Characterization of drug-resistant *Mycobacterium tuberculosis* strains isolated in Nepal

（ネパールにおいて分離された薬剤耐性結核菌株の特徴）

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CONTENTS

ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
INH	Isoniazid
RIF	Rifampicin
EMB	Ethambutol
PZA	Pyrazinamide
RpoB	RNA polymerase β-subunit
KatG	Catalase-peroxidase
FQ	Fluoroquinolone
gyrA	Gene encoding DNA gyrase A subunit
gyrB	Gene encoding DNA gyrase B subunit
MDR-TB	Multidrug-resistant tuberculosis
KAN	Kanamycin
AMK	Amikacin
CAP	Capreomycin
XDR-TB	Extensively drug-resistant tuberculosis
VNTR	Variable number of tandem repeats

rpoB	Gene encoding RNA polymerase β-subunit
RRDR	Rifampicin resistance-determining region
katG	Gene encoding catalase-peroxidase
InhA	Enoyl-acyl carrier protein reductase
GENETUP	German Nepal Tuberculosis Project
STR	Streptomycin
PCR	Polymerase chain reaction
TE	Tris-EDTA
EDTA	Ethylenediaminetetraacetic acid
RIF^r	Rifampicin-resistant
Ser531Leu	An amino acid substitution from serine to leucine at position 531
RIF^s	Rifampicin-susceptible
INH^r	Isoniazid-resistant
INH^s	Isoniazid-susceptible
Ser315Thr	An amino acid substitution from serine to threonine at position 315
WT	Wild type

QRDR	Quinolone resistance-determining region
rrs	Gene encoding 16S rRNA
MLST	Multilocus sequence typing
OFX	Ofloxacin
SpolDB4	The fourth international spoligotyping database
MTB	*Mycobacterium tuberculosis*
Asp94Gly	An amino acid substitution from aspartic acid to glycine at position 94
A1400G	An nucleotide substitution from adenosine to guanosine at position 1400
QUB	Queen's University Belfast

PREFACE

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, which currently presents an immense global health challenge. It ranks as the second leading cause of death from an infectious disease worldwide (67, 68). An estimated one third of the world's population is infected with the tubercle bacilli; with 95% of TB cases occur in developing countries. The World Health Organisation (WHO) estimates that there were 9 million new cases of TB and 1.4 million TB deaths globally in 2011 (68). TB normally affects the lungs (pulmonary TB) and can affect other sites as well (extrapulmonary TB). The disease is spread by the air when people who are sick with pulmonary TB spout bacteria during coughing, sneezing and speaking (9). In general, infection by the pathogen does not necessarily result in the development of clinical symptoms and relatively small proportion (5-10%) of these individuals will progress to active disease each year. The remaining proportion (90-95%) of infected individuals will initially be asymptomatic and undergo latent infection, from which reactivation may occur when immune system of the patient becomes weakened (68).

If patients are not treated properly, TB can be fatal. New cases of drug-susceptible TB are treated with a 6-month regimen of four first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) (68). RIF is a broad spectrum rifamycin derivative that interferes the synthesis of mRNA by binding to the ß subunit of RNA polymerase (RpoB) in bacterial cells (38, 46, 71). INH is a prodrug that requires activation by *M. tuberculosis* catalase-peroxidase (KatG) (72), to generate a range of reactive oxygen species and reactive organic radicals, which then attack multiple targets in the tubercle bacillus. The primary target of the inhibition is the cell wall

mycolic acid synthesis pathway (Figure 1) (62). EMB inhibits the formation of mycobacterial membrane (38, 46). The mechanism of action of PZA is poorly understood. The only known mechanisms are it disrupts membrane energetics and inhibits membrane transport function in *M. tuberculosis* (71).

Fluoroquinolones (FQs) are considered to be important second-line drugs recommended for the treatment of multidrug-resistant TB (MDR-TB) (16, 36, 73). They act by inhibiting DNA supercoiling, thus preventing replication and cell division. They block a type II topoisomerase (called DNA gyrase) of *M. tuberculosis*, a heterotetramer consisting of two A and B subunits coded by the *gyrA* and *gyrB* genes (16, 46 55). Among second-line anti-TB drugs, aminoglycosides (kanamycin [KAN] and amikacin [AMK]) and cyclic peptides (capreomycin [CAP] and viomycin) inhibit protein synthesis by inhibiting the normal function of ribosomes (Figure 1) (32, 46, 54).

The emergence and spread of drug-resistant strains of *M. tuberculosis* throughout the world possess a serious threat to TB control. Resistance to anti-TB drugs arises due to a variety of reasons, such as the failure to detect resistance to TB drugs, interrupting treatment, omitting one or more drugs from the recommended prescription and suboptimal dosage. Thus, *M. tuberculosis* can become resistant to multiple drugs in the period of a few months (11, 23, 69). Essentially, drug resistance arises in areas with improper TB control programmes. A patient who develops active disease with a drug-resistant TB strain can transmit this form of TB to other individuals (69).

