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Characterization of extensively drug-resistant *Mycobacterium tuberculosis* in Nepal

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Running Head: Emergence of XDR-TB in Nepal

Summary

The emergence of extensively drug-resistant tuberculosis (XDR-TB) has raised public health concern for global control of TB. Although molecular characterization of drug resistance-associated mutations in multidrug–resistant isolates in Nepal has been made, mutations in XDR isolates and their genotypes have not been reported previously. In this study, we identified and characterized 13 XDR *Mycobacterium tuberculosis* isolates from clinical isolates in Nepal. The most prevalent mutations involved in rifampicin, isoniazid, ofloxacin, and kanamycin/capreomycin resistance were Ser531Leu in *rpo*B gene (92.3%), Ser315Thr in *katG* gene (92.3%), Asp94Gly in *gyr*A gene (53.9%) and A1400G in *rrs* gene (61.5%), respectively. Spoligotyping and multilocus sequence typing revealed that 69 % belonged to Beijing family, especially modern types. Further typing with 26-loci variable number of tandem repeats suggested the current spread of XDR *M. tuberculosis*. Our result highlights the need to reinforce the TB policy in Nepal with regard to control and detection strategies.

Key words: XDR, tuberculosis, Nepal

1. Introduction

Worldwide emergence of multi- and extensively drug-resistant tuberculosis (MDR and XDR-TB) has become a major obstacle to TB control. XDR-TB is a form of TB caused by *Mycobacterium tuberculosis* (MTB) strains, which is resistant to isoniazid (INH) and rifampicin (RIF), defined as multidrug-resistant MTB (MDR-MTB), as well as fluoroquinolone (FQ) and any of the second-line anti-TB injectable drugs, amikacin (AMK), kanamycin (KAN), or capreomycin (CAP). By the end of 2010, 68 countries had reported at least one case of XDR-TB.[1] XDR-TB is the result of an adverse treatment outcome of MDR-TB; many cases are never diagnosed due to limitations in laboratory capacity to test for second-line drug resistance.[2] Treatment of XDR-TB patients is more challenging and less successful than that of patients with other types of TB.[3] An extremely high death rate from XDR-TB was reported in patients co-infected with HIV in South Africa. [2, 4]

In Nepal, TB is a major public health problem. The incidence of all forms of TB was estimated to be 173/100,000 population while the incidence of new smear-positive cases was at 77/100,000 in 2008. The four surveys conducted between 1996 and 2007 have indicated the fluctuating prevalence of MDR-TB among new cases of between 1.1% and 3.7% (1.1% in 1996, 3.7% in 1999, 1.4% in 2001 and 2.9% in 2007). The latest estimate of MDR-TB is 2.9% and 11.7% among new and recurrent cases, respectively.[1, 5, 6] Although the prevalence of drug-resistance confirming mutations in MDR-TB isolates in Nepal have been reported recently,[6] to the best of our knowledge, no published data on mutations and genotypes of XDR-MTB strains are currently available from Nepal.

Molecular epidemiological studies of *M. tuberculosis* strains have identified variability in the phylogeography of strains globally.[7, 8] Beijing strains are most prevalent globally and also associated with enhanced acquisition of drug resistance; however their resistance patterns varied regionally. [9] Drug resistance in *M. tuberculosis* is commonly caused by mutations in various genes. Previous works have indicated that mutations within 81-bp core region of the RNA polymerase β-subunit gene (*rpo*B) gene are the cause of RIF resistance in more than 90% of cases.[10, 11] In contrast, several different loci are known to be involved in INH resistance, especially *katG* and *inhA*.[6, 10] Mutations in a conserved quinolone resistance-determining region (QRDR) of the *gyrA* or *gyrB* genes encoding DNA gyrase are often involved in fluoroquinolone (FQ) resistance.[12]

Resistance to aminoglycosides (KAN and AMK) and CAP is attributed to mutations in 16s rRNA (*rrs*) gene. [13, 14]

The present study documents drug resistance-associated mutations in XDR isolates from Nepal. To gain an insight into the epidemiology of these isolates, genotyping by using spoligotyping, multilocus sequence typing (MLST) and variable number of tandem repeats (VNTR) were also performed.

