International Journal of Infectious Diseases 63 (2017) 13–20

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

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

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

Yogendra Shah[a,1], Bhagwan Maharjan[a,b,1], Jeewan Thapa[a], Ajay Poudel[c], Hassan Mahmoud Diab[d], Basu Dev Pandey[e,f], Eddie S. Solo[a,g], Norikazu Isoda[a,h], Yasuhiko Suzuki[a,h], Chie Nakajima[a,h,*]

[a] *Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan*
[b] *German Nepal Tuberculosis Project, Nepal Anti-Tuberculosis Association/GENETUP, Kalimati, Kathmandu, Nepal*
[c] *Chitwan Medical College Teaching Hospital, Department of Microbiology, Bharatpur, Chitwan, Nepal*
[d] *Department of Animal Hygiene, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt*
[e] *Leprosy Control Division, Department of Health Services, Ministry of Health, Kathmandu, Nepal*
[f] *Everest International Clinic and Research Center, Kathmandu, Nepal*
[g] *University Teaching Hospital, University of Zambia, Lusaka, Zambia*
[h] *Hokkaido University, GI-CoRE Global Station for Zoonosis Control, Sapporo, Japan*

ARTICLE INFO

Article history:
Received 4 April 2017
Received in revised form 2 June 2017
Accepted 7 June 2017
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Mycobacterium tuberculosis
Multidrug-resistant TB
CAS family
MIRU-VNTR
Nepal

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).

* Corresponding author at: Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan. Tel: +81 11 706 9505. Fax: +81 11 706 7310.
E-mail address: cnakajim@czc.hokudai.ac.jp (C. Nakajima).
[1] Yogendra Shah and Bhagwan Maharjan contributed equally to this work.

http://dx.doi.org/10.1016/j.ijid.2017.06.010

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

Table 1
Demographic and clinical characteristics of patients infected with CAS and non-CAS genotype isolates.

	Number of CAS family	Number of non-CAS family	OR (95% CI)	*p*-Value
Sex				
Male	111	321	1	
Female	34	135	0.728 (0.472–1.123)	0.151
Age (years)				
0–20	14	73	1	
21–40	85	268	1.65 (0.88–3.07)	0.110
41–60	36	99	1.89 (0.95–3.77)	0.066
>60	10	16	3.25 (1.22–8.64)	<0.05[a]
Treatment status				
CAT II failure[b]	122	393	1	
CAT I failure[c]	18	42	1.38 (0.766–2.48)	0.282
New patients	5	21	0.76 (0.283–2.07)	0.813

CAS, Central Asian Strain; OR, odds ratio; CI, confidence interval; MDR, multidrug-resistant; TB, tuberculosis.
[a] A *p*-value of <0.05 was considered significant; determined by Chi-square test or Fisher's exact test.
[b] CAT II failure: patients who were either smear-positive relapse, chronic, MDR contact, relapse, or treatment after default.
[c] CAT I failure: failure in new cases of smear-positive pulmonary TB.

compared (Table 1). Regarding the age group, a significant difference was found between those aged 0–20 years and those aged >60 years ($p < 0.05$, Chi-square test). There were no other factors significantly associated with the CAS isolate infection among MDR-TB patients.

Spoligotype

A total of 145 MDR CAS isolates were divided into 25 spoligotype patterns, and CAS1_Delhi SIT26 was found to be most prevalent (60/145, 41.4%), followed by CAS SIT599 (15/145, 10.3%) and CAS SIT357 (9/145, 6.2%), as listed in Table 2.

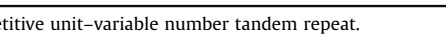
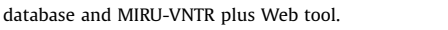
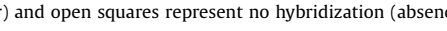
MIRU-VNTR

Allelic diversity of each MIRU-VNTR locus and HGDI are shown in Table 3. Three out of 24 loci (QUB26, MIRU10, and VNTR424) were found to be highly discriminant, 11 loci (MIRU26, VNTR3960, MIRU31, MIRU40, ETR-A, QUB11a, QUB4156, VNTR2401, VNTR1955, MIRU39, and ETR-F) were moderately discriminant, and the remaining 10 loci were poorly discriminant.

The number of MIRU-VNTR patterns with the 24 loci was 107, including 21 clusters that consisted of 54 isolates (clustering rate 37.2%) (Table 4). The cumulative HGDI of VNTR with all 24 loci was equal to that of MIRU-VNTR with the top 15 loci (HGDI 0.9942). As CAS1_Delhi SIT26, the dominant type in the MDR CAS family, consisted of 60 isolates, its MIRU-VNTR allelic diversity was also analyzed in the same manner. The results were similar to those of the total MDR CAS family and the cumulative HGDI reached the maximum with almost the same top 15 loci (**Supplementary Material**, Table S1 in the online version at DOI: 10.1016/j.ijid.2017.06.010).