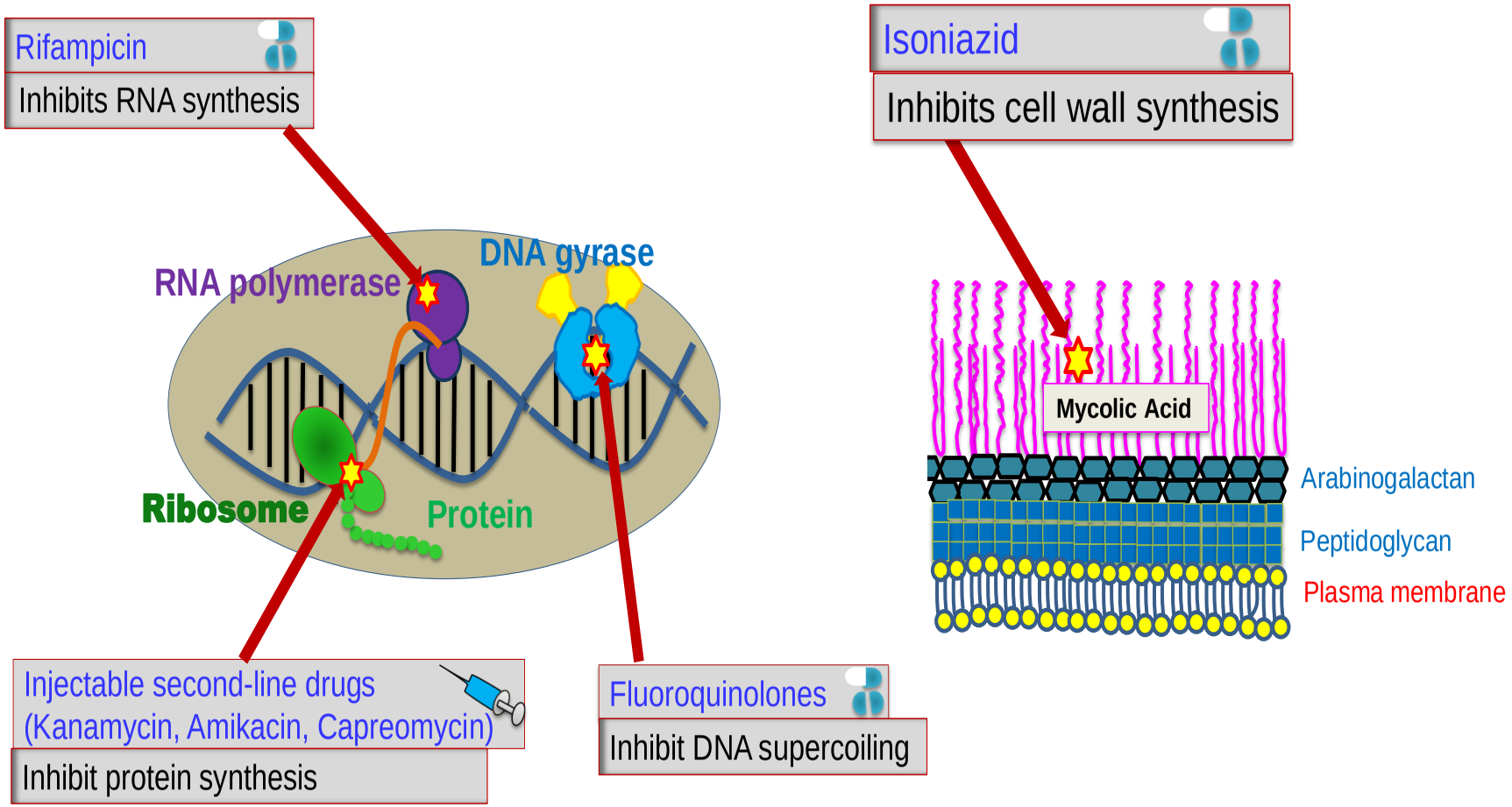

Figure 1. Commonly used TB drugs and their targets.

MDR-TB, which is caused by bacteria that are resistant to least two first-line drugs including INH and RIF and, extensively drug resistant TB (XDR-TB), which is caused by bacteria that are resistant to INH and RIF as well as any FQs and any of the second-line anti-TB injectable drugs (AMK, KAN or CAP) (66, 69). WHO estimates that from 220,000 to 400,000 of MDR-TB cases occur among TB cases notified in the world in 2011. About 60% of these drug-resistant TB cases occur in Brazil, China, India, the Russian Federation and South Africa (that are referred as BRICS countries) (Figure 2) (69). By the end of 2010, 68 countries had reported at least one case of XDR-TB (Figure 3) (66).

Genotyping of *M. tuberculosis* plays an increasing role for understanding the epidemiology and biology of TB (60). Genotyping techniques are important for molecular epidemiological investigations of TB such as defining chains of ongoing transmission and differentiating patient relapse from exogenous re-infection, and defining the evolutionary background of clinical isolates (4, 30). Over the past years, several methods have been developed to discriminate *M. tuberculosis* strains: insertion sequence 6110 restriction fragment length polymorphism (59), spacer oligonucleotide typing (spoligotyping) (24), variable-number tandem repeat (VNTR) (51), single-nucleotide polymorphisms (14) and large sequence polymorphisms (57).

Nepal is a landlocked country in South East Asia, bounded to the north by China and to the south by India, sharing an open border with India. In Nepal, TB is a major public health problem. The incidence of all forms of TB was estimated to be 173/ 100,000

