June 2014

Mycobacterium tuberculosis Central Asian Strain (CAS) lineage strains in Pakistan reveal lower diversity of MIRU loci than other strains

Asho Ali
Aga Khan University

Zahra Hasan
Aga Khan University, zahra.hasan@aku.edu

Sana Jafri
Aga Khan University

Raunaq Inayat
Aga Khan University

Rumina Hasan
Aga Khan University, rumina.hasan@aku.edu

Available at www.sciencedirect.com

ScienceDirect

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

Mycobacterium tuberculosis Central Asian Strain (CAS) lineage strains in Pakistan reveal lower diversity of MIRU loci than other strains

Asho Ali [a,b], *Zahra Hasan* [a], *Sana Jafri* [a], *Raunaq Inayat* [a], *Rumina Hasan* [a,*]

[a] *Department of Pathology & Microbiology, Aga Khan University, Stadium Road, Karachi, Pakistan*
[b] *School of Nursing & Midwifery, Aga Khan University, Stadium Road, Karachi, Pakistan*

ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form
6 March 2014
Accepted 6 March 2014
Available online 10 May 2014

Keywords:
Mycobacterium tuberculosis
Central Asian Strain 1
MIRU-VNTR
Pakistan

ABSTRACT

Mycobacterium tuberculosis (MTB) Central Asian Strain (CAS) lineage strains are predominant in South Asia. Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing is an effective way of determining genetic diversity of strains. A maximum of 24 loci-based MIRU-VNTR typing can be used, however, it is important to investigate the relevance of specific MIRU loci for regional strains for more cost-effective MIRU typing.

MIRU-VNTR typing was performed on MTB strains from Pakistan. Strains were comprised of CAS ($n = 113$) and non-CAS lineages ($n = 87$) – both multi-drug resistant (MDR) and drug susceptible. Hunter Gaston Discriminatory Index (HGDI) for each MIRU loci was interpreted as poor, moderate or highly discriminatory. Results were analyzed using Bionumerics software and miru-vntr*plus* database link.

Clustering analysis revealed 185 different MIRU types. Eight clusters of 2 strains each were present amongst MDR (3 clusters) and drug susceptible (5 clusters) isolates. MDR clusters had orphan and Haarlem strains, whereas drug susceptible strain clusters were comprised of CAS and Beijing lineage strains.

The HGDI for 15 loci-based MIRU typing of all isolates was 0.620, whereas HGDI for CAS was lower than non-CAS lineage strains (p-value: 0.023). HGDI of 8 MIRU-VNTR loci (Qub 26b, 10, 26, 4156, Mtub 04, 16, 31 and ETR-A) were all highly discriminatory. The average HGDI based on these 8 loci was significantly lower for CAS than non-CAS strains (P value: 0.03).

The lower discriminatory index for CAS using both 15 and 8 MIRU loci-based analysis suggests less genetic diversity in these isolates than in other lineages. The eight highly discriminatory MIRU loci for CAS may help in monitoring the transmission of MTB strains in regions with high CAS lineage prevalence.

* Corresponding author. Tel.: +92 21 34864530; fax: +92 21 34934294.
E-mail addresses: asho.ali@aku.edu (A. Ali), zahra.hasan@aku.edu (Z. Hasan), sana.jafri@aku.edu (S. Jafri), raunaq.mahboob@aku.edu (R. Inayat), rumina.hasan@aku.edu (R. Hasan).
http://dx.doi.org/10.1016/j.ijmyco.2014.03.002

Introduction

Mycobacterium tuberculosis (MTB) infects one third of the global population resulting in 4700 deaths each day [1]. The World Health Organization (WHO) estimates show that the incidence of tuberculosis (TB) in Pakistan increased from 181/100,000 to 231/100,000 population between 2006 and 2011 [1,2]. Increasing rates of resistance to anti-tuberculous drugs amongst MTB strains has added to the burden caused by TB [3]. Multi-drug resistant tuberculosis (MDR-TB) is defined as strains resistant to the first-line drugs (rifampicin and isoniazid), while extensive drug resistant tuberculosis (XDR-TB) is defined as MDR strains resistant to fluoroquinolone and also an aminoglycoside drug. Molecular epidemiology can assist TB control through identification of predominant MTB strain types, detection and confirmation of outbreaks, and monitoring of disease transmission, which may be especially important in MDR-TB cases [4]. Genomic diversity of MTB is low as compared with other bacteria, and therefore molecular typing methods are mostly based on polymorphisms in repetitive genomic sequences of MTB [5], including IS*6110*-restriction fragment length polymorphism (IS*6110*-RFLP), spacer-oligonucleotyping (spoligotyping) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing. While IS*6110* RFLP-based typing is used as a gold standard for MTB typing, 24 loci-based MIRU-VNTR typing is demonstrated to have a similar discriminatory power [6,7].