By combining the analysis of spoligotyping and MIRU-VNTR, 18 clusters were found, consisting of 47 isolates with an identical pattern in each cluster. The clustering rate was 32.4%, and the

Table 2
Distribution frequency of diverse CAS family strains.

	Clade[a]	SIT[b]	Spoligotype pattern[c]	Number of isolates (%)
1.	CAS1_Delhi	26		60 (41.4)
2.	CAS	599		15 (10.3)
3.	CAS	357		9 (6.20)
4.	CAS	142		7 (4.82)
5.	CAS	288		7 (4.82)
6.	CAS1_Delhi	471		6 (4.13)
7.	CAS1_Delhi	1312		4 (2.75)
8.	CAS1_Delhi	427		4 (2.75)
9.	CAS	22		3 (2.06)
10.	CAS1_Delhi	25		3 (2.06)
11.	CAS	356		3 (2.06)
12.	CAS	486		3 (2.06)
13.	CAS1_Delhi	429		2 (1.37)
14.	CAS	428		2 (1.37)
15.	CAS	485		2 (1.37)
16	CAS	1093		2 (1.37)
17.	CAS	1266		2 (1.37)
18.	CAS1_Delhi	1343		2 (1.37)
19.	CAS1_Delhi	1590		2 (1.37)
20.	CAS	203		1 (0.68)
21.	CAS	864		1 (0.68)
22.	CAS	1151		1 (0.68)
23.	CAS1_Delhi	1314		1 (0.68)
24.	CAS	1606		1 (0.68)
25.	CAS1_Delhi	1883		1 (0.68)

CAS, Central Asian Strain; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat.
[a] Clades were annotated using the SITVITWEB database.
[b] SIT (spoligotype international types) were assigned by SITVITWEB database and MIRU-VNTR plus Web tool.
[c] Closed squares represent positive hybridization (presence of spacer) and open squares represent no hybridization (absence of spacer).

Table 3
Allelic diversity of each MIRU-VNTR locus for CAS MDR-MTB isolates of Nepal ($n = 145$).

Locus	Allele number[a]											HGDI[c]	Conclusion
	0	1	2	3	4	5	6	7	8	9	10	ND[b]		
QUB26		1		2	4	13	12	89	12			3	0.604	High
MIRU10				2	13	41	83	1	1			3	0.603	High
VNTR424			5	16	88	23	1	1	3		1		0.6	High
MIRU26			1	8	8	20	93	2	12			2	0.56	Moderate
VNTR3690				109	24	11						1	0.465	Moderate
MIRU31			2	1	31	103	5					3	0.448	Moderate
MIRU40		6	23	107	9								0.427	Moderate
ETR-A			1	23	119	2							0.395	Moderate
QUB11a			1			4	2	21	111		3	3	0.394	Moderate
QUB4156	2	2	23		114							4	0.39	Moderate
VNTR2401			119		26								0.351	Moderate
VNTR1955			14	4	116	6	2					3	0.34	Moderate
MIRU39			19	121	3							2	0.322	Moderate
ETR-F			8	124	10							3	0.309	Moderate
MIRU16				23	120							2	0.291	Poor
MIRU20		4	141										0.132	Poor
QUB11b		2	142	1									0.107	Poor
MIRU24	1	144											0.081	Poor
MIRU27			1	140	2							2	0.067	Poor
MIRU4		1	141	1								2	0.054	Poor
MIRU23		1				142	1		1				0.041	Poor
MIRU2			143	1								1	0.027	Poor
ETR-B			145										0	Poor
ETR-C			145										0	Poor

MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat; CAS, Central Asian Strain; MDR-MTB, multidrug-resistant *Mycobacterium tuberculosis*.
[a] Number of tandem repeats.
[b] ND: not determined because of PCR failure.
[c] Hunter Gaston Discriminatory Index: DI > 0.6, highly discriminatory; 0.3 ≤ DI ≤ 0.6, moderately discriminatory; DI < 0.3, poorly discriminatory.

biggest cluster consisted of six isolates, followed by three four-isolate clusters, one three-isolate cluster, and finally 13 clusters consisting of two isolates (Figure 1, Table 5).

Sequence analysis of drug resistance-associated genes

Clustered isolates were analyzed further by sequencing the drug resistance-associated genes (*rpoB*, *katG* coding, and *inhA* promoter region) to identify possible MDR-MTB transmission (Table 5). Among the 47 isolates, all but one had the G944C mutation (i.e., Ser315Thr substitution) in *katG*, which is a well-known INH resistance-associated mutation. In the *rpoB* analysis, 45 isolates had a mutation or deletion in RRDR, and C1349T (Ser531Leu) was dominant (20/47, 42.6%). Among the 18 clusters, 14 had isolates sharing the same *rpoB* mutation, and half of them were the major substitution Ser531Leu. Among the remaining

Table 4
Cumulative HGDI with successive addition of each MIRU-VNTR locus ($n = 145$).

