to EAI3-IND, n = 1; H4 to Ural-1, n = 1).

3.3. MIRU-VNTR typing

All isolates were subjected to 24-locus MIRU-VNTR typing (Fig. 1). Different VNTR loci showed different allelic variabilities (Table 2). All isolates had two alleles present at MIRU 154. Thus, for this locus the HGDI was the lowest (HGDI = 0). The highest level of variation was evidenced for MIRUs 960 and 4052, with 10 allelic types. For these two loci, the HGDIs were of 0.78 and 0.73, respectively.

The vast majority of isolates (n = 123; 96.8%) had unique MIRU-VNTR profiles, yielding a high genetic diversity (GDI = 0.97). Only four (n = 4, 3.2%) isolates were clustered (CR = 3.2%), with two isolates per cluster. However, when a single or double MIRU-VNTR locus variation was tolerated between any two patterns, 30 (CR = 23.6%) and 62 (CR = 48.8%) isolates were clustered, respectively.

3.4. Combined analysis

A combined spoligotyping and MIRU-VNTR analysis resolved 123 (96.8%) unique patterns (GDI = 0.97). The only two clusters comprised four isolates, producing the overall CR of 3.2%.

Clusters of isolates with identical MIRU-VNTR and spoligotyping profiles would have kept their size, even if a tolerance of 1–2 repeat difference had been assumed. However, the CR increased importantly if spoligotyping profiles were identical, and one or two locus differences in the MIRU-VNTR patterns were allowed. The CRs were calculated at 15% (19 isolates clustered) and 28.3% (Chawla et al., 2018), respectively.

4. Discussion

Since the 1990s, when molecular typing methods have become available for the mycobacteriologists, a wide array of advanced tools was developed, allowing for better understanding the key issues in the epidemiology of TB. However, large-scale, molecular epidemiological studies, have only sporadically been performed in low and lower-middle income countries. The present study provides an insight into the genetic diversity of MDR *M. tuberculosis* strains circulating in Pakistan, one of the world's hot-spots for TB, including DR-TB.

At the phylogenetic level, the Central Asian Strain (CAS) family (48.8%) predominated among the isolates under the study. This is in line with previous studies from Pakistan, in which the CAS lineage accounted for similar proportion of isolates (Table 3). This lineage has also been repeatedly identified as one of the major families in countries neighboring Pakistan, such as India (northern part) and Iran (Table 3). In China, the CAS family, has almost exclusively been found in provinces which border India and Pakistan (Dong et al., 2012; Liu et al., 2017). Strains of the CAS family are hypothesized to have originated in Central and Middle Eastern Asia (Brudey et al., 2006).

The second largest phylogenetic group, comprising 12.6% of the analyzed isolates, displayed spoligotypes related to the T family. This lineage contains ubiquitous spoligotypes with ill-defined genealogies (Demay et al., 2012). According to previous reports, the frequencies of the T family isolates were somewhat lower in Pakistan and India yet higher in Iran (Table 3).

Finally, 7.9% of the studied isolates, represented the Beijing family. The frequencies of this family among TB isolates in Pakistan varied in past studies, ranging from 3% to 8.8% (Table 3). Similar numbers have been documented in India and Iran (Table 3). The prevalence of the Beijing family is remarkably high on the Asian continent, particularly in northern China (ca. 90%) (Wan et al., 2011; Yin et al., 2016). The Beijing strains are of high epidemiological importance since they have been associated with an increased acquisition of drug resistance, enhanced virulence, and high transmissibility (Jagielski et al., 2016).

In general, the CAS, T, and Beijing clades, continue to have the dominant role in shaping the genetic structure of the *M. tuberculosis* population in Pakistan. The three lineages represented more than two-

Table 1
Spoligotype-based diversity and SITVIT/SITVIT2 database comparison of 127 *M. tuberculosis* isolates under the study.