Studies from Asia, Europe and Africa have shown a variable association between drug resistance and MTB lineages. Beijing strains have been shown to be associated with MDR and XDR-TB in several studies [8–10]. However, other studies from Africa do not show any association between the Beijing strain and drug resistance of isolates [11]. In Pakistan, the Beijing and Haarlem strains have been associated with MDR-TB [12–14].

Although 24 loci-based MIRU typing is highly discriminatory, it is costly for resource-limited endemic settings. Genetic diversity of strains varies between geographical regions and certain MIRU loci are shown to be more discriminatory than others; therefore, specific MIRU loci are potentially more useful for typing predominant strains [6,15,16]. For Beijing strains in South Korea and China, a set of 8 loci of the 24-loci MIRU-VNTR standard set or alternately a set of 10 out of the 15-loci MIRU-VNTR standard set of alleles has been proposed for typing MTB strains [6,16].

In Pakistan, from the 12 loci-based MIRU typing standard set, 7 MIRU loci, i.e., 10, 16, 26, 27, 31, 39 and 40, were reported as being highly discriminatory for CAS1 strains [17]. Information on 15 loci MIRU typing on MTB isolates from Pakistan was not available; therefore, to identify a smaller but comprehensive set of loci for typing of local strains, the utility of 15 loci typing for both drug susceptible and drug resistant isolates was investigated.

Material and methods

Strain selection

The Aga Khan University (AKU) laboratory has a wide drug susceptibility testing (DST) network; it receives specimens collected through more than 180 collection units located in major cities and towns across the country. All specimens from collection units are sent to the main clinical laboratory at Aga Khan University Hospital (AKUH), Karachi, for TB cultures and DST. Over the past 4 years, AKU received 12,000–15,000 specimens annually for MTB culture with a 15–20% positivity rate. MTB susceptibility testing is further validated by the WHO Supranational Laboratory quality assurance program. MTB strains ($N = 200$) were randomly selected from the mycobacterial strain bank of the clinical laboratory of the AKUH collected during the time period 2006–2009. Of these, there were drug susceptible ($n = 99$) strains, MDR ($n = 41$) and XDR strains ($n = 60$). MTB strain typing received approval from the Ethical Review Committee of the AKU. These strains were previously spoligotyped for a cross-sectional study from Pakistan [12,18].

Strain types and drug susceptibility pattern

The strains ($n = 200$) included Central Asian Strain (CAS) (n: 113, 56.5%) and non-CAS(n: 87, 43.5%), including: Beijing, East African Indian (EAI) and Haarlem ($n = 10$, 5% each), T family ($n = 12$, 6%), Ural family ($n = 7$, 3.5%), LAM ($n = 2$, 1%), X3 ($n = 1$, 0.5%), unique clusters ($n = 9$, 4.5%), and orphan strains ($n = 28$, 14%). Of these, 99 (49.5%) isolates were susceptible to all first- and second-line anti-tuberculosis drugs: 41 (20.5%) were MDR and 60 (30%) were XDR (Table 1).

Culture and drug susceptibility testing

MTB strains were isolated from the specimens using Lowenstein–Jensen media and MGIT (Becton Dickinson, Franklin Lakes, NJ, USA). MTB was identified using BACTEC NAP TB differentiation test (Becton Dickinson), growth on para-nitrobenzoic acid containing media, nitrate reduction, and niacin accumulation [19]. Drug susceptibility testing of these isolates was previously performed using an agar proportion method on enriched Middlebrook 7H10 medium (BBL Microbiology Systems, Cockeysville, MD, USA) at the following concentrations: rifampicin 1 μg/mL, isoniazid 0.2 μg/mL, streptomycin 2 μg/mL and 10 μg/mL, and ethambutol 5 μg/mL. Pyrazinamide sensitivity was determined by using BACTEC 7H12 medium, pH 6.0, at 100 μg/mL (BACTEC PZA test medium, Becton Dickinson). MDR TB strains were further tested with capreomycin 10 μg/mL, ciprofloxacin 2 μg/mL, ethionamide 5 μg/mL, amikacin 5 μg/mL, and kanamycin 6 μg/mL. Reference strain *M. tuberculosis* H37Rv was used as a control with each susceptibility testing batch [20]. The hospital and its clinical laboratory are accredited by the Joint Commission of International Accreditation (JCIA) and designated as a technical partner of the National TB program (NTP). The laboratory participates in the College of American Pathologists' proficiency testing program for mycobacterial identification and sensitivity.

Mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) typing

Selected isolates were MIRU typed by PCR amplification of the 15 MIRU-VNTR loci as previously described by Supply et al. [7].

Table 1 – Distribution of spoligotypes and drug susceptibility profile of *Mycobacterium tuberculosis* isolates (*N*: 200).