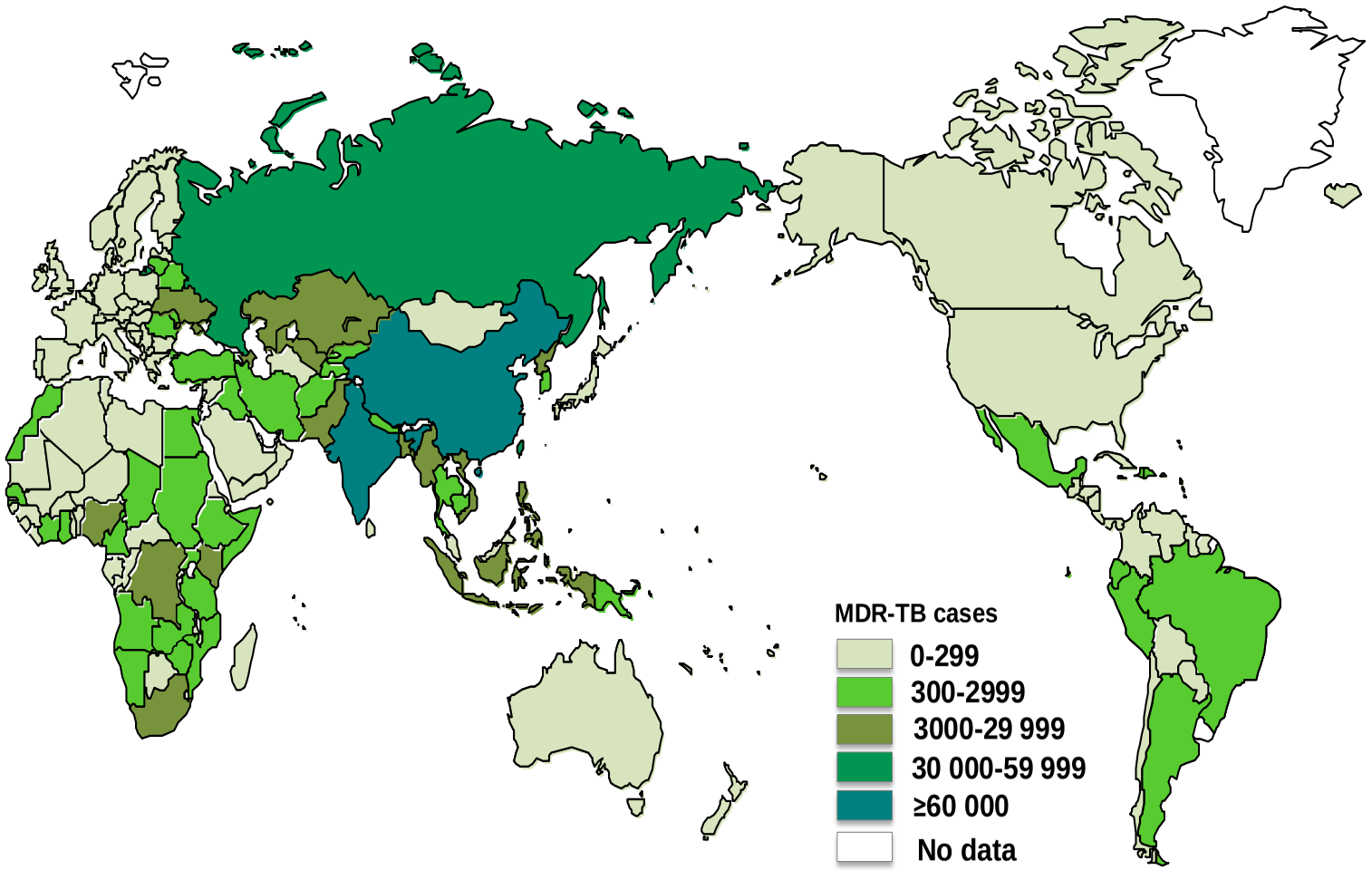

Figure 2. Number of MDR-TB cases estimated to occur among notified pulmonary TB cases, 2011 (69).

Figure 3. Distribution of countries and territories reporting to WHO at least one case of XDR-TB as of January 2010 (66).

population while the incidence of new smear-positive cases was at 77 per 100,000 in 2008. The four surveillances 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 (28, 44, 45).

Rapid determination of the antimicrobial susceptibility pattern in clinical isolates of *M. tuberculosis* is important for the early administration of appropriate therapeutic agents for the prevention of additional resistance development (41). In this context, the molecular characterization of drug resistance by identifying mutations in associated genes will be applicable for developing a potential rapid molecular drug susceptibility test as an alternative to conventional methods (31, 43).

The present thesis consists of two chapters; in chapter I, I have investigated the type and frequency of drug resistance-conferring mutations that occurred among *M. tuberculosis* clinical isolates that were phenotypically MDR by DNA sequencing. I have also compared the frequency of different mutations with those in isolates circulating in the surrounding countries. In chapter II, I have described drug resistance-associated mutations in XDR isolates and analyzed the genetic background of these isolates by using a molecular approach.

CHAPTER I

Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal

Introduction

The collection of data from different countries has indicated that resistance to RIF in >90% of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the *rpoB* gene, called the RIF resistance-determining region (RRDR) (21, 46, 48, 56). In contrast, INH resistance is mediated by mutations in several genes, most frequently within the *katG* gene, encoding a catalase-peroxidase which transforms INH into its active form (19, 26, 46), and in the promoter region of *inhA*, encoding a putative enzyme involved in mycolic acid biosynthesis. An upregulation mutation in the *inhA* promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism (46).

In this chapter I, I sought to determine the prevalence of resistance-associated mutations in three specific genes *(rpoB, katG,* and *the inhA* promoter region) of *M. tuberculosis* isolates in Nepal and to compare the frequency of different mutations with those in isolates circulating in the surrounding countries.

Materials and Methods

Isolates.

In total, 109 and 49 samples were randomly selected from MDR and non-MDR clinical isolates, respectively, in isolates bank at the German Nepal Tuberculosis Project (GENETUP) over a 3-year period from 2007 and 2010. The isolates were recovered from 158 patients living in nine different cities of Nepal, six of which have an open border with northern India. Of 109 MDR isolates, the numbers of isolated from each city were as follows: Kathmandu (n = 70), Biratnagar (n = 8), Bhairahawa (n = 8), Pokhara (n = 7), Birgunj (n = 4), Nepalgunj (n = 4), Dhangadi (n = 4), Butwal (n = 3), and Sarlahi (n = 1). Of the non-MDR isolates, 48 were obtained from patients in Kathmandu, and 1 was obtained from Biratnagar. Histories of previous TB treatment were available in 94.5% of the MDR and 42.9% of the non-MDR patients. A drug susceptibility test was performed using Löwenstein-Jensen medium by a conventional proportional method with the following critical drug concentrations of INH, RIF, streptomycin (STR), and EMB: 0.2, 40, 4, and 2 μg/ml, respectively (7).

DNA extraction.

DNAs were prepared for polymerase chain reaction (PCR) by mechanical disruption, as described previously (54). 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; BioSpec Products, Inc., OK). Mycobacterial cells were disrupted by shaking with 0.5 ml of chloroform on a cell disrupter (Micro Smash; Tomy Seiko Co., Ltd., Tokyo, Japan) for 1 min. After centrifugation, the DNAs in

the upper layer were concentrated by ethanol precipitation and dissolved in 100 µl of TE buffer.

Species differentiation multiplex PCR.

M. tuberculosis species were identified on the isolates by a multiplex PCR with primer pairs designed to amplify three genetic regions (*cfp*32, RD9, and RD12), as described previously (39).

Sequencing of the *rpoB* and *katG* encoding regions and the *inhA* promoter region.