SITVIT		SITVIT2*	Spoligotype description		No. of isolates (%)	Total (%)
Subfamily (family)	ST	Subfamily (ST)	Octal	Binary		
CAS1_DELHI (CAS)	1942		703377740003771	■■■□□□□■■□■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	29 (22.8%)	53 (41.7%)
	26		703777740003771	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	12 (9.4%)	
	141		703767740003771	■■■□□□□■■■■■■■□■■■■■■■□□□□□□□□□□□□■■■■■■■■■	5 (3.9%)	
	1314		703377740003171	■■■□□□□■■□■■■■■■■■■■■■□□□□□□□□□□□□■■□□■■■■■	4 (3.1%)	
	428		703777740003371	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■□■■■■■■	1 (0.8%)	
	1343		703737740003771	■■■□□□□■■■■■□■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	1 (0.8%)	
	1091		703777740002771	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■□■■■■■■■	1 (0.8%)	
T1 (T)	53		777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	11 (8.6%)	14 (11%)
	154		757777777760771	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1 (0.8%)	
	334		577777777760771	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1 (0.8%)	
	2496		777367777760771	■■■■■■■■■□■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1 (0.8%)	
Beijing (Beijing)	1		000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	6 (4.7%)	10 (7.8%)
	621		000000000002771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■□■■■■■■■	4 (3.1%)	
CAS (CAS)	1151		700377740001771	■■■□□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■	3 (2.4%)	8 (6.3%)
	1422	CAS1_Delhi	703777000000771	■■■□□□□■■■■■■■■■■■□□□□□□□□□□□□□□□□□□■■■■■■■	2 (1.6%)	
	2419		703000000000371	■■■□□□□■■□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■	1 (0.8%)	
	2726	CAS1_Delhi	703777600000771	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■	1 (0.8%)	
	2391	CAS1-Kili	703377400003771	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■■■■	1 (0.8%)	
MANU2 (MANU)	54		777777777763771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	4 (3.1%)	
EAI5 (EAI)	126		477777777413771	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	2 (1.6%)	3 (2.4%)
	355	EAI3-IND	477777777413031	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	1 (0.8%)	
T2 (T)	118	T1	777767777760771	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	2 (1.6%)	
LAM10_CAM (LAM)	61		777777743760771	■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■■■■	2 (1.6%)	
H1 (H)	47		777777774020771	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	2 (1.6%)	
X1 (X)	221		777766777760771	■■■■■■■■■■■■■■□■■□■■■■■■■■■■■■■■□□□□■■■■■■■	1 (0.8%)	
H4 (H)	777	Ural-1	777777777420771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	1 (0.8%)	
CAS2 (CAS)	288		700377740003771	■■■□□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	1 (0.8%)	
Unique patterns			703767740003031	■■■□□□□■■■■■■■□■■■■■■■□□□□□□□□□□□□■■□□□□■■■	26 (20.5%)	
			777767677413731	■■■■■■■■■■■■■■□■■■■■□■■■■■■■□□□□■□■■■■■□■■■		
		Manu1	777767777773771	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□■■■■■■■■■		
			737177777760771	■■■□■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■		
		CAS (2756)	703777700000171	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□■■■■■		
			743777740003771	■■■■□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■		
			700377740000371	■■■□□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■		
		CAS	703777600000371	■■■□□□□■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□■■■■■■		
			703775000000771	■■■□□□□■■■■■■■■■□■□□□□□□□□□□□□□□□□□□■■■■■■■		
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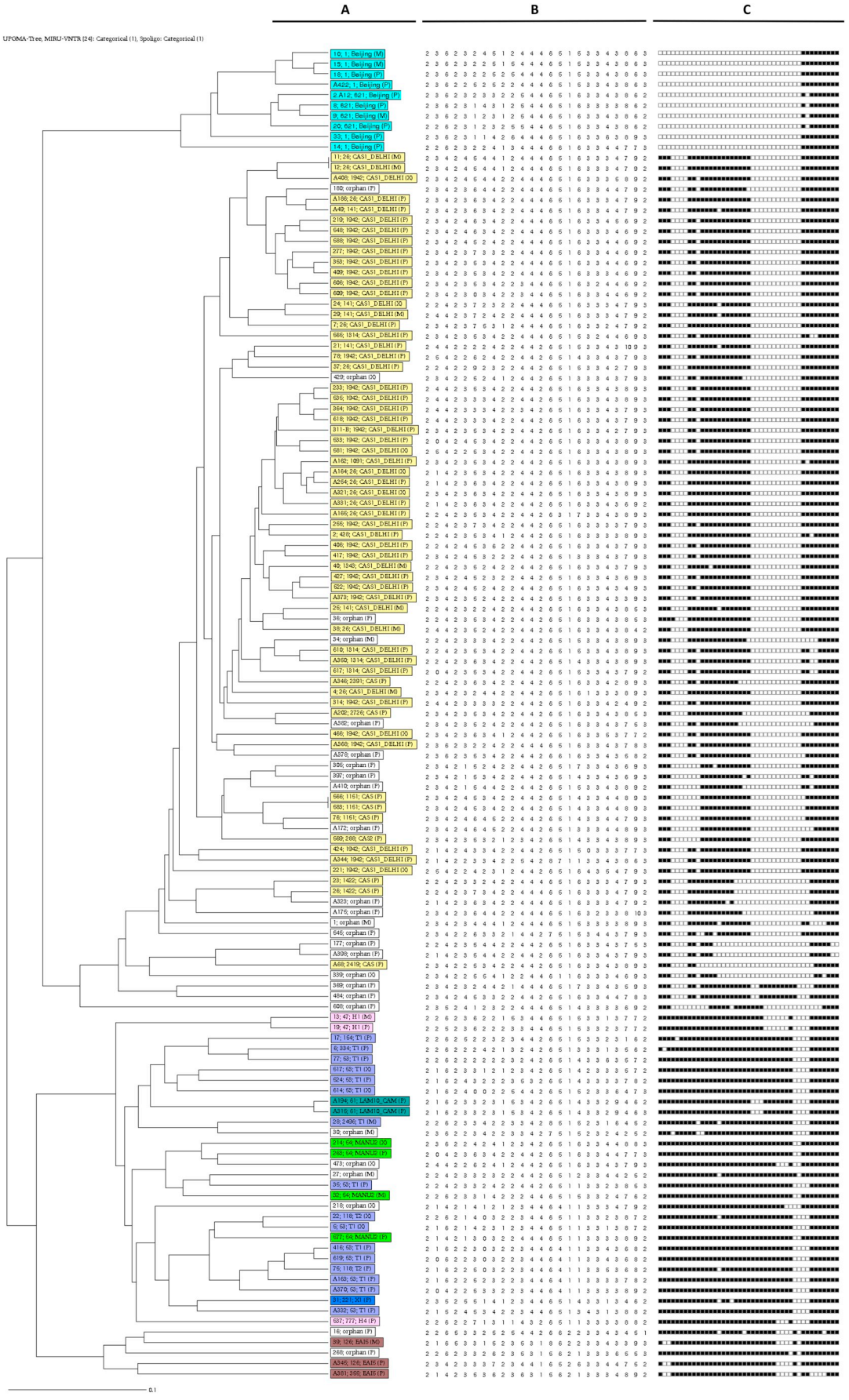
TOTAL					127 (100%)	