Spoligotype	No. of strain	Drug resistance profile		
		Susceptible	MDR	XDR
CAS	113	58	20	35
Non-CAS	87	41	21	25
Beijing	10	3	2	5
EAI	10	9	–	1
Haarlem	10	1	9	–
T	12	7	1	4
Ural	5	2	1	2
LAM	2	–	2	–
X3	1	–	–	1
Unique cluster	9	9	–	–
Orphan strains	28	10	6	12
Total	200	99	41	60

Distribution of MTB spoligotypes and drug susceptibility profiles exhibiting around 50% susceptible and 50% MDR and XDR isolates among the selected MTB strains.

PCRs were carried out in 25 μl volume using 0.4 μM specific primers, 0.5 mM dNTPs mix, 1 mM $MgCl_2$, 1× PCR buffer, 4% DMSO and 1U of Super TthTaq DNA polymerase. Approximately 40–60 ng of template DNA was added for each reaction. The following cycling condition for PCR was used: 15 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 59 °C, and 1 min 30 s at 72 °C, which was followed by an extension of 72 °C. The PCR products were electrophoresed on a 3% agarose gel and sized with a 100-bp ladder (Promega). All the reactions were performed in duplicates using standard positive and negative controls supplied in the MIRU-VNTR validation kit. Sizing of the PCR fragments and assignment of the various VNTR alleles were also done using standard protocol (Philip Supply INSERM U629, Institut de Biologie/Institut Pasteur de Lille-May 2005, www.genoscreen.com).

Analysis of allelic diversity of MIRU-VNTR loci and clustering

All the MIRU loci were individually interpreted as poorly, moderately and highly discriminatory based on their Hunter Gaston Discriminatory Index (HGDI) as described previously [21,22]. HGDI was defined as: highly discriminatory, >0.6; moderately discriminatory (0.6–0.3); and poorly discriminatory, <0.3. Genetic relatedness among the isolates were estimated by the unweighted pair group method with arithmetic averages (UPGMA) using the international database link for MIRU typing (www.miruvntr-plus) and a dendrogram that was generated for cluster analysis. A cluster was defined as two or more MTB strains exhibiting zero distance cut-off and identical MIRU-VNTR and spoligotype patterns. The 15 loci MIRU patterns were also compared with the patterns from the MIRU-VNTR*plus* database to identify MTB strain lineages and relatedness.

Statistical analysis

HGDI of MIRU loci for MTB isolates were analyzed using SPSS version 19 software. Pearson's chi-square or Fisher-exact test was used to calculate the statistical significance. A p value <0.05 was considered significant.

Results

Demographic information

MTB isolates and XDR isolates were collected from four provinces: Sind, Punjab, Khyber Pakhtunkhwa (KPK) and Baluchistan (Table 2). MTB isolates were obtained from both male and female patients, with a mean age of 31.8 years. The proportion of male and female patients was 52% (mean age = 32.7 - years) and 48% (mean age = 30.8 years), respectively.

Cluster analysis

Data from 15 loci based MIRU patterns obtained for 200 MTB strains were entered in an international database link

Table 2 – Demographic information of patients from whom MTB samples were collected.

Sampling location	Gender		Mean age (Years)	Total sample (N = 200)
	Male	Female		
Sind	88	81	30.2	169
Punjab	10	11	41.7	21
KPK*	5	2	35.6	7
Baluchistan	1	2	45	3
Total	104	96		

* KPK = Khyber Pakhtunkhwa.

