Molecular Characterization of Multidrug-Resistant *Mycobacterium tuberculosis* Isolated in Nepal

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Keywords: Multi drug-resistant, *Mycobacterium tuberculosis*, *rpoB*, *katG*, *inhA*

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Running title: Drug resistance determining mutations in *MTB* in Nepal

ABSTRACT

Despite being one of the first countries globally to introduce multidrug-resistant tuberculosis (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 *Mycobacterium tuberculosis* is required. In the present study, we 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 collected in Nepal, by DNA sequencing. Mutations affecting the 81-bp rifampicin (RIF) resistance-determining region (RRDR) of *rpoB* were identified in 106 of 109 (97.3%) RIF-resistant isolates. The codons most frequently affected were 531, 526 and 516 with percentages of 58.7%, 15.6% and 15.6%, respectively. Of 113 isoniazid (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-based tests to monitor drug-resistant strains in this country.

INTRODUCTION

With an estimated 9 million new cases and 2 million deaths every year, tuberculosis (TB) represents one of the most serious infectious diseases worldwide (35). The increasing spread of multidrug-resistant TB (MDR-TB), resistant to at least two drugs including isoniazid (INH) and rifampicin (RIF), and the recent emergence of

extensively drug-resistant TB (XDR-TB), with additional resistance to a fluoroquinolone (FQ) and at least one of the three injectable second-line drugs, possess a significant threat to tuberculosis control (19, 35). Lack of adequate treatment, often due to irregular drug supply, inappropriate regimens or poor patient compliance is associated with the emergence of drug-resistant *M. tuberculosis* (9, 13). In 2008, approximately 440,000 cases of MDR-TB were estimated throughout the world, and 58 nations had reported to WHO at least one case of XDR-TB (19, 21, 35). Among the countries listed in the WHO report, India and China had the highest burden of MDR-TB, together accounting for almost half of the world's total cases (19, 35). In Nepal, 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 (14, 35). According to the national drug resistance survey conducted in 2006, the prevalence of MDR-TB in Nepal among new and retreatment cases were 2.9 and 11.7%, respectively (14).

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. Every year, a large number of people of Nepal and India cross the border for various purposes, such as work, study, trade, pilgrimage, cultural visits, and so on. According to the 2001 census of Nepal, 762,181 people were abroad, with 78% in India. The census recorded 116,571 foreign citizens residing in Nepal, of whom 88% were Indians (20). However, this information doesn't adequately cover the short-term and short-distance mobility that could significantly contribute TB epidemics in Nepal. Since drug resistance rates on one side of the border impact the other side of the border (33), a high proportion of MDR-TB in Nepal may reflect the possible dissemination of infection from surrounding two countries, mainly from India.

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 (21). In this context, 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 (16, 23).

The collection of data from different countries has indicated that resistance to RIF in more than 90% of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the RNA polymerase β-subunit gene (*rpoB*), called the RIF resistance-determining region (RRDR) (8, 24, 26, 30). 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 (6, 11, 24) and in the promoter region of *inhA*, encoding a putative enzyme involved in mycolic acid biosynthesis. An up-regulation mutation in the *inhA* promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism (24).

The present study aimed to determine the prevalence of resistance-associated mutations in three specific genes (*rpoB, katG* and the *inh*A 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 years period from 2007 and 2010. The isolates were recovered from 158 patients living in 9 different cities of Nepal, six of which have open boarder with North 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 non-MDR isolates, 48 were obtained from patients in Kathmandu

and 1 was obtained from Biratnagar. The histories of previous TB treatment were presented in 94.5% MDR and 42.9 % non-MDR patients. Drug susceptibility test (DST) was performed using Löwenstein-Jensen medium by a conventional proportional method with the following critical drug concentrations for INH, RIF, streptomycin (STR), and ethambutol (EMB): 0.2, 40, 4 and 2 µg/ml, respectively (2).

DNA extraction. DNAs were prepared for PCR by mechanical disruption, as described previously (29). Briefly, the colonies were suspended in TE buffer consisting of 10mM 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., OK, USA). 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 DNAs in the upper layer were concentrated by ethanol precipitation and dissolved in 100 µl TE buffer.