2. Materials and methods

2.1. *M. tuberculosis* isolates

A total of 109 MDR *M. tuberculosis* clinical isolates were randomly selected from isolates bank at German Nepal Tuberculosis Project (GENETUP), Nepal, collected over a 3-year period from 2007 to 2010. Each isolates were recovered from individual patients with pulmonary TB.

2.2. Antibiotic susceptibility testing

Testing for susceptibility to first- and second-line drugs was carried out at GENETUP using the conventional proportional method on Löwenstein-Jensen medium according to the World Health Organization guidelines[15] with the following critical drug concentrations: INH (Cat No. 2261/0801; Fatol Arzneimittel GmbH, Schiffweiler, Germany); 0.2 µg/ml, RIF (Cat No. 004030; Fatol); 40 µg/ml, STR (Cat No. S6501; Sigma-Aldrich, St. Louis, MO); 4 µg/ml, EMB (Cat No. 1237/0806; Fatol); 2 µg/ml, ofloxacin (OFX; Cat No. O8757; Sigma-Aldrich); 2 µg/ml, KAN (Cat No. 60615 ; Sigma-Aldrich); 30 µg/ml and CAP (Cat No. C4142 ; Sigma-Aldrich); 40 µg/ml.

2.3. DNA extaction

DNA was prepared for PCR by mechanical disruption, as described previously.[6] Briefly, the colonies were suspended in TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA in a 2 ml screw-cap vial, one-fourth of which was filled with 0.5 g glass beads (0.1 mm) (Bio Spec Products Inc., Bartlesville, OK). Mycobacterial cells were disrupted by shaking with 0.5 ml chloroform on a cell disrupter (Micro smash; Tomy Seiko Co. Ltd., Tokyo, Japan) for 1 min. After centrifugation, the DNA in the upper layer was concentrated by ethanol precipitation and dissolved in 100 µl TE buffer.

2.4. PCR amplification and DNA sequencing of drug resistance-associated genes

PCR reactions were performed in a 20 µl mixture consisted of 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 µM of each primer (Primers for rrs, gyrA and gyrB in Table 1 and those in Poudel A et al. [6] for *rpoB*, *katG* and *inhA* gene segment amplification). One U GoTaq DNA Polymerase (Promega, Madison, WI), GoTaq buffer and 1 µl DNA

template. The reactions were carried out in a thermal cycler (Bio-Rad Laboratories, Ipswich, MA) under the following conditions: initial denaturation at 96 °C for 60s followed by 35 cycles of denaturation at 96 °C for 10 s, annealing at 55° C for 10 s and extension at 72 °C for 30 s with a final extension at 72 °C for 5 min. PCR products were sequenced according to the manufacturer's instructions with the same primers used for PCR and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., Carlsbad, CA) using an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.5. Phylogenetic markers

Spoligotyping was performed according to the standard protocol.[16] and the spoligotype in the binary format was compared with the SpolDB4 database.[17] Another molecular epidemiological investigation was performed by PCR amplification of the 26 variable *M. tuberculosis* microsatellites and assigned an allele number based on the number of repeats as described previously.[18] A combined spoligotype-VNTR UPGMA3 dendrogram was computed and drawn using Bionumerics 6.0 version software (Applied Maths, Sint-Marten-Latems, Belgium). MLST targeting 10 chromosomal positions were performed according to Filliol *et al*.[19]

3. Results

3.1. Drug-susceptibility patterns

Among 109 MDR-MTB isolates obtained, 13 were found to be XDR (Table 2). Three of the patients having XDR-TBs (84, 90 and 123) were naive for MDR treatment. Of the remaining 96 isolates, 41, 1, and 1 were mono-resistant to OFX, KAN, and CAP, respectively, and categorized as pre-XDR-MTB.

3.2. Geographical distribution of XDR *M.tuberculosis* isolates

The XDR-MTB isolates were originated from patients living in five main cities of Nepal (Figure 1): Kathmandu (n = 7), Pokhara (n = 3), Butwal (n = 1), Bhairahawa (n = 1) and Dhangadhi (n = 1). The number of XDR-TB in Kathmandu correlates well with its high population.