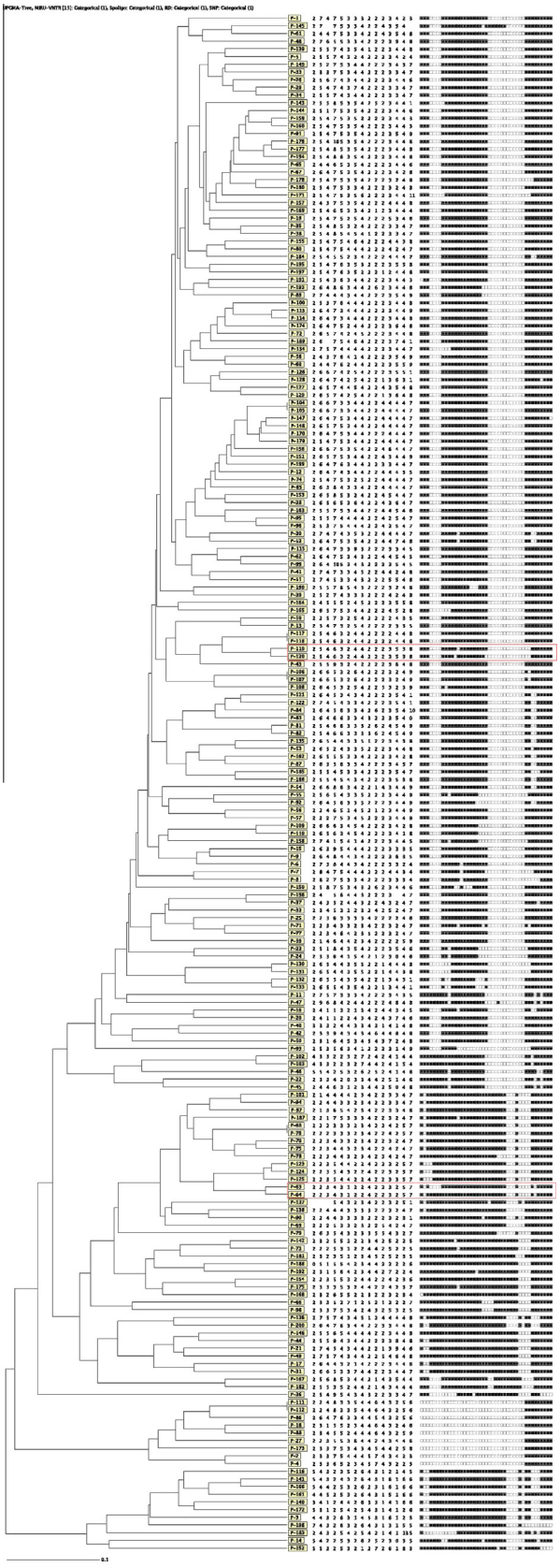

Fig. 1 – Fifteen loci MIRU-VNTR dendrogram of 200 MTB isolates. Dendrogram generated by unweighted pair group method with arithmetic averages (UPGMA) using www.miru-vntrplus.org link. MIRU-VNTR typing differentiated isolates into 7 clusters, of these, 5 clusters had similar MIRU and spoligotype profiles (identified as cluster 1 to cluster 5 in the figure), whereas the remaining two clusters had similar MIRU profiles but different spoligotype patterns (identified in a box in the figure). The remaining 186 strains revealed non-matching MIRU profiles.

Table 3 – Allelic diversity of each of the 15 MIRU-VNTR loci for MTB isolates (*N*: 200).

MIRU loci	Allele number															Allelic diversity	Rank	Discriminatory index (DI)[#]
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	ND[*]			
Qub 26b	1	10		8	11	19	25	51	53	19	1	1			1	0.826	1	High
10		3	26	23	20	56	53	15	2	1					1	0.807	2	High
26		3	16	6	19	37	22	74	16	5	2					0.797	3	High
4156	3	16	46	19	71	29	7	3	3	2					1	0.784	4	High
Mtub04		6	46	38	63	38	6	2		1						0.777	5	High
16		11	12	44	78	36	14		1						4	0.749	6	High
31			6	40	49	80	25									0.727	7	High
ETRA		2	14	43	115	15	9	2								0.613	8	High
40	1	9	27	120	38	4		1								0.586	9	Moderate
Mtub39			11	125	46	11	5	2								0.552	10	Moderate
Mtub21		1	18	24	130	21	4		1					1		0.546	11	Moderate
Mtub30		19	134	4	39	4										0.505	12	Moderate
ETRC		4	135	8	47	6										0.488	13	Moderate
Qub 11b		6	154	10	10	7	10	3								0.399	14	Moderate
4	1		184	2	4	7		1							1	0.144	15	Poor
																Average = 0.620		

The table shows average as well as individual allelic diversity index of MIRU loci for MTB isolates. Eight MIRU loci were characterized as highly discriminant (HGDI ⩾ 0.6), six as moderately discriminant (HGDI < 0.6 > 0.3) and one poorly discriminant (HGD1 < 0.3).

[*] ND = no amplification detected.

DI; high (>0.6), moderate (>0.3 < 0.6), poor (<0.3).

(www.miru-vntrplus.org) for cluster analysis. Five clusters of two strains each were found with identical MIRU and spoligotype patterns (Fig. 1). Clusters 1, 2 and 3 were comprised of drug susceptible CAS strains, Cluster 4 was comprised of Haarlem strains resistant to ethambutol, rifampicin, pyrazinamide, amikacin and capreomycin and Cluster 5 comprised of drug susceptible Beijing strains (Supplementary File 1). There were also two clusters of two strains each which had identical MIRU profiles but different spoligotype patterns; one was a cluster of MDR strains and the other, of a non-MDR and a susceptible strain (Fig. 1 and Supplementary File 1). The remaining 186 strains had differing MIRU patterns.

Furthermore, none of the MIRU profile of our strains matched with the strains in the international MIRU-VNTR*plus* database (Supplementary File 2).

Allelic diversity of individual 15 MIRU loci

Allelic diversity for each of the 15 MIRU loci was also determined for all isolates. The allelic diversity of the standard15

Table 4 – Comparative Hunter Gaston Discriminatory Index (HGDI) of individual 15 MIRU loci for CAS and non-CAS MTB isolates.