*Only differences in the designations between SITVIT and SITVIT2 databases were noted.

thirds (69.3%) of the isolates in this work and similar percentage (63.3–73.7%) of isolates from previous Pakistani studies (Hasan et al., 2006; Tanveer et al., 2008; Hasan et al., 2010; Ali et al., 2014).

Neither of the family was specifically associated with MDR, pre-XDR or XDR TB phenotype ($P > .05$). However, the CAS family accounted for a slightly higher proportion of the pre-XDR- (51.6%) than XDR- (43.8%) and MDR-TB (38.9%) isolates (Supplementary Table 3). The high prevalence of the CAS family among DR-TB has also been observed in other studies. Strains of this clade predominated, yet without statistical significance, among XDR- and MDR-TB isolates from Pakistan (Hasan et al., 2006; Tanveer et al., 2008; Hasan et al., 2010; Ali et al., 2014) or MDR-TB isolates from India (Dhatwalia et al., 2016). In this study, the Beijing family was more prevalent among MDR- than pre-XDR-, and XDR-TB isolates (16.7% vs 7.5% vs 0%) (Supplementary Table 3). Several studies from across the world, including Pakistan, have documented the association between the Beijing genotype and MDR phenotype (Almeida et al., 2005; Hasan et al., 2006; Tanveer et al., 2008; Farnia et al., 2006).