Species differentiation multiplex PCR. MTC species were identified on the isolates by a multiplex PCR with primer pairs designed to amplify three genetic regions (*cfp32*, RD9 and RD12), as described previously (18).

Sequencing of *rpoB* and *katG* encoding regions and *inh*A promotor region. PCR reactions were performed in a 20 µl mixture containing 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 µM of each primer (Table 1), 1 U of GoTaq DNA Polymerase (Promega, WI, USA), GoTaq buffer and 1 µl DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories, CA, USA) under the following conditions: denaturation at 96°C for 60s 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 primers TB *rpoB* S, TB *katG* S

and TB *inhA* S for *rpoB, katG* and *inhA*, respectively, and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., CA, USA) 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) (5).

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 none-MDR isolates consisted of 41 fully susceptible and 2, 3 and 1 isolates with mono-resistant 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 *cfp32*, RD9 and RD12 by multiplex PCR and were classified as *M. tuberculosis* (data not shown).

Mutations in the *rpo*B gene. Mutations in the RRDR of the *rpoB* gene were identified in 106 of 109 RIF-resistant 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 3 (2.8%) had a mutation in codon 533. An Insertion of Phe between codons 514 and 515 was observed in 2 (1.8%) isolates, one of which had an additional point mutation affecting codon 531. Two isolates carried double mutations in two separate codons: 513 and 526; and 516 and 533, respectively. No mutations were detected in the remaining 3 (2.8%) RIF-resistant and 49 RIF-susceptible isolates.

Mutations in *katG* encoding region and *inhA* promoter region. Out of 113 phenotypically INH-resistant isolates, 99 (87.6%) had *katG* mutations, the vast majority of which was a commonly described substitution *KatG* Ser315Thr (Table 4). Only one isolate had a Ser to Asn substitution at *KatG*315. *Kat*G Gly299Ser and *Kat*G Asp329Ala mutations were detected in two INH-resistant 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-resistant isolates; 12 of which had a mutation at -15 in *inhA* promoter. Among the isolates with mutation in *inhA* promoter, 3 had additional mutation in *katG*315 and one each had additional mutation in *katG*285, *KatG*289 and *katG*289 plus *katG*296. No mutations in either region were identified in 7 (6.2%) INH-resistant and 45 INH-susceptible isolates.

DISCUSSION

Anti-tuberculosis 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 (24, 26). Although a large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported from different countries, until now no study has been managed to reveal the range of mutation in clinical samples from Nepal, one of the highest TB prevalent countries. Hence, in the present study, we 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 (7, 24). This hypothesis is supported by the finding in this study that 100% of the RIF-resistant isolates were MDR. Consistent with previous studies that around 95% of RIF-resistant *M. tuberculosis* isolates worldwide have mutations within the 81-bp core

region of the *rpoB* gene, we found mutations in this region in 97.3% of RIF-resistant 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 (15, 27, 28), China (4, 10, 12, 36) and other geographical regions (3, 31) (Table 5). Although low frequencies of mutations in codon 516 in clinical isolates have been reported from various parts of China (4, 12, 36), we found a higher frequency of this mutation (15.6%), which was comparable to that of North India (20.5%) (27).

Phenotypically RIF-resistant isolates with no *rpoB* mutations in our study were 2.8 %, similar to those reported previously (3, 10, 12, 26, 28). Therefore, this finding suggested that majority of RIF-resistant isolates in Nepal could be rapidly detected by screening for the most common genetic alterations in RRDR of the *rpo*B 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 *kat*G, particularly in codon 315, and the promoter region of *inh*A (6, 11, 16, 24). Accordingly, we found that 87.6 % and 12.4 % of phenotypically INH-resistant clinical isolates had point mutations in *kat*G and the *inh*A promoter region, respectively, and the frequencies were similar to those reported by other researchers (1, 3, 8). However, no deletion or insertion in *kat*G was detected in any isolates in this study. This result confirmed previous reports from different geographic regions of the rarity of this event in causing INH resistance (4, 8, 10, 11, 12, 16, 22). The seven (6.2%) INH-resistant *M. tuberculosis* isolates had no resistant-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*, *ndh*) (6, 8, 26).