VNTR alias	Individual HGDI	Number of patterns	Number of clusters	Number of clustered isolates	Clustering rate (%)	Number of isolates in each cluster	Cumulative HGDI
QUB26	0.604						0.604
MIRU10	0.603	18	9	130	89.6	2–52	0.8187
VNTR424	0.6	35	16	121	83.4	2–39	0.8937
MIRU26	0.56	55	20	104	71.7	2–27	0.9453
VNTR3690	0.465	63	20	96	62.2	2–13	0.9674
MIRU31	0.448	70	22	90	62.0	2–11	0.9767
MIRU40	0.427	79	23	82	56.6	2–8	0.9846
ETR-A	0.395	86	25	79	54.4	2–7	0.9874
QUB11a	0.394	90	25	75	51.7	2–6	0.9899
QUB4156	0.39	92	26	73	50.3	2–5	0.9917
VNTR2401	0.351	93	26	71	48.9	2–5	0.9923
VNTR1955	0.34	95	23	62	42.7	2–5	0.9927
MIRU39	0.322	97	22	61	42.0	2–5	0.9927
ETR-F	0.309	97	22	60	41.3	2–5	0.9931
MIRU16	0.291	98	22	60	41.3	2–5	0.9942
MIRU20	0.132	100	21	60	41.3	2–5	0.9942
MIRU24	0.107	102	22	58	40.0	2–5	0.9942
QUB11b	0.081	103	22	57	39.3	2–5	0.9942
MIRU27	0.067	105	21	55	37.9	2–4	0.9942
MIRU4	0.054	106	21	55	37.9	2–4	0.9942
MIRU23	0.041	107	21	54	37.2	2–4	0.9944
MIRU2	0.027	107	21	54	37.2	2–4	0.9942
ETR-B	0	107	21	54	37.2	2–4	0.9942
ETR-C	0	107	21	54	37.2	2–4	0.9942

HGDI, Hunter Gaston Discriminatory Index; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat.