MIRU loci	HGDI of		*P*-value
	CAS strains (*n* = 113)	Non-CAS strains (*n* = 87)	
Qub 26	0.807	0.841	0.235
10	0.699	0.835	0.00[*]
26	0.686	0.854	0.00[*]
4156	0.699	0.775	0.004[*]
Mtub4	0.742	0.721	0.004[*]
16	0.672	0.755	0.001[*]
31	0.712	0.745	0.056
ETR-A	0.425	0.75	0.046[*]
40	0.565	0.613	0.095
Mtub39	0.495	0.616	0.037[*]
Mtub21	0.394	0.691	0.0001[*]
Mtub30	0.424	0.597	0.0702
ETR-C	0.168	0.619	0.045[*]
Qub 11b	0.215	0.591	0.002[*]
4	0.035	0.29	0.004[*]
DI of 15 loci set	0.517	0.691	0.023[**]

HGDI for CAS and other than CAS strains displayed in ascending order reveals comparatively lower DIs for CAS1 strains. This difference was found significant (*p*-value = 0.023).

[*] Allelic variability for respective locus between CAS and other than CAS strains is significantly different.

[**] Average DI of CAS and other than CAS strains was significantly different.

Table 5 – Comparative HGDIs of 8 highly discriminatory MIRU loci for all MTB strains, CAS and non-CAS MTB strains.

Loci	All strains (*N* = 200)	CAS strains (*n* = 113)	Non-CAS strains (*n* = 87)	*P*-value
Qub 26	0.826	0.807	0.841	0.235
10	0.807	0.699	0.835	0.00*
26	0.797	0.686	0.854	0.00*
4156	0.784	0.699	0.775	0.004*
Mtub04	0.777	0.742	0.721	0.004*
16	0.749	0.672	0.755	0.001*
31	0.727	0.712	0.745	0.056
ETR A	0.613	0.425	0.75	0.046*
DI of 8 loci set	0.76	0.680	0.784	0.03*

* DI of 8 highly discriminatory loci set of CAS and other than CAS strains was higher than 15 loci set for CAS and non-CAS strains and it was significantly different.

Table 6a – Comparative HGDIs of 15 MIRU loci of Sensitive, MDR and XDR CAS and Non-CAS MTB strains.

Loci	CAS sensitive (*n* = 58)	Non-CAS sensitive (*n* = 41)	CAS MDR (*n* = 20)	Non-CAS MDR (*n* = 21)	CAS XDR (*n* = 35)	Non-CAS XDRs (*n* = 25)
Qub 26	0.767	0.861	0.821	0.609	0.845	0.846
10	0.631	0.834	0.70	0.652	0.8	0.836
26	0.672	0.693	0.721	0.719	0.705	0.883
4156	0.585	0.770	0.594	0.519	0.796	0.873
Mtub04	0.735	0.703	0.721	0.538	0.758	0.763
16	0.669	0.786	0.531	0.638	0.722	0.790
31	0.673	0.693	0.668	0.728	0.707	0.756
ETR A	0.335	0.791	0.531	0.633	0.507	0.586
40	0.600	0.534	0.468	0.547	0.568	0.763
Mtub39	0.496	0.606	0.568	0.552	0.467	0.716
Mtub21	0.439	0.761	0.194	0.685	0.431	0.516
Mtub30	0.448	0.625	0.568	0.490	0.384	0.600
ETR C	0.163	0.619	0.278	0.471	0.112	0.680
Qub 11b	0.227	0.607	0.268	0.423	0.161	0.706
4	0.068	0.428	0.00	0.00	0.00	0.296
DI of 15 loci set	0.500	0.687	0.508	0.546	0.530	0.707
P-value	0.006*		0.613		0.037*	

* DI of 15 loci set between CAS and other than CAS is significantly different.

Table 6b – Comparative HGDIs of 8 highly discriminatory MIRU loci for sensitive, MDR and XDR CAS and non-CAS MTB strains.

Loci	CAS sensitive (*n* = 58)	Non-CAS sensitive (*n* = 41)	CAS MDR (*n* = 20)	Non-CAS MDR (*n* = 21)	CAS XDR (*n* = 35)	Non-CAS XDRs (*n* = 25)
Qub 26	0.767	0.861	0.821	0.609	0.845	0.846
10	0.631	0.834	0.70	0.652	0.8	0.836
26	0.672	0.693	0.721	0.719	0.705	0.883
4156	0.585	0.770	0.594	0.519	0.796	0.873
Mtub 04	0.735	0.703	0.721	0.538	0.758	0.763
16	0.669	0.786	0.531	0.638	0.722	0.790
31	0.673	0.693	0.668	0.728	0.707	0.756
ETR-A	0.335	0.791	0.531	0.633	0.507	0.586
DI of 8 loci set	0.633	0.766	0.660	0.629	0.730	0.791
P-value*	0.023		0.494		0.235	

* DI of 8 loci set between sensitive CAS and non-CAS isolates was significantly different (*P* value = 0.023). Whereas difference in DI of MDR and XDR isolates was insignificant.

MIRU loci set for all isolates was 0.620 which was highly discriminatory, at 0.620 (Table 3). Eight out of 15 loci (Qub 26b, 10, 26, 4156, Mtub 04, 16, 31 and ETR-A) were highly discriminatory. Six loci were moderately discriminatory, while MIRU 4 was poorly discriminatory.