PCRs were performed in a 20 µl mixture containing 0.25 mM (each) deoxynucleoside triphosphates, 0.5 M betaine, 0.5 µM concentrations of each primer (Table 1), 1 U of GoTaq DNA polymerase (Promega, WI), GoTaq buffer, and 1 µl of DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories, CA) under the following conditions: denaturation at 96°C for 60 s, followed by 35 cycles of amplification at 96°C for 10 s, 55°C for 10 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The presence of PCR products was confirmed by agarose gel electrophoresis. PCR products were sequenced according to the manufacturer's protocol with the primers TB *rpoB* S, TB *katG* S, and TB *inhA* S for *rpoB*, *katG*, and *inhA*, respectively, and the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies Corp., CA) using an ABI Prism 3130xl Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared to wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9).

Table 1. Primers used for PCR amplification and sequencing of drug resistance associated genes in *M. tuberculosis*

Locus	Primer	Nucleotide sequence (5'-3')	Target region (position)	Product size (bp)
rpoB	TB *rpoB* S	CAGGACGTGGAGGCGATCAC	1519-1599[a]	278
	TB *rpoB* AS	GAGCCGATCAGACCGATGTTGG		
katG	TB *katG* S	ATGGCCATGAACGACGTCGAAAC	823-1140	392
	TB *katG* AS	CGCAGCGAGAGGTCAGTGGCCAG		
inhA	TB *inhA* S	TCACACCGACAAACGTCACGAGC	-50 to -1	231
	TB *inhA* AS	AGCCAGCCGCTGTGCGATCGCCA		

[a] Corresponding *E. coli* numbering was used for *rpoB*

Results

Drug susceptibility patterns.

Of the 109 MDR isolates, 102 were resistant to three or more first-line anti-TB drugs (Table 2). Forty-nine non-MDR isolates consisted of 41 fully susceptible and 2, 3, and 1 isolates with monoresistance against INH, STR, and EMB, respectively. Two isolates were resistant to both INH and STR.

Species identification.

All 158 isolates showed three amplified bands corresponding to *cfp*32, RD9, and RD12 by multiplex PCR and were classified as *M. tuberculosis* (Figure 4).

Mutations in the *rpoB* gene.

Mutations in the RRDR of the *rpoB* gene were identified in 106 of 109 RIF-resistant (RIF^r) isolates (Table 3). A single nucleotide alteration in codon 531, resulting in the amino acid substitution of Ser to Leu, was most prevalent and observed in 62 isolates (56.9%). The second most affected codons were 516 and 526, which were found in 17 (15.6%) isolates each, and had 3 and 6 types of amino acid substitutions, respectively. Five (4.6%) isolates had a mutation in codon 513, and three (2.8%) had a mutation in codon 533. An insertion of Phenylalanine between codons 514 and 515 was observed in two (1.8%) isolates, one of which had an additional point mutation affecting codon 531. Two isolates carried double mutations in two separate codons, i.e., codons 513 and 526 and codons 516 and 533, respectively. No mutations were detected in the remaining 3 (2.8%) RIF^r and 49 RIF-susceptible (RIF^S) isolates.

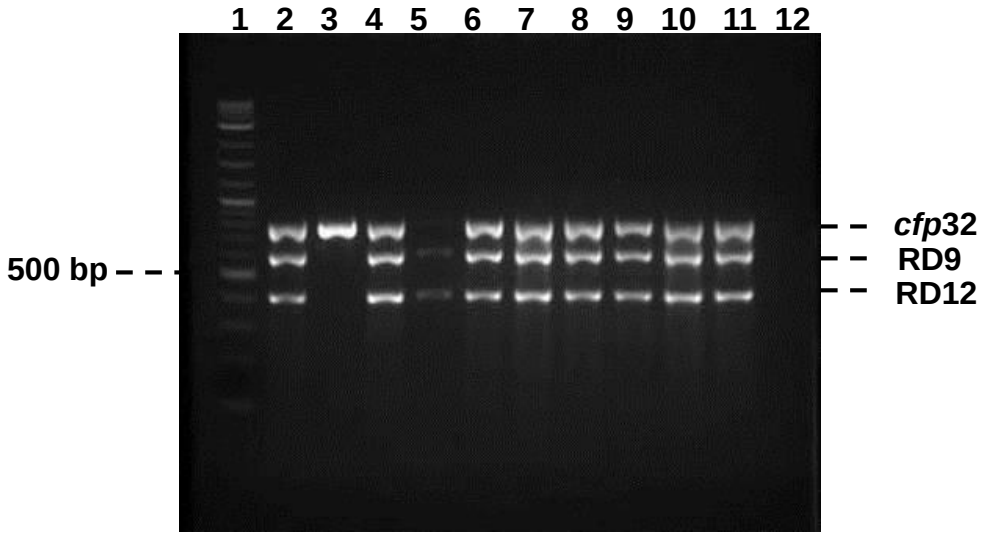

Figure 4. *M. tuberculosis* complex-discrimination multiplex PCR. PCR products were analyzed on 2.0% agarose gel electrophoresis followed by ethidium bromide stain. Lanes: 1, 50-bp ladder; 2, *M. tuberculosis*; 3, *M. bovis*; 4 to 11, *M. tuberculosis*; 12, negative control.

Mutations in *katG* encoding region and *inhA* promoter region.

Of 113 phenotypically INH^r isolates, 99 (87.6%) had *katG* mutations, the vast majority of which was the commonly described substitution KatG (Ser315Thr) (Table 4). Only one isolate had a Ser to Asn substitution at KatG position 315 (*katG* 315). KatG (Gly299Ser) and KatG (Asp329Ala) mutations were detected in two INH^r isolates. One isolate showed double mutations in two separate *katG* codons: Thr275Ala and Ser315Thr. Mutations in the *inhA* promoter region were observed in 14 (12.4%) INH^r isolates; 12 of which had a mutation at −15 in the *inhA* promoter. Among the isolates with mutation in *inhA* promoter, three had additional mutation in *katG* 315, and one each had additional mutations in *katG* 285, *katG* 289, and *katG* 289 plus *katG* 296. No mutations in either region were identified in 7 (6.2%) INH^r and 45 INH^s isolates.