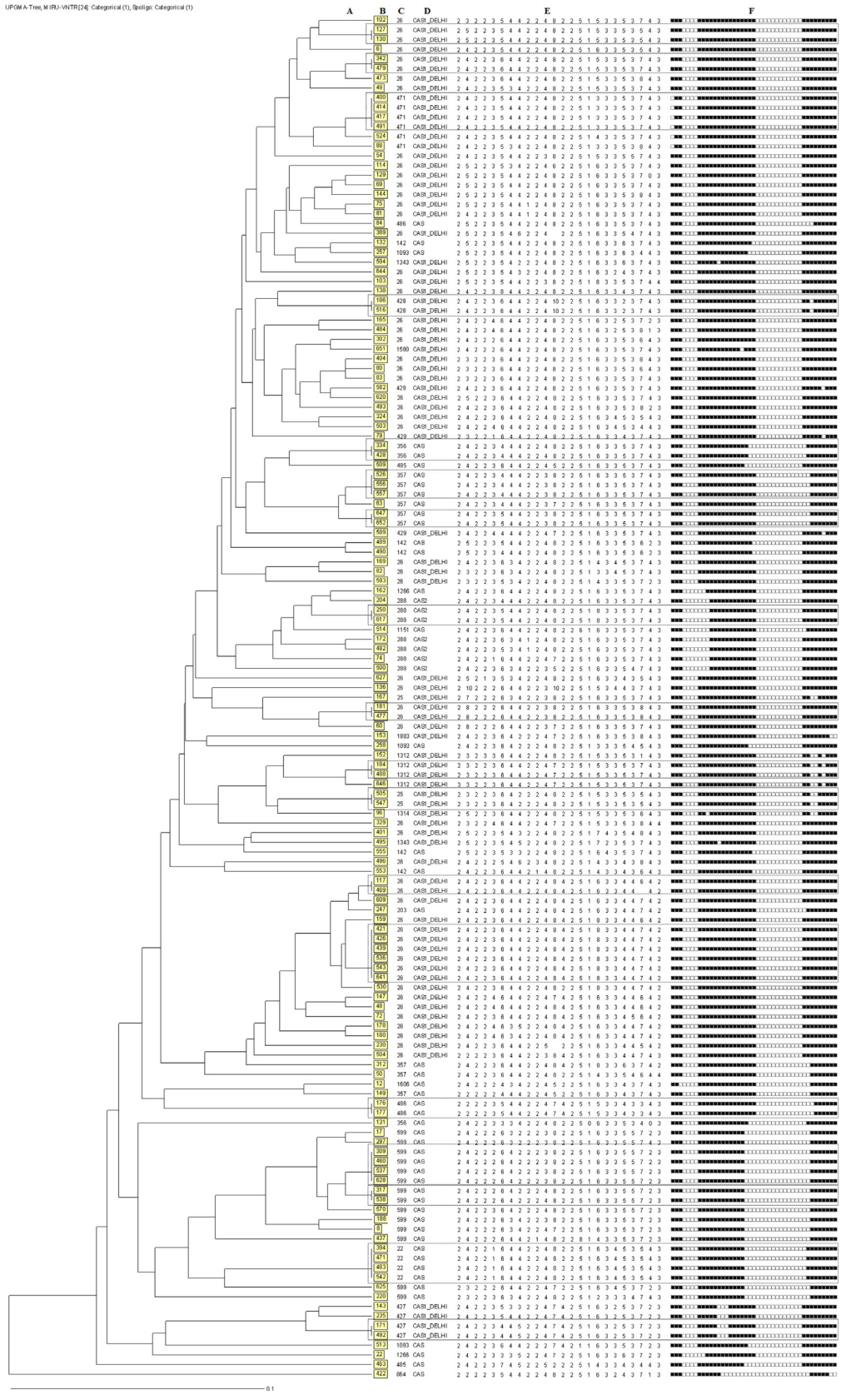


Figure 1. 24-locus MIRU-VNTR and spoligotyping-based dendrogram of 145 multidrug-resistant Central Asian Strain (MDR CAS) isolates from Nepal. (A) The dendrogram was generated by UPGMA (http://www.miru-vntrplus.org). (B) Strain identification number. (C) SIT (spoligotype international type) number. (D) Clade annotated by SITVIT database. (E) MIRU-VNTR results of 24 loci. The order of loci, left to right, is as follows: MIRU2, VNTR424, ETR-C, MIRU4, MIRU40, MIRU10, MIRU16, VNTR 1955, MIRU20, QUB11b, ETR-A, QUB11a, VNTR 2401, ETR-B, MIRU23, MIRU24, MIRU26, MIRU27, ETR-F, MIRU31, VNTR 3690, QUB26, QUB4156, and MIRU39. (F) Spoligotype pattern. Isolate clusters (identical patterns of MIRU-VNTR and spoligotyping) are enclosed in open boxes.

Table 5
Demographic information of patients and genetic characteristics of the 47 MDR-MTB clustered isolates belonging to the CAS genotype.