Allelic diversity of 15 MIRU loci for CAS and other than CAS strains

Allelic diversity for each of the 15 MIRU loci was analyzed separately for CAS and other than CAS strains (Table 4, Supplementary Files 3 and 4). For CAS strains, 7 out of 15 loci (Qub 26b, 10, 26, 4156, Mtub 04, 16 and 31) were highly discriminatory. Loci ETR-A, 40, Mtub39, Mtub21 and Mtub30 were moderately discriminatory, while ETR-C, Qub11 and MIRU 4 were poorly discriminatory. For non-CAS strains, 12 out of 15 loci (10, 16, 26, 31, 40, Mtub 04, 4156, Qub 26b, ETR-A, ETR-C, Mtub21 and Mtub39) were highly discriminatory; loci Qub11, and Mtub 30 were moderately discriminatory; and MIRU 4 remained poorly discriminatory.

The HGDI of standard 15 MIRU loci set for CAS was significantly lower as compared with non-CAS isolates (*P* value = 0.023). For each MIRU loci, allelic variability was compared between CAS and non-CAS isolates and showed that HGDI of loci 26, 4156, Mtub4, 16, ETR-A, Mtub39, Mtub21, ETR-C, Qub11b and 4 was significantly different between groups.

Allelic diversity of 8 highly discriminatory MIRU loci

Allelic diversity of Pakistani isolates was further analyzed using only the 8 highly discriminatory loci (Qub26, MIRU 10, 16, 4256, Mtub 04, 16, 31 and ETR-A). The average HGDI based on these 8 loci for all MTB isolates was 0.76 (Table 5). Separately, the average HGDI of CAS and non-CAS strains was 0.680 and 0.784, respectively (Table 5).

Allelic diversity of 15 and 8 MIRU loci for susceptible and drug resistant CAS and non-CAS isolates

To investigate a possible association between MIRU types and drug susceptibility, discriminatory loci analysis was performed for both standard 15 loci set and selected 8 loci set typing by comparing MIRU profiles of drug susceptible, MDR and XDR strains belonging to both CAS and non-CAS lineages. Based on 15 loci typing, the average DI for CAS strains, drug susceptible, MDR and XDR strains was comparable (HGDI: 0.500, 0.508 and 0.530, respectively) (Table 6a). Similarly, for non-CAS isolates, HGDI for susceptible, MDR and XDR isolates was also comparable (HGDI: 0.687, 0.546 and 0.707, respectively). Based on 8 loci MIRU typing, the average DI for CAS susceptible, MDR and XDR strains was 0.633, 0.660 and 0.730, respectively, and HGDI for non-CAS strains was 0.766, 0.629 and 0.791, respectively (Table 6b).

Discussion

In this study, 15 loci-based MIRU-VNTR typing was used to identify similarities between 200 clinical MTB isolates from Pakistan comprising CAS and non-CAS lineage strains. It was found that the discriminatory power of MIRU-based analysis varied depending on the lineage of MTB strains. Also, it was observed that MIRU-VNTR together with spoligotyping enhanced the discrimination of MTB strains in the population, supporting previous reports [23]. Comparative analysis of MIRU-VNTRs for CAS and non-CAS isolates revealed the CAS lineage to be less diverse. The least diverse VNTRs (HGDI < 0.1) for CAS strains were loci 4, Qub11b and ETR-C. This finding is consistent with other studies which suggested that low diversity in these loci may be due to greater genetic stability for strains, as found for the CAS1 lineage [16,24–26]. ETR-C was found to be highly discriminatory in this study of non-CAS strains. Conversely, Qub11b was poorly discriminatory for CAS, but moderately discriminatory for Beijing strains. Consistent with these findings, Qub11b has been reported as being highly discriminatory for other than CAS, including Beijing strains from Russia and China; conversely, it has also been reported as poorly discriminatory for Beijing strains from Japan [16,25,27,28].

In regions with a high prevalence of Beijing strains, inclusion of Mtub 29 and the replacement of ETR-C with MIRU 39 in the standard 15 loci MIRU-VNTR typing has been recommended for improving the discriminatory power of MIRU-VNTR [25,29]. In an earlier study based on 12 loci MIRU-VNTR typing, loci 27 and 39 (in addition to 10, 16 and 31) were identified as being highly discriminatory for CAS strains [17]. In a study of CAS strains from India, 7 of the above-mentioned 8 loci (minus ETR-A), in addition to Mtub 21 and MIRU 40, are proposed for typing while MIRU 4, Qub 11b and ETR-C are not recommended [30]. This data shows that for CAS lineage strains, MIRU loci Qub 26b, 10, 26, 4156, Mtub 04, 16, 31 and ETR-A are highly discriminatory and may be suitable for typing of CAS strains using fewer loci.