Table 2. Drug susceptibility profile of 109 multidrug-resistant *M. tuberculosis* isolates

Characteristics	Resistance pattern[a]	Number of isolates
MDR	INH + RIF	7
	INH + RIF + EMB	6
	INH + RIF + STR	17
	INH + RIF + EMB + STR	79
None-MDR	None	41
	INH	2
	STR	3
	EMB	1
	INH + STR	2

[a]INH, isoniazid; RIF, rifampicin; STR, streptomycin; EMB, ethambutol.

Table 3. Distribution of mutations in the *rpo*B RRDR of 109 rifampicin-resistant and 49 rifampicin-susceptible *M. tuberculosis* isolates from Nepal

Mutated codon (s)	Amino acid change (s)[a]	Nucleotide change	No. (%) of isolates	
			RIF^r (n = 109)	RIF^s (n = 49)
511	Leu→Pro	CTG→CCG	1 (0.9)	0 (0.0)
513	Gln→Leu	CAA→CTA	2 (1.8)	0 (0.0)
	Gln→Lys	CAA→AAA	2 (1.8)	0 (0.0)
514	Phe (ins)	TTC→TTCTTC	1 (0.9)	0 (0.0)
516	Asp→Val	GAC→GTC	13 (11.9)	0 (0.0)
	Asp→Phe	GAC→TTC	2 (1.8)	0 (0.0)
	Asp→Tyr	GAC→TAC	1 (0.9)	0 (0.0)
526	His→Tyr	CAC→TAC	5 (4.6)	0 (0.0)
	His→Arg	CAC→CGC	4 (3.7)	0 (0.0)
	His→Asp	CAC→GAC	3 (2.8)	0 (0.0)
	His→Cys	CAC→TGC	2 (1.8)	0 (0.0)
	His→Gly	CAC→GGC	1 (0.9)	0 (0.0)
	His→Leu	CAC→CTC	1 (0.9)	0 (0.0)
531	Ser→Leu	TCG→TTG	61 (56.0)	0 (0.0)
	Ser→Gln	TCG→CAG	1 (0.9)	0 (0.0)
	Ser→Val	TCG→GTG	1 (0.9)	0 (0.0)
533	Leu→Pro	CTG→CCG	2 (1.8)	0 (0.0)
531 and 514	Ser→Leu and Phe (ins)	TCG→TTG and TTC→TTCTTC	1 (0.9)	0 (0.0)
513 and 526	Gln→Lys and His→Asp	CAA→AAA and CAC→GAC	1 (0.9)	0 (0.0)
516 and 533	Asp→Ala and Leu→Pro	GAC→GCC and CTG→CCG	1 (0.9)	0 (0.0)
Wild type[b]	None	None	3 (2.8)	49 (100.0)

[a]Phe (ins), Phe insertion.

[b]No mutations in the sequenced region.

Table 4. Distribution of mutations in *katG* gene and the *inhA* promoter region of 113 INH^r and 45 INH^s *M. tuberculosis* isolates from Nepal

Locus	Amino acid change (s)	Nucleotide change (s)	No. (%) of isolates	
			INH^r (n = 113)	INH^s (n = 45)
katG 315	Ser→Thr	AGC→ACC	86 (76.1)	0 (0.0)
	Ser→Thr	AGC→ACT	1 (0.9)	0 (0.0)
	Ser→Asn	AGC→AAC	1 (0.9)	0 (0.0)
katG 299	Gly→Ser	GGC→AGC	1 (0.9)	0 (0.0)
katG 329	Asp→Ala	GAC→GCC	1 (0.9)	0 (0.0)
katG 341	Trp→Gly	TGG→GGG	1 (0.9)	0 (0.0)
katG 275 and *katG* 315	Thr→Ala and Ser→Thr	ACC→GCC and AGC→ACC	1 (0.9)	0 (0.0)
inhA -15	NA[b]	C→T	6 (5.3)	0 (0.0)
inhA -8	NA	T→C	1 (0.9)	0 (0.0)
katG 285 and *inhA* -15	Gly→Asp and NA	GGC→GAC and C→T	1 (0.9)	0 (0.0)
katG 289 and *inhA* -15	Glu →Ala and NA	GAG→GCG and C→T	1 (0.9)	0 (0.0)
katG 289, *katG* 296 and *inhA* -15	Glu→Ala, Met→Val and NA	GAG→GCG, ATG→GTG and C→T	1 (0.9)	0 (0.0)
katG 315 and *inhA* -12	Ser→Thr and NA	AGC→ACC and T→A	1 (0.9)	0 (0.0)
katG 315 and *inhA* -15	Ser→Thr and NA	AGC→ACC and C→T	3 (2.7)	0 (0.0)
Wild type[a]	None	None	7 (6.2)	45 (100)

[a]No mutations in sequenced regions of *katG* and *inhA* promoter.

[b] Not applicable.

Discussion

Antituberculosis drug resistance poses a significant threat to human health, which usually develops due to the alteration of drug targets by mutations in *M. tuberculosis* chromosomal genes (46, 48). Although a large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported from different countries, no study until now has managed to reveal the range of mutation in clinical samples from Nepal, one of the countries with the high TB prevalence. Hence, in the present study, I attempted to identify the molecular basis of the drug resistance of *M. tuberculosis* circulating in Nepal.

RIF resistance is often considered as a surrogate marker for checking MDR-TB (20, 46). This hypothesis is supported by the finding in the present study that 100% of the RIF^r isolates were MDR. Consistent with previous studies that ca. 95% of RIF^r *M. tuberculosis* isolates worldwide have mutations within the 81-bp core region of the *rpoB* gene, I found mutations in this region in 97.3% of RIF^r isolates. The most frequently mutated codon in our study was codon 531 (58.7%), which was similar to those reported in clinical isolates from India (29, 49, 53), China (18, 22, 27, 70), and other geographical regions (8, 58) (Table 5). Although low frequencies of mutations in codon 516 in clinical isolates have been reported from various parts of China (18, 27, 70), I found a higher frequency of mutations in this codon (15.6%), which was comparable to that of northern India (20.5%) (49).