ID	Demographic information of patients					Genotype of isolates		DST	Mutations in drug resistance-associated genes[e]				
	Location	Age	Sex	Year	Categoly[a]	SIT[b]	MIRU-VNTR[c]	results[d]	RpoB in codon	*rpoB* in nucleotide	KatG	*inhA*	
127	Nepalgunj	24	F	2008	cat II failure	26	252235442248225153353543	R,I	Gln 513 His, del: 514~51	del: 1296~1304 attcatgga	Ser 315 Thr	WT	Cluster 1
130	Nepalgunj	30	F	2008	cat II failure	26	252235442248225153353543	R,I,E	Gln 513 His, del: 514~51	del: 1296~1304 attcatgga	Ser 315 Thr	WT	
342	Janakpur	28	M	2012	cat II failure	26	242236442248225153353743	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	C -15 T	Cluster 2
479	Nepalgunj	41	M	2012	cat II failure	26	242236442248225153353743	R,I,S,E	-	-	Ser 315 Thr	-	
400	Pokhara	50	F	2008	cat II failure	471	242235442248225133353743	R,I,S,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 3
414	Pokhara	82	M	2011	cat II failure	471	242235442248225133353743	R,I,S,E	del: 517	del: 1306~1308 cag	Ser 315 Thr	WT	
417	Pokhara	33	M	2012	relapse	471	242235442248225133353743	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
491	Nepalgunj	34	F	2012	cat I failure	471	242235442248225133353743	R,I,S	Asp 516 Tyr	G 1303 T	Ser 315 Thr	WT	
186	Dhangahi	22	F	2011	cat II failure	428	242236442241022516332374	R,I	Asp 516 Tyr	G 1303 T	Ser 315 Thr	WT	Cluster 4
516	Dhangahi	22	M	2012	cat II failure	428	242236442241022516332374	R,I	Asp 516 Tyr	G 1303 T	Ser 315 Thr	WT	
334	Janakpur	18	M	2009	cat II failure	356	242234442248225163353743	R,I,S,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 5
428	Bhairahawa	24	M	2008	cat II failure	356	242234442248225163353743	R,I,S	Gln 513 Leu	A 1295 T	Ser 315 Thr	WT	
526	Kathmandu	40	F	2008	cat II failure	357	242234442238225163353743	R,I,S,E	Asp 516 Val	A 1304 T	Ser 315 Thr	WT	
556	Kathmandu	34	M	2009	cat II failure	357	242234442238225163353743	R,I,E	Asp 516 Phe	G 1303 T, A 1304 T	Ser 315 Thr	WT	Cluster 6
557	Kathmandu	40	M	2009	cat II failure	357	242234442238225163353743	R,I,E	Asp 516 Phe	G 1303 T, A 1304 T	Ser 315 Thr	WT	
647	Kathmandu	24	M	2010	cat II failure	357	242235442238225163353743	R,I,S	Asp 516 Val	A 1304 T	Ser 315 Thr	WT	Cluster 7
652	Kathmandu	35	M	2011	cat I failure	357	242235442238225163353743	R,I	Asp 516 Phe	G 1303 T, A 1304 T	Ser 315 Thr	WT	
250	Kathmandu	36	M	2009	cat II failure	288	242235442248225183353743	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 8
617	Kathmandu	20	M	2010	cat II failure	288	242235442248225183353743	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
181	Dhangahi	31	F	2010	cat II failure	26	282226442238225163353843	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 9
477	Nepalgunj	62	M	2012	defaulter	26	282226442238225163353843	R,I	WT	none	WT	WT	
184	Dhangahi	32	F	2010	cat II failure	1312	232236442247225153353743	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 10
488	Nepalgunj	25	M	2012	cat II failure	1312	232236442247225153353743	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
505	Dhangahi	32	M	2011	cat II failure	25	232236422248225153353543	R,I	Leu 511 Pro	T 1289 C	Ser 315 Thr	WT	Cluster 11
547	Kathmandu	36	F	2009	cat II failure	25	232236422248225153353543	R,IS,E	Leu 511 Pro	T 1289 C	Ser 315 Thr	WT	
117	Butwal	34	M	2011	cat II failure	26	242236442248425163344642	R,I,S,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 12
469	Nepalgunj	30	M	2009	cat II failure	26	242236442248425163344-42	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
421	Bhairahawa	61	M	2008	cat II failure	26	242236442248425183344742	R,I,S,E	His 526 Asp	C 1333 G	Ser 315 Thr	T -8 C	Cluster 13
426	Bhairahawa	30	M	2009	cat II failure	26	242236442248425183344742	R,I,S,E	His 526 Asp	C 1333 G	Ser 315 Thr	T -8 C	
439	Butwal	31	M	2012	cat II failure	26	242236442248425183344742	R,I	His 526 Tyr	C 1333 T	Ser 315 Thr	WT	
536	Kathmandu	30	F	2009	cat II failure	26	242236442248425183344742	R,I,S,E	His 526 Asp	C 1333 G	Ser 315 Thr	WT	
543	Kathmandu	73	M	2009	cat II failure	26	242236442248425183344742	R,I,S	His 526 Asp	C 1333 G	Ser 315 Thr	WT	
641	Kathmandu	16	F	2010	cat I failure	26	242236442248425183344742	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
176	Mahendranagar	34	M	2009	cat II failure	486	222235442247425153343343	R,I,S,E	Ser 531 Gln	T 1348 C, C 1349 A	Ser 315 Thr	WT	Cluster 14
177	Mahendranagar	55	F	2010	cat II failure	486	222235442247425153343343	R,I,S,E	Ser 531 Gln	T 1348 C, C 1349 A	Ser 315 Thr	WT	
309	Kathmandu	18	F	2012	cat I failure	599	242226422238225163355723	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 15
460	Butwal	18	M	2012	cat I failure	599	242226422238225163355723	R,I,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
537	Kathmandu	25	M	2009	cat II failure	599	242226422238225163355723	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
628	Kathmandu	21	M	2010	cat I failure	599	242226422238225163355723	R,I,S,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
317	Kathmandu	36	M	2012	cat I failure	599	242226422248225163355723	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 16
538	Kathmandu	16	F	2009	MDR contact	599	242226422248225163355723	R,I,S	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	
394	Dharan	25	F	2011	new	22	242216442248225163453543	R,I,S,E	Gln 513 Leu	A 1295 T	Ser 315 Thr	WT	Cluster 17
471	Nepalgunj	23	M	2010	cat II failure	22	242216442248225163453543	R,I,S,E	Gln 513 Leu	A 1295 T	Ser 315 Thr	WT	
483	Nepalgunj	22	M	2012	cat I failure	22	242216442248225163453543	R,I,S,E	Gln 513 Leu	A 1295 T	Ser 315 Thr	WT	
542	Kathmandu	63	M	2009	cat II failure	22	242216442248225163453543	R,I,S,E	Gln 513 Leu	A 1295 T	Ser 315 Thr	WT	
171	Nepalgunj	20	M	2011	cat II failure	427	242234452247425163253723	R,I,S,E	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	Cluster 18
492	Nepalgunj	28	M	2012	relapse	427	242234452247425163253723	R,I	Ser 531 Leu	C 1349 T	Ser 315 Thr	WT	

MDR-MTB, multidrug-resistant *Mycobacterium tuberculosis*; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat; M, male; F, female.
[a]CAT II failure: patients who were either smear-positive relapse, chronic, MDR contact, relapse, or treatment after default. CAT I failure: failure in new cases of smear-positive pulmonary TB.
[b]SIT (spoligotype international types) were assigned by SITVITWEB database and MIRU-VNTR plus Web tool.
[c]Order of loci: MIRU2, VNTR424, ETR-C, MIRU4, MIRU40, MIRU10, MIRU16, VNTR1955, MIRU20, QUB11b, ETR-A, QUB11a, VNTR2401, ETR-B, MIRU23, MIRU24, MIRU26, MIRU27, ETR-F, MIRU31, VNTR3690, QUB26, QUB4156, and MIRU39.
[d]Drug susceptibility test results. Isolates showed resistance against R (rifampicin), I (isoniazid), S (streptomycin), E (ethambutol).
[e]Amino acid substitutions are shown in the codon number. Del: deletion; –: PCR failed; WT: wild-type sequence. KatG Ser 315 Thr substitution was by *katG* G944C mutation.