Strains in clusters were predominantly comprised mainly of CAS drug susceptible strains, but also of Haarlem and Beijing strains. Moreover, clusters with a similar MIRU profile but different spoligotypes identified in this study is consistent with findings from India [23]. The lower clustering of resistant strains is encouraging and suggests that drug resistance may be due to the acquisition of mutations rather than transmission of drug resistant strains.

CAS strains are also common in countries neighboring Pakistan, such as Iran, which has reported 24% of CAS isolates, while studies from India indicate between 46% and 84% of CAS (ST26) strains [15,31,32]. Therefore, the proposed use of a CAS-selective 8 loci MIRU typing set may allow improved discriminatory power and provide a cost-effective MTB genotyping method for tracing infection in hospital and community settings in all regions where CAS strains are predominant.

Conclusions

The lower diversity of MIRU loci for CAS lineage as compared with other than CAS lineages shows greater genetic stability amongst CAS strains; 8 MIRU loci identified in this study as being highly discriminatory in an MTB population with predominant CAS strains may offer an affordable typing method

to investigate MTB transmission within hospital and community settings.

Conflict of interest

None declared.

Acknowledgements

We would like to express our gratitude to Dr. Mahnaz Tanveer for establishing spoligotyping methodology and Ms Rabia Laiq for conducting Spoligotyping and MIRU typing in the initial phase of the study. We would also like to thank the Clinical Laboratory of Aga Khan University Hospital for providing samples used in the study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmyco.2014.03.002.

REFERENCES

[1] WHO, World Health Organization Report, Global Tuberculosis Control, 2011.
[2] WHO, World Health Organization Report, Global Tuberculosis Control, 2008.
[3] R. Hasan, K. Jabeen, V. Mehraj, F. Zafar, F. Malik, Q. Hassan, I. Azam, M.M. Kadir, Trends in *Mycobacterium tuberculosis* resistance, Pakistan, 1990–2007, Int. J. Infect. Dis. 13 (6) (2009) e377–382.
[4] M.M. Gutacker, B. Mathema, H. Soini, E. Shashkina, B.N. Kreiswirth, E.A. Graviss, et al, Single-nucleotide polymorphism-based population genetic analysis of *Mycobacterium tuberculosis* strains from 4 geographic sites, J. Infect. Dis. 193 (1) (2006) 121–128.
[5] S. Sreevatsan, X. Pan, K.E. Stockbauer, N.D. Connell, B.N. Kreiswirth, T.S. Whittam, et al, Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination, Proc. Natl. Acad. Sci. U.S.A. 94 (18) (1997) 9869–9874.
[6] I.C. Shamputa, J. Lee, C. Allix-Beguec, E.J. Cho, J.I. Lee, V. Rajan, et al, Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea, J. Clin. Microbiol. 48 (2) (2010) 387–394.
[7] P. Supply, C. Allix, S. Lesjean, M. Cardoso-Oelemann, S. Rusch-Gerdes, E. Willery, et al, Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*, J. Clin. Microbiol. 44 (12) (2006) 4498–4510.
[8] A.D. Calver, A.A. Falmer, M. Murray, O.J. Strauss, E.M. Streicher, M. Hanekom, et al, Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa, Emerg. Infect. Dis. 16 (2) (2010) 264–271.
[9] N.R. Gandhi, P. Nunn, K. Dheda, H.S. Schaaf, M. Zignol, D. van Soolingen, et al, Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis, Lancet 375 (9728) (2010) 1830–1843.
[10] S. Niemann, R. Diel, G. Khechinashvili, M. Gegia, N. Mdivani, Y.W. Tang, *Mycobacterium tuberculosis* Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia, J. Clin. Microbiol. 48 (10) (2010) 3544–3550.
[11] H.M. Said, M.M. Kock, N.A. Ismail, M. Mphahlele, K. Baba, S.V. Omar, et al, Molecular characterization and second-line anti-TB drug-resistance patterns of multidrug resistant tuberculosis isolates from the northern region of South Africa, J. Clin. Microbiol. 50 (2012) 2857–2862.
[12] R. Hasan, K. Jabeen, A. Ali, Y. Rafiq, R. Laiq, B. Malik, et al, Extensively drug-resistant tuberculosis, Pakistan, Emerg. Infect. Dis. 16 (9) (2010) 1473–1475.
[13] M. Tanveer, Z. Hasan, A.R. Siddiqui, A. Ali, A. Kanji, S. Ghebremicheal, et al, Genotyping and drug resistance patterns of *M. tuberculosis* strains in Pakistan, BMC Infect. Dis. 8 (2008) 171.
[14] Z. Hasan, M. Tanveer, A. Kanji, Q. Hasan, S. Ghebremichael, R. Hasan, Spoligotyping of *Mycobacterium tuberculosis* isolates from Pakistan reveals predominance of Central Asian Strain 1 and Beijing isolates, J. Clin. Microbiol. 44 (5) (2006) 1763–1768.
[15] A. Chatterjee, N. Mistry, MIRU-VNTR profiles of three major *Mycobacterium tuberculosis* spoligotypes found in western India, Tuberculosis (Edinb) 93 (2) (2013) 250–256.
[16] J. Wang, Y. Liu, C.L. Zhang, B.Y. Ji, L.Z. Zhang, Y.Z. Shao, et al, Genotypes and characteristics of clustering and drug susceptibility of *Mycobacterium tuberculosis* isolates collected in Heilongjiang Province, China, J. Clin. Microbiol. 49 (4) (2011) 1354–1362.
[17] A. Ali, Z. Hasan, M. Tanveer, A.R. Siddiqui, S. Ghebremichael, G. Kallenius, et al, Characterization of *Mycobacterium tuberculosis* Central Asian Strain 1 using mycobacterial interspersed repetitive unit genotyping, BMC Microbiol. 7 (2007) 76.
[18] A. Ayaz, Z. Hasan, S. Jafri, R. Inayat, R. Mangi, A.A. Channa, et al, Characterizing *Mycobacterium tuberculosis* isolates from Karachi, Pakistan: drug resistance and genotypes, Int. J. Infect. Dis. 16 (4) (2012) e303–309.
[19] E.W.A.S. Koneman, V.R. Dowell, H.M. Sommers, Color Atlas and Text book of Diagnostic Microbiology, JB Lippincott Company, Philadelphia, 1983.
[20] G.L.B.-E.B. Woods, E.P. Desmond, G.S. Hall, L. Heifets, G.E. Pfyffer, et al., Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes: approved standard Wayne, PA, NCCLS, 2003. Document No. M24-A. 2003.
[21] P.R. Hunter, M.A. Gaston, Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity, J. Clin. Microbiol. 26 (11) (1988) 2465–2466.
[22] C. Sola, I. Filliol, E. Legrand, S. Lesjean, C. Locht, P. Supply, N. Rastogi, Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics, Infect. Genet. Evol. 3 (2) (2003) 125–133.
[23] M. Varma-Basil, S. Kumar, J. Arora, A. Angrup, T. Zozio, J.N. Banavaliker, et al, Comparison of spoligotyping, mycobacterial interspersed repetitive units typing and IS6110-RFLP in a study of genotypic diversity of *Mycobacterium tuberculosis* in Delhi, North India, Mem. Inst. Oswaldo Cruz 106 (5) (2011) 524–535.
[24] I. Comas, S. Homolka, S. Niemann, S. Gagneux, Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies, PLoS ONE 4 (11) (2009) e7815.
[25] W.W. Jiao, I. Mokrousov, G.Z. Sun, Y.J. Guo, A. Vyazovaya, O. Narvskaya, et al, Evaluation of new variable-number tandem-repeat systems for typing *Mycobacterium tuberculosis* with Beijing genotype isolates from Beijing, China, J. Clin. Microbiol. 46 (3) (2008) 1045–1049.