Table 5. Frequency of the mutations in *rpoB* RRDR in RIF[r] *M. tuberculosis* isolates in India and China reported by seven groups

Mutated codon	% Mutations in different geographic regions[a]							
	North India (n = 93)	India 1[b] (n = 149)	India 2[c] (n = 44)	South China (n = 60)	East China 1[d] (n = 242)	China[e] (n = 72)	East China 2 (n = 53)	This study (n = 109)
511	9.7	1.3	6.0	3.3	3.3	1.4		0.9
513		0.7	2.0	2.6	2.9	1.4		4.6
516	20.5	11.5	4.0	5.0	7.4	4.2	7.5	15.6
518	7.5		2.0			1.4		
522	5.4			2.6	1.7	2.8		
526	20.4	22.0	19.0	11.6	19.4	36.1	30.2	15.6
531	38.7	59.0	53.0	58.3	61.2	37.5	58.5	58.7
533		4.0	2.0	5.0	5.0	1.4		2.8
Others	10.8	1.3	13.7		2.1	4.2		1.8
None		2.0	2.0	10.0	3.7	9.7	7.5	2.8

[a]The values include isolates with mutations at multiple codons. Source references for the various regions were as follows: northern India (49), India 1 (53), India 2 (29), southern China (18), eastern China 1 (27), China (70), and eastern China 2 (22).

[b]Includes northern India (*n* = 110) and southern India (*n* = 39).

[c]Includes southern India (*n* = 35), northern India (*n* = 6), and western India (*n* = 3).

[d]Collected only in Shanghai (*n* = 242).

[e]Includes southern China (*n* = 26), northern China (*n* = 16), and eastern China (*n* = 30).

Phenotypically RIF^r isolates with no *rpoB* mutations in my study were 2.8%, similar to those reported previously (8, 27, 48, 53). Therefore, this finding suggested that majority of RIF^r isolates in Nepal could be rapidly detected by screening for the most common genetic alterations in RRDR of the *rpoB* gene, although the prevalence of isolates lacking mutations also needs to be considered.

Previous studies indicated that INH resistance was mediated by mutations in several genes, most commonly *katG*, particularly in codon 315, and the promoter region of *inhA* (19, 26, 31, 46). Accordingly, I found that 87.6 and 12.4% of phenotypically INH^r clinical isolates had point mutations in *katG* and in the *inhA* promoter region, respectively, and the frequencies were similar to those reported by other researchers (3, 8, 21). However, no deletion or insertion in *katG* was detected in any isolates in the present study. This result confirmed previous reports from different geographic regions of the rarity of this event in causing INH resistance (18, 21, 22, 26, 27, 31, 42). The seven (6.2%) INH^r *M. tuberculosis* isolates had no resistance-associated alterations in the two targets analyzed, indicating that resistance in these isolates could be due to mutations present outside of the sequenced area or in other genes (e.g. *kasA* and *ndh*) (19, 21, 48).

It has been postulated that the amino acid substitution KatG (Ser315Thr) is favored by the bacteria because this alteration was elucidated to spoil INH activation and, on the other hand, to retain 30 to 40% of the catalase-peroxidase activity necessary for virulence (47); however, the prevalence of the KatG (Ser315Thr) substitution in *M. tuberculosis* isolates around the world varies, especially with regard to the prevalence of TB. In general, a higher prevalence of this substitution has been observed in high TB burden regions, often with the predominance of Beijing and MDR *M. tuberculosis* strains,

compared to regions where the prevalence of TB is intermediate or low (22, 35). The present study documented the prevalence of the KatG Ser315Thr substitution in 81.4% of INH^r isolates, which was not as high as those reported in INH^r isolates in northeastern Russia (93.6%) (35) but was comparable to those in Lithuania and Germany (85.7 and 88.4%, respectively) (3, 21). The occurrence of the KatG Ser315Thr alteration among Nepalese isolates was higher than that reported in India (31, 42) and China (18, 22, 27) (Table 6).

Van Soolingen et al. (61) reported that strains with amino acid substitutions in KatG 315 are more likely to develop resistance to other drugs. In this respect, I found a correlation between this alteration and resistance to other drugs: 100% of the isolates with a KatG 315 substitution showed resistance to RIF. Meanwhile, this mutation was found among 92 in 109 (84.4%) of MDR and none in four non-MDR INH^r isolates. This is consistent with the finding of previous studies in which substitutions in codon 315 of *katG* are more common in MDR isolates (19, 48, 58). Several studies from different countries have shown that ca. 10 to 34% of INH^r cases have mutations in the *inhA* promoter region (26, 64). In contrast, I identified mutations in only 12.4% of INH^r isolates, the majority of which was a C to T mutation at position −15.

Since Nepal shares an open border with northern India, there is a large amount of population movement between these countries (40). Patients from northern India usually come to Nepal because of cheaper TB treatment facilities in Nepal; thus, I postulated the frequent air-born transmission of TB between these points (63). By comparing data with neighboring countries, I observed a similarity between Nepalese and northern Indian RIF^r isolates in the occurrence of mutations in codons 531, 526, and 516 of the *rpoB* gene

(Table 5). In contrast, the frequency of KatG (Ser315Thr) substitution and C to T mutations at position −15 in the *inhA* promoter between Nepalese and northern Indian INH^r isolates showed a significant difference (Table 6). This discrepancy might not suggest transport but the possible emergence of MDR-TB in Nepal. For confirmation, molecular typing of strains circulating in Nepal and northern India seems to be necessary.

Table 6. Frequency of the mutations in *katG* 315 or/and *inhA* promoter region -15 in INH^r isolates in India and China reported by five groups

Locus	% Mutations in different geographic regions[a]					
	North India (n = 121)	South India (n = 70)	South China (n = 50)	East China 1 (n = 131)	East China 2[c] (n = 242)	This study (n = 113)
katG 315	55.4	64.3	60.0	61.8	72.7	82.3
inhA -15	25.6	11.4	8.0	21.4	8.3	10.6
Others[b]	27.3	28.6	36.0	18.3	21.5	10.8

[a]Values include isolates with mutations at both loci. Source references for the various regions were as follows: northern India (31), southern India (42), southern China (18), eastern China 1 (22), and eastern China 2 (27).