At the level of individual STs, the most prevalent was ST 1942 (CAS), with every fourth (22.8%) isolate assigned to this type. Interestingly, in previous studies from Pakistan, this type was absent. The most common was ST 26, representing as much as 47% of drug-susceptible (Hasan et al., 2006), 40.5% of MDR (Hasan et al., 2006), and 42.1% of XDR (Hasan et al., 2010) *M. tuberculosis* isolates from Pakistan (Hasan et al., 2006; Tanveer et al., 2008; Hasan et al., 2010). The STs 1942 and 26 differ only by spacer 10 (lacking in ST 1942). The



(caption on next page)

Fig. 1. Genotyping results for 127 *M. tuberculosis* isolates under the study. A, isolate ID; ST (Shared Type); subfamily; (susceptibility profile; M, MDR; P, pre-XDR; X, XDR) B, MIRU-VNTR typing pattern; C, spoligotyping pattern. Colors on the dendrogram denote spoligotype families. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed change in the frequencies of the two STs may reflect the proneness of ST 26 to a deletion of the 10 direct variant repeat within the DR locus and/or the spread of ST 1942 from outside Pakistan, most probably from India, where recently, every tenth isolate (10.2%) has been described as ST 1942 (Chawla et al., 2018). However, any epidemiological links to India could not be identified.

Finding single isolates belonging to such types as ST 61 (LAM) (n = 2) and ST 221 (X) (n = 1), characteristic mainly for the African continent (Demay et al., 2012; Uzoewulu et al., 2016; Titanji and Assam, 2016; Maguga-Phasha et al., 2017), and ST 47 (n = 1), and never observed in Pakistan before, may suggest these cases have been imported from outside the country.

Numerous studies, including those from Central and Middle Eastern Asia have estimated the proportion of TB transmission based on clustering of strains' MIRU-VNTR profiles, with a cluster defined as strains sharing identical profiles (Mears et al., 2015). When using the same approach, the transmission rate (TR) in this study was calculated at 3.2%, and 0–9% among MDR-TB isolates in India, Pakistan and Iran