3.3. Mutations identified in the *rpoB, katG, inhA, gyrA, gyrB* and *rrs* genes

Sequence analysis identified the most frequent mutations conferring Ser to Leu amino acid substitution at position 531 (Ser531Leu) in *rpo*B (12/13), Ser315Thr in *kat*G

(12/13), Asp94Gly in *gyr*A (7/13), and a mutation from A to G at nucleotide position 1400 (A1400G) in *rrs* (9/13). Other mutations with lower rates were seen in *rpo*B (Asp516Val; 1/13), *inhA* regulatory region (C-15T; 1/13), *gyrA* (Ser91Pro; 1/13, Asp94Ala; 2/13, Asp94Asn; 1/13, Asp94His; 1/13, and Asp94Tyr; 1/13), and *rrs* (C1401T and G1483T; two each), while none had mutations in the quinolone resistance-determining region of *gyr*B (Table 2).

3.4. Spoligotyping and MLST

Among XDR-TB isolates, Spoligotyping revealed the predominance of Beijing family strains (9/13). In addition, 1 strain of CAS family, 2 strains of T family (T1 and T2) and 1 strain of undefined type were also identified. MLST confirmed 8 isolates with Bejing spoligotype belonged to modern types (Table 2).

3.5. Cluster analysis by VNTR

VNTR typing grouped the isolates into seven unique patterns and two clusters (Figure 2). Each cluster contained three isolates of the Beijing family. Among the clustered isolates, 86 and 103 in a cluster (cluster 1) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-C1401T), whereas 84 carried a distinct mutation in *rrs* (A1400G). Similarly, 118 and 161 in another cluster (cluster 2) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-A1400G) and 123 showed a distinct mutation pattern (C-15T at *inh*A regulatory region instead of *katG*-Ser315Thr for INH resistance, *gyrA*-Asp94Asn for FQ resistance, and *rrs*-G1483T for KAN/CAP resistance).

4. Discussion

In this study, we investigated drug resistance-associated mutations and genotypes of XDR-MTB isolates in Nepal. This study also raises concerns over the high proportion of pre-XDR-TB in Nepal. The high rate of pre-XDR-MTB isolates implied the inappropriate usage of drugs, especially FQs, including OFX. OFX is the most commonly prescribed antibiotic for respiratory tract infection in Nepal and this might lead to the emergence of pre-XDR-TB with resistance to OFX. As drug resistance in *M. tuberculosis* is due to the stepwise accumulation of mutations in the genome, this pool of pre-XDR-MTB isolates are always at the risk of developing XDR-TB.

Sequence analysis of the hot spot regions of various genetic loci showed that the most common mutations among XDR isolates were Ser531Leu of *rpo*B, Ser315Thr of

*kat*G, Asp94Gly of *gyr*A and A1400G of *rrs* for RIF, INH, OFX and KAN/CAP resistance, respectively. Other studies have also reported similar mutations among XDR-TB isolates from different countries. [20, 21, 22, 23] As mutations such conferring amino acid substitutions, Ser531Leu in *rpo*B and Ser315Thr in *kat*G with low fitness costs are known to dominate the drug-resistant isolates. [24]

Genotyping of the isolate by spoligotyping and MLST pointed out the predominance of strains belonging to the modern type Beijing genotypes. The similar involvement of XDR-MTB by modern type Beijing genotypes has been reported from South Africa, [4] India, [20] and China, [23] while the ancient type Beijing family predominates in Japan. [25] Over-representation of Beijing genotype in XDR-MTB in this study compared to the lower prevalence of this genotype in non-MDR and MDR isolates (33 and 51 %, respectively; data not shown) supported the previous study that this genotype has been associated with drug resistance, [4, 26] because of its higher mutation rates and lower fitness costs with specific mutations. [24] The significantly low average age of patients suffering from Beijing genotype MTB compared to patients suffering from MTB with other genotypes (23.1 ± 4.8 vs 39.5 ± 4.9 years old; Table 2) may suggest the higher transmissibility of Beijing genotype XDR-MTB among the young generation because of their frequent movement. [27]