[b]Includes both other mutations and no mutations.

[c]Collected only in Shanghai (*n* = 242).

Summary

Despite the fact that Nepal is one of the first countries globally to introduce MDR-TB case management, the number of MDR-TB cases is continuing to rise in Nepal. Rapid molecular tests applicable in this setting to identify resistant organisms would be an effective tool in reversing this trend. To develop such tools, information about the frequency and distribution of mutations that are associated with phenotypic drug resistance in *M. tuberculosis* is required. In the present study, I investigated the prevalence of mutations in *rpoB* and *katG* genes and the *inhA* promoter region in 158 *M. tuberculosis* isolates (109 phenotypically MDR and 49 non-MDR isolates collected in Nepal) by DNA sequencing. Mutations affecting the 81-bp RRDR of *rpoB* were identified in 106 of 109 (97.3%) RIF-resistant isolates. Codons 531, 526, and 516 were the most commonly affected, at percentages of 58.7, 15.6, and 15.6%, respectively. Of 113 INH-resistant isolates, 99 (87.6%) had mutations in the *katG* gene, with Ser315Thr being the most prevalent (81.4%) substitution. Mutations in the *inhA* promoter region were detected in 14 (12.4%) INH-resistant isolates. The results from this study provide an overview of the current situation of RIF and INH resistance in *M. tuberculosis* in Nepal and can serve as a basis for developing or improving rapid molecular tests to monitor drug-resistant strains in this country.

CHAPTER II

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

Introduction

Drug resistance in *M. tuberculosis* is commonly caused by mutations in various genes. Mutations in a conserved quinolone resistance-determining region (QRDR) of the *gyrA* or *gyrB* genes encoding DNA gyrase are often involved in FQ resistance (55, 16). Resistance to aminoglycosides (KAN and AMK) and CAP is attributed to mutations in 16S rRNA (*rrs*) gene (32, 54). Molecular epidemiological studies of *M. tuberculosis* strains have identified variability in the phylogeography of strains globally (12,13). Beijing strains are most prevalent globally and also associated with enhanced acquisition of drug resistance; however their resistance patterns varied regionally (17).

This Chapter II documents drug resistance-associated mutations in XDR isolates from Nepal. To gain an insight into the epidemiology of these isolates, I performed genotyping by using spoligotyping, multilocus sequence typing (MLST) and VNTR.

Materials and Methods

M. tuberculosis isolates.

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

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 WHO guidelines (65) 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.

DNA extraction.

DNA was prepared for PCR by mechanical disruption, as described previously (45). 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.

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 7 and those in Poudel A *et al*. (45). 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 60 s 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 3130xl 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).

Table 7. 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 gyr*B* S	CGGCACGTAAGGCACGAGAG	1373-1770	398
	TB *gyrB* AS	GAACCGGAACAACAACGTCAAC		
rrs	TB *rrs* S	AGTCCCGCAACGAGCGCAACCC	1350 – 1550	665
	TB *rrs* AS	GATGCTCGCAACCACTATCCA		

Phylogenetic markers.

Spoligotyping was performed according to the standard protocol (24) and the spoligotype in the binary format was compared with the fourth international spoligotyping database (SpolDB4) (5). 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 (52). 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.* (14).

Results

Drug-susceptibility patterns.

Among 109 MDR *M. tuberculosis* (MTB) isolates obtained, 13 were found to be XDR (Table 8). 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.

Geographical distribution of XDR *M. tuberculosis* isolates.

The XDR-MTB isolates were originated from patients living in five main cities of Nepal (Figure 5): 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.

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 RpoB (12/13), Ser315Thr in KatG (12/13), Asp94Gly in GyrA (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 RpoB (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 *gyrB* (Table 8).

Table 8: 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	*rpoB*	*katG*	*inhA* regulatory region	*gyrA*	*gyrB*	*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

Figure. 5. Geographical location of XDR-TB isolation. Cities where XDR-MTB has been isolated are indicated by a closed circle.

Spoligotyping and MLST.

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

Cluster analysis by VNTR.

VNTR typing grouped the isolates into seven unique patterns and two clusters (Figure 6). 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 *inhA* regulatory region instead of *katG*-Ser315Thr for INH resistance, *gyrA*-Asp94Asn for FQ resistance, and *rrs*-G1483T for KAN/CAP resistance).