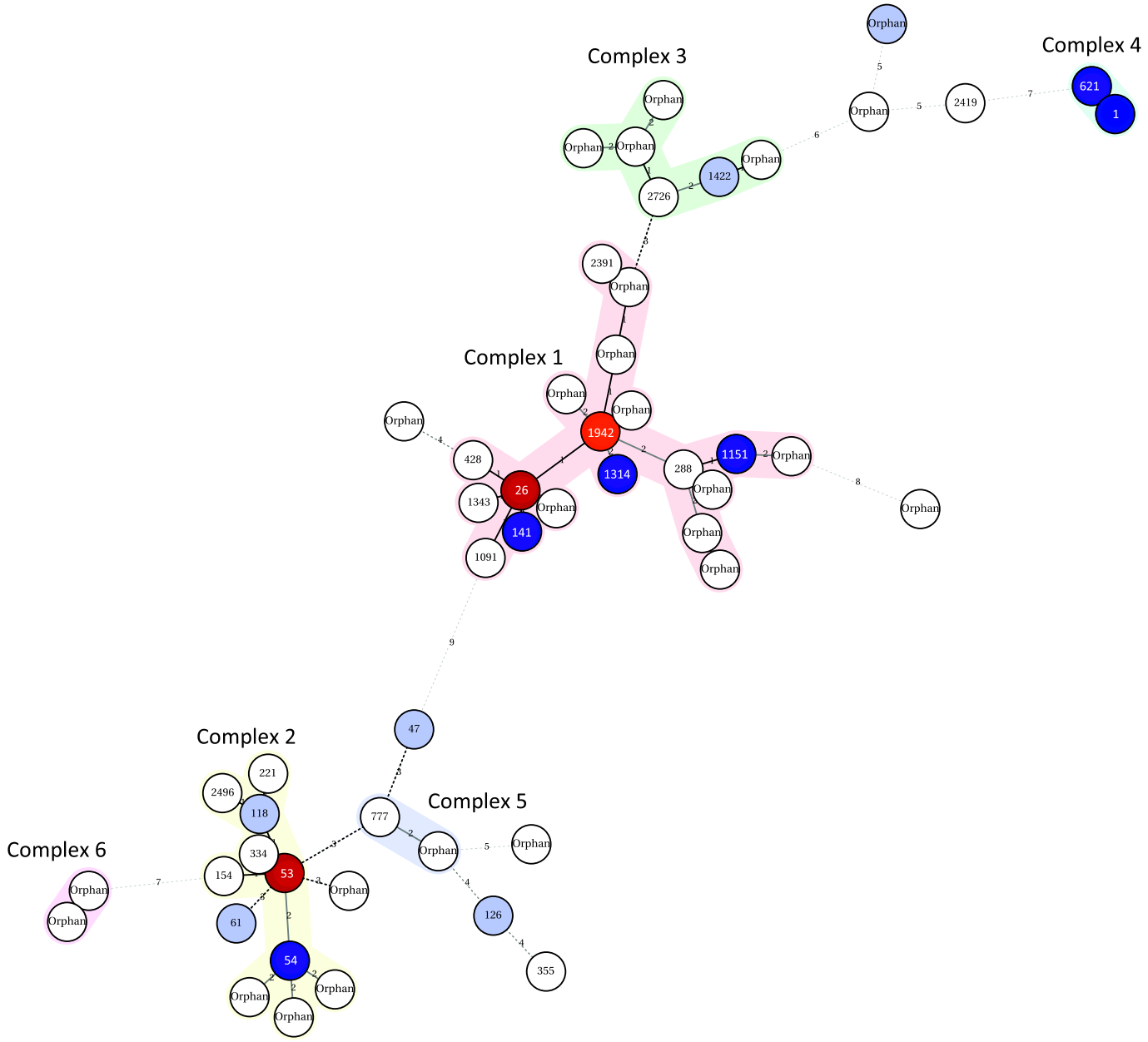


Fig. 2. Minimum spanning tree illustrating the potential evolutionary relationships of the *M. tuberculosis* spoligotypes identified in this study. The lengths of the branches indicate the levels of changes induced by loss or gain of spoligotype spacers in the 43-oligonucleotide format to induce a shift from one allele to another. Solid lines represent a single (black) or double (grey) spacer change, while dotted lines represent 3 or more changes (a precise number of changes is indicated on the line). The colors of the circles are proportional to the number of clinical isolates in the study: white, 1 isolate; sky blue, 2 isolates; blue, 3 to 5 isolates; navy blue, 6 isolates; brown, 11 or 12 isolates and red, 29 isolates. The remaining colors on the MST denote complexes for closely related isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Allelic diversity of the MIRU-VNTR loci and their discriminatory power.

No.	Locus	No. of isolates with a specific number of tandem repeats											Discriminatory power
		0	1	2	3	4	5	6	7	8	9	10	HGDI index	
1	MIRU 154	0	0	127	0	0	0	0	0	0	0	0	0.00	poor
2	MIRU 424	5	21	31	58	9	3	0	0	0	0	0	0.70	**high**
3	MIRU 577	0	0	0	0	88	4	35	0	0	0	0	0.45	moderate
4	MIRU 580	0	0	125	0	0	2	0	0	0	0	0	0.03	poor
5	MIRU 802	0	7	22	75	21	2	0	0	0	0	0	0.60	moderate
6	MIRU 960	2	5	13	24	6	48	21	7	0	1	0	0.78	**high**
7	MIRU 1644	6	7	40	54	18	2	0	0	0	0	0	0.70	**high**
8	MIRU 1955	0	0	12	27	76	8	3	1	0	0	0	0.59	moderate
9	MIRU 2059	0	26	101	0	0	0	0	0	0	0	0	0.33	moderate
10	MIRU 2163b	0	3	103	12	0	8	1	0	0	0	0	0.33	moderate
11	MIRU 2165	0	0	4	22	92	8	1	0	0	0	0	0.44	moderate
12	MIRU 2347	0	0	0	5	122	0	0	0	0	0	0	0.08	poor
13	MIRU 2401	0	4	72	0	51	0	0	0	0	0	0	0.52	moderate
14	MIRU 2461	0	0	0	0	0	2	120	2	3	0	0	0.11	poor
15	MIRU 2531	0	1	0	1	6	112	6	1	0	0	0	0.22	poor
16	MIRU 2687	0	122	5	0	0	0	0	0	0	0	0	0.08	poor
17	MIRU 2996	0	14	3	5	14	21	67	3	0	0	0	0.67	**high**
18	MIRU 3007	1	1	4	119	2	0	0	0	0	0	0	0.12	poor
19	MIRU 3171	0	0	3	123	1	0	0	0	0	0	0	0.06	poor
20	MIRU 3192	0	6	9	30	77	3	2	0	0	0	0	0.57	moderate
21	MIRU 3690	0	0	4	81	35	2	3	0	0	2	0	0.52	moderate
22	MIRU 4052	0	1	2	2	8	6	18	42	47	0	1	0.73	**high**
23	MIRU 4156	0	0	0	0	1	12	15	11	11	76	1	0.61	**high**
24	MIRU 4348	0	1	56	70	0	0	0	0	0	0	0	0.51	moderate

High discriminatory power of each loci was marked in bold.