clusters, cluster 1 isolates shared the same three-codon deletion in which the deletion pattern was identical. In the biggest cluster, cluster 13, four isolates shared the same substitution His526Asp and two of them had a mutation T-8C in the *inhA* promoter region. In cluster 6 and cluster 14, two isolates each shared a rare substitution, Asp516Phe (GAC → TTC) and Ser531Gln (TCG → CAG), respectively, both of which require a double mutation in a codon. In cluster 17, all four isolates shared a relatively rare substitution, Gln513Leu; moreover, the substitution Leu511Pro shared in cluster 11 was also rare. Most of the isolates sharing rare mutations were restricted to the same geographic locations (Table 5); however, none of these patients had been in personal contact with the others sharing the genetically same bacteria. Other demographic features can also be found in Table 5.

Discussion

This study is the first to demonstrate the genetic characteristics of MDR CAS isolates in Nepal and their high diversity is shown. The CAS family is reported as being highly prevalent among TB patients in certain regions of the Indian subcontinent, namely, Pakistan and the northern half of India (Hasan et al., 2006; Singh et al., 2007; Singh et al., 2015). Geographically, Nepal is located between two greatly TB-burdened countries, India and China, which contribute one third of the world's TB cases. Historically, Nepal has had an open border with India, and both Nepalese and Indian citizens often visit the bordering area for work, pilgrimage, and education. This regular interaction may provide the opportunity for direct transmission of the CAS family MTB between the Indian and Nepalese populations.

In the present study, the ratio of MDR-TB patients infected with the CAS family was higher in the age group >60 years than in the younger groups (odds ratio 3.25 compared to the 0–20 years age group; $p < 0.05$, Table 1). This association between the CAS family and elderly people might be related to the historical prevalence of CAS in Nepal and caused by reactivation of latent TB infection. However, when comparing the patient age distribution of clustered ($n = 47$) and non-clustered ($n = 98$) isolates, it was found that the average age of the clustered isolate-infected patients was significantly lower than that of the non-clustered group (*t*-test,

$p < 0.05$). This may suggest that younger generations were more likely to be infected with clustered isolates, indicating that these cases were the outcome of recent transmissions (**Supplementary Material**, Figure S2 in the online version, at http://dx.doi.org/10.1016/j.ijid.2017.06.010). This phenomenon may be explained by the higher social activity of younger generations compared with older generations, which likely contributes to a higher transmission risk.

It was found that spoligotyping alone could not identify diversity in the CAS family in Nepal due to the dominant cluster of CAS1_Delhi SIT26 contributing 60 isolates (Table 2). However, all 145 MDR CAS family isolates were successfully differentiated into 116 patterns including 18 clusters (clustering rate 32.4%) by combining spoligotyping and 24-locus MIRU-VNTR analysis (Figure 1). The optimization of these MIRU-VNTR loci was further investigated. As expected, it was possible to select an affordable locus set, as suggested by the authors of previous studies (Diab et al., 2016; Wang et al., 2011). Based on the cumulative HGDI analysis of 24 loci with clustering rates (Table 4), a set of 15 loci was shown to have the same discriminatory power as 24 loci. To obtain higher discriminatory power, two of the 24 loci reported by Supply et al. (2006) were replaced: Mtub29 and Mtub34, with ETR-F and QUB11a. Both of the newly replaced loci were listed among the top 15 discriminant loci that successfully worked for the Nepali MDR CAS cases (Table 4; **Supplementary Material**, Table S1). To save time and cost, smaller locus numbers of VNTR are better than larger numbers, even though MIRU-VNTR analysis may be more feasible at local research centers than other typing methods.

In addition, it was possible to discriminate clustered MDR isolates by conducting sequencing analysis of drug resistance-associated genes, *katG*, *rpoB*, and *inhA* promoter region. The *katG* 315 substitution is an INH resistance-associated mutation well known for its low fitness cost (van Soolingen et al., 2000). However, in highly TB-burdened countries, the majority of INH-resistant MTB isolates have this mutation, thus it cannot be used as a transmission marker, although it can still be used as a genetic marker of INH resistance (Aye et al., 2016; Poudel et al., 2012; Rahim et al., 2012). In contrast, *rpoB* mutations can be a good marker for MDR-TB transmission, as the variation in RIF resistance-associated mutation in RRDR is much higher than *katG* in highly TB-burdened countries (Aye et al., 2016; Poudel et al., 2012; Rahim et al., 2012). Having a mutation in RRDR strongly suggests that the isolate is RIF-resistant. RNA polymerase is an essential enzyme for bacteria, thus the majority of non-synonymous mutations in RRDR deteriorate, and frequently observed mutations in RIF-resistant MTB are limited. Even such 'acceptable' amino acid substitutions still exert adverse effects on the enzyme function; therefore, RRDR mutated survivors tend to have additional mutations known as 'compensatory mutations' inside the RRDR or nearby RNA polymerase components (de Vos et al., 2013).