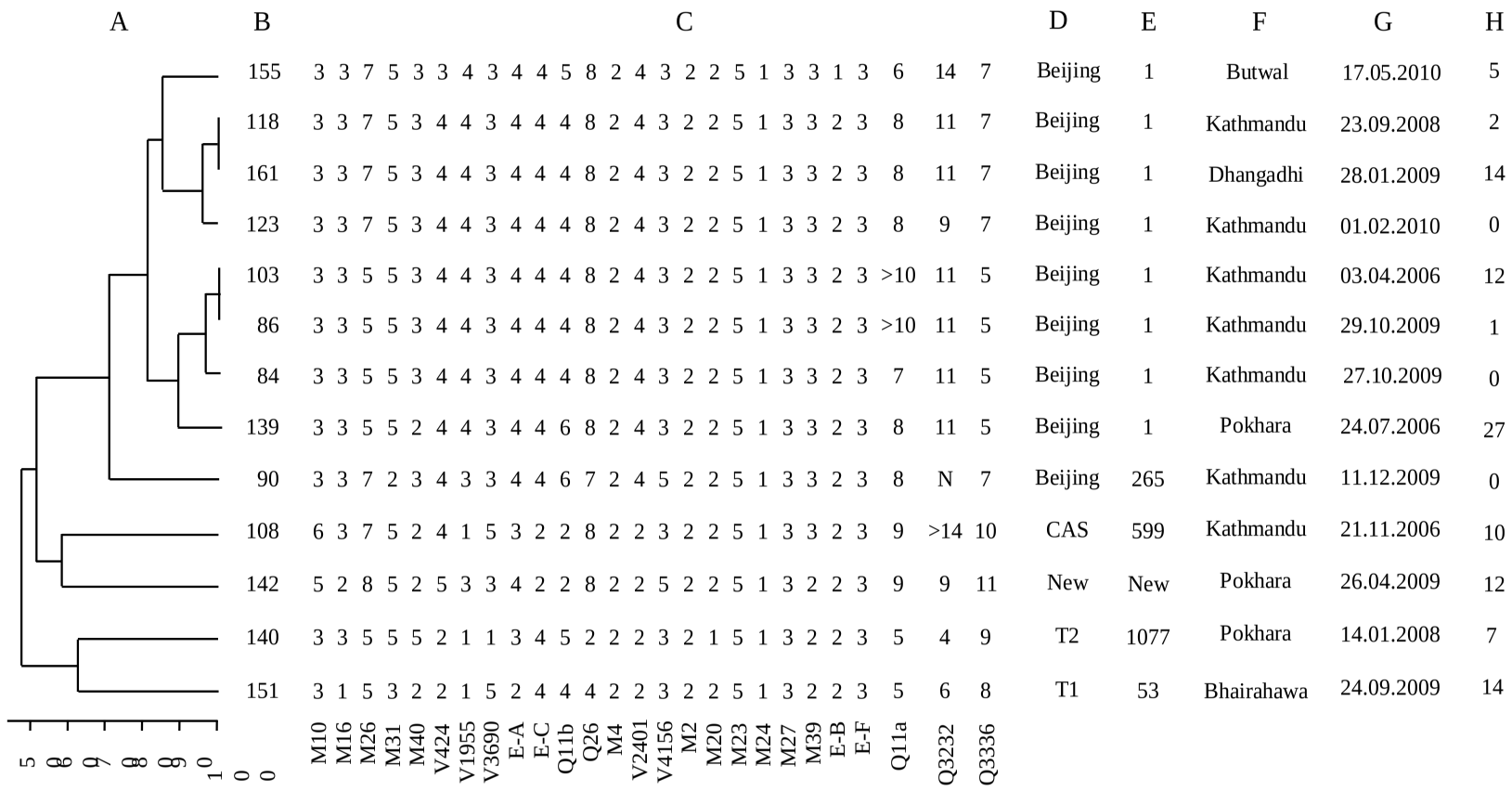

Figure 6. Dendrogram and schematic representation of VNTR typing and spoligotyping results obtained with 13 XDR-TB isolates in Nepal. Column A: dendrogram (UPGMA method, distance matrix average of spoligotyping-based and VNTR) built with Bionumerics version 6, B: strain identification, C: 26 loci VNTR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

Discussion

In this study, I 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 *rpoB*, Ser315Thr of *katG*, Asp94Gly of *gyrA* and a mutation from A to G at nucleotide position 1400 (A1400G) of *rrs* for RIF, INH, OFX and KAN/CAP resistance, respectively. Other studies have also reported similar mutations among XDR-MTB isolates from different countries (1, 2, 25, 50). As mutations such conferring amino acid substitutions, Ser531Leu in RpoB and Ser315Thr in KatG with low fitness costs are known to dominate the drug-resistant isolates (15).

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 (34), India (1) and China (50), while the ancient type Beijing family predominates in Japan (37). 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 (34,10), because of its higher mutation rates and lower fitness costs with specific mutations (15). 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 (6).

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, I 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 5). 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 (46, 54). 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 (28). Both the possibility of direct transmission and acquired resistance should be considered equally for XDR-TB in Nepal.

Summary

The emergence of XDR-TB has raised public health concern for global control of TB. Although molecular characterization of drug resistance-associated mutations in MDR isolates in Nepal has been made, mutations in XDR isolates and their genotypes have not been reported previously. In this study, I identified and characterized 13 XDR-TB isolates from clinical isolates in Nepal. The most prevalent mutations involved in RIF, INH, OFX, and KAN/CAP resistance were Ser531Leu in *rpoB* gene (92.3%), Ser315Thr in *katG* gene (92.3%), Asp94Gly in *gyrA* gene (53.9%) and A1400G in *rrs* gene (61.5%), respectively. Spoligotyping and MLST 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-TB. My result highlights the need to reinforce the TB policy in Nepal with regard to control and detection strategies.

CONCLUSION

MDR- and XDR-TB are emerging problems in Nepal; however no study until now has been managed to determine the prevalence of different drug resistance-conferring mutations among MDR and XDR clinical isolates. In addition, information regarding the genetic diversity of drug-resistant isolates including XDR in this country is lacking. Thus, the molecular characterization of MDR- and XDR-TB isolates in Nepal is necessary for the TB control.

In this regards, in chapter I, I investigated the prevalence of resistance-associated mutations in three specific genes (*rpoB*, *katG*, and the *inhA* promoter region) of *M. tuberculosis* isolates in Nepal and compared the frequency of different mutations with those reported in isolates in surrounding countries. Similar to the neighboring countries, mutations in codons 531, 526, and 516 of the *rpoB* gene and codon 315 of *katG* were the most frequent mutations among MDR isolates from Nepal. Moreover, I postulated the frequent air-born transmission of TB between Nepal and India, since there is a large amount of population movement across the open border between these countries.

In chapter II, I characterized the prevalence and pattern of mutations occurred to the drug target loci in XDR-TB isolates in Nepal. In addition, the genetic background of these isolates was analyzed by spoligotyping and MIRU-VNTR and MLST. The most frequent mutations among XDR isolates were Ser531Leu of *rpoB*, Ser315Thr of *katG*, Asp94Gly of *gyrA* and A1400G of *rrs* for RIF, INH, OFX and KAN/CAP resistance, respectively. The majority of isolates were belonged to the modern type Beijing family. Infections of this family were more common among younger generation than those belonging to other spoligotype families. Furthermore, the identical pattern of VNTR and

drug resistance-associated mutations suggested the possible transmission of Beijing genotype XDR-TB among people in Nepal.

I believe that the finding in my study expands our current knowledge of the molecular mechanisms of drug resistance and also serves as basic information for developing or improving rapid molecular tests to monitor drug-resistant strains. Moreover, my findings emphasize the urgent need to identify patients suffering from XDR-TB and to treat them in isolated wards for a better control program to prevent the spread of this incurable disease.

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