(Devi et al., 2015; Ali et al., 2014; Khosravi et al., 2017). The low clustering rate indicate that patients developed TB due to reactivation of a latent infection or a transmission in a distant past, rather than a recent one. Noteworthy, up to two changes in the 24-loci MIRU-VNTR patterns have been observed in isolates from epidemiologically linked TB patients and in sequential isolates from the same patient (Supply et al., 2006; Li et al., 2018). Thus, the amount of transmission depends on the stringency of the adopted criteria. With a tolerance of a single or double locus variation between the MIRU-VNTR patterns, strain clustering increased to up to 28%, suggesting that transmission does play some role in the incidence of MDR-TB in Pakistan. The same effect, that is an increase of TR (by up to 57% when using more relaxed criteria for clustering of MIRU-VNTR patterns had been reported previously (Augustynowicz-Kopeć et al., 2012; Sloot et al., 2013; Sharma et al., 2017).

Only four patients under the study harbored genetically indistinguishable *M. tuberculosis* isolates. The clustered patients were from the city of Lahore (cluster one, $n = 2$) or Hujra Shah Muqeem (cluster two, $n = 2$). Apart from the domicile, no other epidemiological links were revealed to support a direct transmission between the patients within clusters.

As for the allelic diversity, six MIRU loci (i.e. 424, 960, 1644, 2996, 4052, and 4156) were found to demonstrate high discriminatory power (Supplementary Table 4). Except MIRU 1644, these loci have been described as moderately or highly discriminatory in other studies from different geographical settings (Sankar et al., 2013; Bouklata et al., 2015; Hoza et al., 2016; Shah et al., 2017). Four of these loci (i.e. 424, 960, 2996 and 4052) have consistently been described as highly discriminatory in the past studies from Pakistan (Ali et al., 2007; Ali et al., 2014; Yasmin et al., 2014). Seven loci (i.e. 154, 580, 2347, 2461, 2687, 3007, 3171) were poorly discriminatory in this study, and moderately or poorly discriminatory under previous investigations (Sankar et al., 2013; Bouklata et al., 2015; Hoza et al., 2016; Shah et al., 2017). The use of the six most polymorphic loci had significantly lowered the discriminatory power of the typing system, when compared with a full, 24-loci panel (GDI = 0.79 vs GDI = 0.97; CR = 3.2% vs 20.5%). Therefore, in the MIRU-VNTR typing, the use of only six highly discriminatory loci might be insufficient for estimating strain relatedness and thus assessing the transmission of TB in Pakistan.

5. Conclusions

In conclusion, this work provides a brief description of the genetic diversity of MDR and (pre-)XDR *M. tuberculosis* strains circulating in Punjab, Pakistan with a consensus methodological standard, that is combination of spoligotyping and 24-loci MIRU-VNTR-typing. There are three major findings from the study. First, a triad of CAS, T, and Beijing clades accounted for over two-thirds of tubercle bacilli studied, confirming their pivotal role in shaping the genetic structure of the TB population in Pakistan. Second, the combination of spoligotyping and MIRU-VNTR typing yielded, depending on the stringency of interpretative criteria, a clustering of up to 28.3%, suggesting that recent

Table 3
Prevalences of the CAS, T, and Beijing clades - an overview of previous reports from the region.

Contry/region	Clade			References
	CAS	T	Beijing	
Pakistan	55.4–61%	2.5–7%	3–8.8%	Hasan et al., 2006; Tanveer et al., 2008; Hasan et al., 2010; Ali et al., 2014
North India	41.9–59.1%	5.1–7.2%	3.3–10.8%	Purwar et al., 2011; Varma-Basil et al., 2011; Sankar et al., 2013; Sharma et al., 2017
Iran	24–37.9%[a]	12.8–18.2%[b]	1.4–8.1% [b]	aMozafari et al., 2013; Haeili et al., 2015; Feyisa et al., 2016 b[Mozafari et al., 2013]; Haeili et al., 2013;Feyisa et al., 2016
This study	48.8%	12.6%	7.9%	–

transmission does contribute to the persistence of MDR-TB in Pakistan. Third, in the MIRU-VNTR typing, the use of six highly discriminatory loci (i.e. 424, 960, 1644, 2996, 4052, and 4156) might be of importance as a first screening tool assessing transmission of TB in Pakistan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.029.

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