Although the numbers of isolates were small, complete matches of VNTR, including three hypervariable loci (QUB 11a, QUB 3232, QUB 3336) and drug resistance-associated mutations between two isolates in each cluster, suggested the possible transmission of XDR-TB in Nepal. MDR treatment of a patient who was the source of strain No. 103 started 3rd, April 2006 and the duration of MDR treatment was 12 month. In contrast, that of strain No. 86 started 29th, October 2009 and the duration of MDR treatment was 1 month. By these facts, we arrived at the idea that patient with strain No. 103 might be a source of transmission of XDR-TB and that with strain No. 86 might be a recipient. Alternatively, there might be common transmission source(s) to these patients. Situation was different in another cluster. MDR treatment of a patient who was the source of strain No. 118 started 23rd, September 2008 and the duration of MDR treatment was 2 month and primary XDR-TB was suspected. In contrast, that of strain No. 161 started 28th, January 2009 and the duration of MDR treatment was 14 month. The existence of common infection source of these strains was supposed. The transmission of XDR-TB was also speculated from the fact that three patients (from whom strain No. 84, 90 and 123 were isolated) were naive for MDR-TB treatment. It is

interesting that transmission of XDR-TB were speculated not only within Kathmandu but also between Kathmandu and Dhangadhi, apart more than 650 km (Figure 1). As Kathmandu is the capital of Nepal and people come and go frequently from different parts of Nepal, transmission between people living in Kathmandu and those living far from Kathmandu might be possible. Indeed, the patient from whom strain No. 161 was isolated has a history of traveling to Kathmandu. The possibility of transmission of XDR-TB seemed to be high, especially in cluster 1 (including strains No. 86 and 103), because the *rrs*-C1401T mutation carried by both strain No. 86 and 106 was rare between KAN/CAP-resistant isolates.[10, 13] On the other hand, care should be taken when concluding XDR-TB transmission in cluster 2 (including strains No. 118 and 161) as both of the mutations, *gyrA*-Asp94Gly and *rrs*-A1400G, have been reported to be rather common in OFX- and KAN/CAP-resistant MTB, respectively, and the distance between the two cities is great. The high rate of pre-XDR-TB in MDR-TB might suggest the acquisition of XDR phenotype during successive transmission as these strains belong to the Beijing family, known to have higher mutation-acquiring capacity. The high number of MDR-TB patients who stop treatment in Nepal could also explain this high drug resistance acquisition rate. [5] Both the possibility of direct transmission and acquired resistance should be considered equally for XDR-TB in Nepal.

5. Conclusion

The majority of XDR-MTB isolates in this study belonged to the Beijing family. Infections of this family were more common among younger generation than those belonging to other spoligotype families. In addition, the identical pattern of VNTR and drug resistance-associated mutations suggested the possible transmission of Beijing genotype XDR-MTB among people in Nepal. Our findings emphasize the urgent need to identify patients suffering from XDR-TB with Beijing genotype MTB and to treat them in isolated wards for a better control program to prevent the spread of this incurable disease.

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Conflict of interest statements: All authors have no competing interests.

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Figure Legends

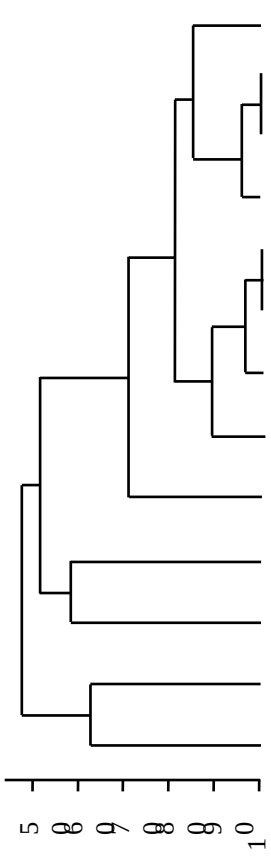
Figure 1. Geographical location of XDR-TB isolation. Cities where XDR-MTB has been isolated are indicated by a closed circle.

Figure 2. Dendrogram and schematic representation of VNTR typing and spoligotyping results obtained with 13 XDR-TB isolates in Nepal. Column A: dendrogram (UPGMA method, distance matrix average of spoligotyping-based and VNTR) built with Bionumerics version 6, B: strain identification, C: 26 loci VNTR results, D: spoligotyping-based defined clades; E: spoligotyping international type, F: geographical location, G: MDR treatment start date and H: Duration of MDR treatment.