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%–40% of the catalase-peroxidase activity necessary for virulence (25); however, the prevalence of *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 (10, 17). The present study documented the prevalence of KatG Ser315Thr substitution in 81.4% of INH-resistant isolates, which was not as high as those reported in INH-resistant isolates in north eastern Russia (93.6%) (17), but was comparable to those in Lithuania and Germany (85.7% and, 88.4%, respectively) (1, 26). The occurrence of KatG Ser315Thr alteration among Nepalese isolates was higher than that reported in India (16, 22) and China (4, 10, 12) (Table 6).

Van Soolingen et al. (32) reported that strains having amino acid substitutions in *KatG*315 are more likely to develop resistance to other drugs. In this respect, we found a correlation between this alteration and resistance to other drugs: 100% of isolates with *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-resistant isolates. This is consistent with the finding of previous studies in which substitutions in codon 315 of KatG are more common in MDR isolates (6, 26, 31). Several studies from different countries have shown that about 10% to 34% of INH-resistant cases have mutations in the *inhA* promoter region (11, 34). In contrast, this study identified mutations in only 12.4 % of INH-resistant isolates, the majority of which was C to T at -15.

As Nepal shares an open border with north India, there is large population movement between these countries (20). Patients from north India usually come to Nepal because of cheaper TB treatment facilities in Nepal; thus, we postulated the frequent air-born transmission of TB between these points (33). By comparing data with neighboring countries, we observed a similarity between Nepalese and north Indian RIF-resistant isolates in the occurrence of mutations in codons 531, 526 and 516 of the *rpo*B gene (Table 5). In contrast, the frequency of *KatG* Ser315Thr substitution and C to T mutations at -15 in *inhA* promoter between Nepalese and north Indian INH-resistant 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 north India seems to be necessary.

In conclusion, this study provides valuable information on mutations occurring at *rpo*B, *kat*G gene and promoter region of *inh*A in Nepalese clinical isolates of *M. tuberculosis*. It expands our current knowledge of the molecular mechanisms of drug resistance and also assists in improving current molecular-based techniques for the diagnosis of MDR tuberculosis in Nepal. Such methods promise rapid detection rates compared to those achieved by methods based solely on culture of the isolates.

ACKNOWLEGEMENTS

This study was supported in part by J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT), the Global Center of Excellence (COE) Program, "Establishment of International Collaboration Centers for Zoonosis Control" from MEXT, a grant from U.S.-Japan Cooperative Medical Science Programs to Y. S.,

and Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) to Y. S. and C. N.

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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	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～ -1	231
	TB *inhA* AS	AGCCAGCCGCTGTGCGATCGCCA		

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

Table 2. Drug susceptibility profile of 109 multi drug- 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	Amino acid change	Nucleotide change	No. (%) of RIF[r] isolates (n=109)	No. (%) of RIF[s] isolates (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)[a]	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)[a]	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-resistant and 45 INH -susceptible *M. tuberculosis* isolates from Nepal

Locus	Amino acid change	Nucleotide change	No. (%) of INH^{r} isolates (n=113)	No. (%) of INH^{s} isolates (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.

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

	% Mutations in different geographic regions[a]							
Mutated codon(s)	North India (reference 27; n=93)	India[b] (reference 28; n=149)	India[c] (reference 15; n=44)	South China (reference 4; n=60)	East China[d] (reference 12; n=242)	China[e] (reference 36; n=72)	East China (reference 10; 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] Including isolates having mutations at multiple codons.

[b] North India (n=110) and South India (n=39).

[c] South India (n=35), North India (n=6) and West India (n=3).

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

[e] South China (n=26), North China (n=16) and East China (n=30).

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

	% Mutations in different geographic regions[a]					
Locus	North India (reference 16; n=121)	South India (reference 22; n=70)	South China (reference 4; n=50)	East China (reference 10; n=131)	East China[c] (reference 12; 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] Including isolates having mutations at both loci.

[b] Including other mutations and no mutations.

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