[26] I. Mokrousov, Genetic geography of *Mycobacterium tuberculosis* Beijing genotype: a multifacet mirror of human history? Infect. Genet. Evol. 8 (6) (2008) 777–785.
[27] I. Mokrousov, O. Narvskaya, A. Vyazovaya, J. Millet, T. Otten, B. Vishnevsky, et al, *Mycobacterium tuberculosis* Beijing genotype in Russia: in search of informative variable-number tandem-repeat loci, J. Clin. Microbiol. 46 (11) (2008) 3576–3584.
[28] Y. Murase, S. Mitarai, I. Sugawara, S. Kato, S. Maeda, Promising loci of variable numbers of tandem repeats for typing Beijing family *Mycobacterium tuberculosis*, J. Med. Microbiol. 57 (Pt 7) (2008) 873–880.
[29] A. Zhou, M. Nawaz, X. Xue, P.C. Karakousis, Y. Yao, J. Xu, Molecular genotyping of *Mycobacterium tuberculosis* in Xi'an, China, using MIRU-VNTR typing, Int. J. Tuberc. Lung Dis. 15 (4) (2011) 517–522.
[30] V. Vadwai, A. Shetty, C. Rodrigues, Do diverse *Mycobacterium tuberculosis* strains circulate in Mumbai?, Asia-Pac J. Trop. Dis. 2 (2012) S563–S566.
[31] M. Haeili, D. Darban-Sarokhalil, A.A. Fooladi, S. Javadpour, A. Hashemi, F. Siavoshi, et al, Spoligotyping and drug resistance patterns of *Mycobacterium tuberculosis* isolates from five provinces of Iran, Microbiologyopen 2 (6) (2013) 988–996.
[32] R. Stavrum, V.P. Myneedu, V.K. Arora, N. Ahmed, H.M. Grewal, In-depth molecular characterization of *Mycobacterium tuberculosis* from New Delhi – predominance of drug resistant isolates of the 'modern' (TbD1) type, PLoS ONE 4 (2) (2009) e4540.