Poudel *et al.* Fig. 1



Poudel *et al* Fig. 2



A	B	C																								D	E	F	G	H
		M10	M16	M26	M31	M40	V424	V1955	V3690	E-A	E-C	Q11b	Q26	M4	V2401	V4156	M2	M20	M23	M24	M27	M39	E-B	E-F	Q11a	Q3232	Q3336					
	155	3	3	7	5	3	3	4	3	4	4	5	8	2	4	3	2	2	5	1	3	3	1	3	6	14	7	Beijing	1	Butwal	17.05.2010	5
	118	3	3	7	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	8	11	7	Beijing	1	Kathmandu	23.09.2008	2
	161	3	3	7	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	8	11	7	Beijing	1	Dhangadhi	28.01.2009	14
	123	3	3	7	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	8	9	7	Beijing	1	Kathmandu	01.02.2010	0
	103	3	3	5	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	>10	11	5	Beijing	1	Kathmandu	03.04.2006	12
	86	3	3	5	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	>10	11	5	Beijing	1	Kathmandu	29.10.2009	1
	84	3	3	5	5	3	4	4	3	4	4	4	8	2	4	3	2	2	5	1	3	3	2	3	7	11	5	Beijing	1	Kathmandu	27.10.2009	0
	139	3	3	5	5	2	4	4	3	4	4	6	8	2	4	3	2	2	5	1	3	3	2	3	8	11	5	Beijing	1	Pokhara	24.07.2006	27
	90	3	3	7	2	3	4	3	3	4	4	6	7	2	4	5	2	2	5	1	3	3	2	3	8	N	7	Beijing	265	Kathmandu	11.12.2009	0
	108	6	3	7	5	2	4	1	5	3	2	2	8	2	2	3	2	2	5	1	3	3	2	3	9	>14	10	CAS	599	Kathmandu	21.11.2006	10
	142	5	2	8	5	2	5	3	3	4	2	2	8	2	2	5	2	2	5	1	3	2	2	3	9	9	11	New	New	Pokhara	26.04.2009	12
	140	3	3	5	5	5	2	1	1	3	4	5	2	2	2	3	2	1	5	1	3	2	2	3	5	4	9	T2	1077	Pokhara	14.01.2008	7
	151	3	1	5	3	2	2	1	5	2	4	4	4	2	2	3	2	2	5	1	3	2	2	3	5	6	8	T1	1077	Bhairahawa	24.09.2009	14

Table 1. Nucleotide sequence of primers used for PCR and sequencing.

Locus	Primer	Nucleotide sequence (5'-3')	Target region	Product size (bp)
gyrA	TB *gyrA* S	AGCGCAGCTACATCGACTATGCG	220-339	321
	TB *gyrA* AS	CTTCGGTGTACCTCATCGCCGCC		
gyrB	TB gyrB S	CGGCACGTAAGGCACGAGAG	1373-1770	398
	TB *gyrB* AS	GAACCGGAACAACAACGTCAAC		
rrs	TB *rrs* S	AGTCCCGCAACGAGCGCAACCC	1350 – 1550	665
	TB *rrs* AS	GATGCTCGCAACCACTATCCA		

Table 2: Antimicrobial susceptibility profile and mutation pattern of the different drug-target genes or regions among XDR isolates.

Strain No.	Drug susceptibility profile*							Mutation pattern in different drug-target genes or regions†						Spoligotype based clade with ST	Geographical location	age of patient
	RFP	INH	STR	EMB	OFX	KAN	CAP	*rpo*B	*kat*G	*inh*A regulatory region	*gyr*A	*gyr*B	*rrs*			
84	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt§	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	21
86	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	16
90	R	R	R	R	R	R	R	Asp516Val	Ser315Thr	wt	Ser91Pro	wt	A1400G	Beijing (Ancient)	Kathmandu	26
103	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	24
108	R	R	S	S	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	CAS	Kathmandu	40
118	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	25
123	R	R	R	R	R	R	R	Ser531Leu	wt	C-15T	Asp94Asn	wt	G1483T	Beijing (Modern)	Kathmandu	21
139	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	Beijing (Modern)	Pokhara	25
140	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	T2	Pokhara	33
142	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Tyr	wt	G1483T	New	Pokhara	45
151	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94His	wt	A1400G	T1	Bhairahawa	40
155	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Butwal	18
161	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Dhangadhi	32

*INH, isoniazid; RFP, rifampicin; STR, streptomycin; EMB, ethambutol; OFX, ofloxacin; KAN, kanamycin; CAP, capreomycin; R, resistant; S, susceptible.

† Mutations in rpoB, katG and gyrA are presented as amino acid changes with codon position; mutations in rrs gene and inhA promoter region are presented as nucleotide changes with mutation position.

§ WT, wild type.