In this study, two double mutations that occurred in one codon were found. One of them, Asp516Phe (GAC → TTC), found in clusters 6 and 7, may be an example of the aforementioned compensatory mutations, as the possible parent mutant Asp516Val (GAC → GTC) was found in both clusters (Table 5). As clusters 6 and 7 were different by only one locus with high polymorphism (MIRU10) in the lineage in MIRU-VNTR (Table 3), and all isolates were obtained from the same geographic location, the original MDR-MTB bearing Asp516Val might be spread throughout this area. As a result, some of the descendent bacteria may have acquired an additional mutation in the same codon, possibly due to the above-mentioned compensatory mutation, which may have been most suitable for the Asp516Val mutant. Since the variety of the compensatory mutation is also high in RRDR mutant cases (de Vos et al., 2013), the combined analysis of these regions may improve the detection rate of MDR-MTB transmissions.

Several complete matches of genotype, geographic location, and drug resistance-associated mutations were found between two or more isolates in some clusters, suggesting possible transmissions of MDR-MTB between individuals (Table 5). In particular, sharing a rare *rpoB* mutation in the cluster strongly suggested the acquisition of MDR-MTB via person-to-person contact. It was not possible to identify the exact epidemiological link between these patients, which suggests that a common transmission source may exist in the geographic area. In some cases, isolates were obtained from patients living in distant areas. For example, in the case of cluster 17, four isolates were collected in three different areas, Dharan (East), Nepalgunj (Mid West), and Kathmandu (Central), which are as far apart as 200–600 km. In this cluster, the first case was an old man in Kathmandu in 2009, and the remaining were young people visiting hospitals in each area later on. In the case of the patient in the Eastern area of Dharan, it was a new case of MDR-MTB that showed the same four-drug resistance as the others prior to TB treatment (Table 5). As Kathmandu is the capital city, people frequently visit from different areas of Nepal, and thus the city could be a site for transmission of infectious diseases (Poudel et al., 2013). This hypothesis, however, requires validation by further whole genome sequencing analysis.

The application of combined analysis of spoligotyping and MIRU-VNTR typing together with *rpoB* sequencing is considered an effective approach to determine the molecular epidemiology of MDR-MTB. The molecular analysis demonstrated that the MDR CAS family is highly diverse in Nepal, which suggests that the bacteria progressively acquired drug resistance and ultimately became MDR in each patient. Nonetheless, the characteristics of some clusters showed evidence of actual MDR-MTB transmission.

A large MDR-MTB outbreak would be more likely to occur among the Nepalese population if transmission trends observed in the present study grew out of control. Thus, the results highlight the importance of laboratory diagnosis of TB, intensified finding of cases, and timely and appropriate treatment of TB patients to cut the transmission chain. It is believed that the proposed 15-locus MIRU-VNTR typing scheme is well suited to assess the population structure of the MDR CAS family and trace back the transmission dynamics in Nepal. The results from the present study will likely contribute to improve epidemiological surveillance, which in turn could lead to the implementation of more effective control measures against the spread of MDR-MTB in Nepal and surrounding countries.

Funding

This study was supported by the Leading Program "Fostering Global Leaders in Veterinary Science toward Contributing to 'One Health"' from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), U.S.–Japan Cooperative Medical Science Programs from the Japan Agency for Medical Research and Development (AMED), Japanese Society for the Promotion of Science (JSPS) ROMPAKU Program, MEXT/JSPS KAKENHI grant number 15K08724, and Research Program on Emerging and Re-emerging Infectious Diseases from AMED.

Ethics statement

This study was approved by the Nepal Health Research Council (NHRC).

Conflict of interest

None.

Acknowledgements

We would like to thank the staff of the German Nepal Tuberculosis Project (GENETUP), Kathmandu, Nepal for helping with the collection and processing of samples. We would also like to thank Yukari Fukushima and Haruka Suzuki in the Division of Bioresources, Hokkaido University Research Center for Zoonosis Control for their technical support.

References

Ali A, Hasan Z, Tanveer M, Siddiqui AR, Ghebremichael S, Kallenius G, et al. Characterization of *Mycobacterium tuberculosis* Central Asian Strain 1 using mycobacterial interspersed repetitive unit genotyping. BMC Microbiol 2007;7:76.

Aye KS, Nakajima C, Yamaguchi T, Win MM, Shwe MM, Win AA, et al. Genotypic characterization of multi-drug-resistant *Mycobacterium tuberculosis* isolates in Myanmar. J Infect Chemother 2016;22:174–9.

Beggs ML, Eisenach KD, Cave MD. Mapping of IS*6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. J Clin Microbiol 2000;38:2923–8.

Comas I, Homolka S, Niemann S, Gagneux S. Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. PLoS One 2009;4:e7815.

Crawford JT. Genotyping in contact investigations: a CDC perspective. Int J Tuberc Lung Dis 2003;7:S453–7.

Demay C, Liens B, Burguière T, Hill V, Couvin D, Millet J, et al. SITVITWEB - A publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. Infect Genet Evol 2012;12:755–66.

de Vos M, Müller B, Borrell S, Black PA, van Helden PD, Warren RM, et al. Putative compensatory mutations in the *rpoC* gene of rifampin-resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. Antimicrob Agents Chemother 2013;57:827–32.

Diab HM, Nakajima C, Kotb SA, Mokhtar A, Khder NF, Abdelaal AS, et al. First insight into the genetic population structure of *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in Egypt. Tuberculosis 2016;96:13–20.

Filliol I, Driscoll JR, Soolingen D Van, Kreiswirth BN, Kremer K, Vale G, et al. Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. J Clin Microbiol 2003;41:1963–70.

Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 2006;103:2869–73.

Glynn JR, Vynnycky E, Fine PE. Influence of sampling on estimates of clustering and recent transmission of *Mycobacterium tuberculosis* derived from DNA fingerprinting techniques. Am J Epidemiol 1999;149:366–71.

Hasan R, Jabeen K, Ali A, Rafiq Y, Laiq R, Malik B, et al. Extensively drug-resistant tuberculosis, Pakistan. Emerg Infect Dis 2010;16:1473–5.

Hasan Z, Tanveer M, Kanji A, Hasan Q, Ghebremichael S, Hasan R. Spoligotyping of *Mycobacterium tuberculosis* isolates from Pakistan reveals predominance of Central Asian Strain 1 and Beijing isolates. J Clin Microbiol 2006;44:1763–8.

Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. J Clin Microbiol 1988;26:2465–6.

Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997;35:907–14.

Malla B, Stucki D, Borrell S, Feldmann J, Maharjan B, Shrestha B, et al. First Insights into the Phylogenetic Diversity of *Mycobacterium tuberculosis* in Nepal. PLos One 2012;7:1–8.

Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, et al. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc Natl Acad Sci USA 2001;98:1901–6.

National tuberculosis programme Nepal. Annual report 2009/2010, 2010. http://www.nepalntp.gov.np/theme/images/uploads/1358935947nnual-Report-2010.pdf.

National tuberculosis programme Nepal. Annual report 2013/2014, 2014. http://www.nepalntp.gov.np/theme/images/uploads/NTP_Annual_Report_2070-71_final.pdf.

Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey BD, Maharjan B, et al. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. Antimicrob Agents Chemother 2012;56:2831–6.

Poudel A, Maharjan B, Nakajima C, Fukushima Y, Pandey BD, Beneke A, et al. Characterization of extensively drug-resistant *Mycobacterium tuberculosis* in Nepal. Tuberculosis 2013;93:84–8.

Rahim Z, Nakajima C, Raqib R, Zaman K, Endtz HP, van der Zanden AG, et al. Molecular mechanism of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* from Bangladesh. Tuberculosis 2012;92:529–34.

Singh J, Sankar MM, Kumar P, Couvin D, Rastogi N, Singh S. Genetic diversity and drug susceptibility profile of *Mycobacterium tuberculosis* isolated from different regions of India. J Infect 2015;71:207–19.

Singh UB, Arora J, Suresh N, Pant H, Rana T, Sola C, et al. Genetic biodiversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in India. Infect Genet Evol 2007;7:441–8.

Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P, et al. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: Association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol 2003;3:125–33.

Stavrum R, Myneedu VP, Arora VK, Ahmed N, Grewal HMS. In-depth molecular characterization of *Mycobacterium tuberculosis* from New Delhi - Predominance of drug resistant isolates of the "modern" (TbD1-) type. PLoS One 2009;4:2–8.

Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 2006;44:4498–510.

van Soolingen D, de Haas PE, van Doorn HR, Kuijper E, Rinder H, Borgdorff MW. Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. J Infect Dis 2000;182:1788–90.

Varma-Basil M, Kumar S, Arora J, Angrup A, Zozio T, Banavaliker JN, et al. Comparison of spoligotyping, mycobacterial interspersed repetitive units typing and IS*6110*-RFLP in a study of genotypic diversity of *Mycobacterium tuberculosis* in Delhi, North India. Mem Inst Oswaldo Cruz 2011;106:524–35.

Wang J, Liu Y, Zhang CL, Ji BY, Zhang LZ, Shao YZ, et al. Genotypes and characteristics of clustering and drug susceptibility of *Mycobacterium tuberculosis* isolates collected in Heilongjiang Province, China. J Clin Microbiol 2011;49:1354–62.

Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTRplus: A web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Res 2010;38:326–31.

World health organization. Guidelines for surveillance of drug resistance in tuberculosis, 2009. http://apps.who.int/iris/bitstream/10665/44206/1/9789241598675_eng.pdf.

World Health Ogranization. Global tuberculosis report 2016. WHO/HTM/TB/2016.13. ISBN 978 92 4 156539 4.

Yasmin M, Gomgnimbou MK, Siddiqui RT, Refrégier G, Sola C. Multi-drug resistant *Mycobacterium tuberculosis* complex genetic diversity and clues on recent transmission in Punjab, Pakistant. Infect Genet Evol 2014;27:6–14.