AAC Journals.ASM.org

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

Ajay Poudel,[a] Chie Nakajima,[a] Yukari Fukushima,[a] Haruka Suzuki,[a] Basu Dev Pandey,[b] Bhagwan Maharjan,[c] and Yasuhiko Suzuki[a,d]

Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan[a]; Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, Nepal[b]; German Nepal Tuberculosis Project (GENETUP), Kathmandu, Nepal[c]; and JST/JICA-SATREPS, Tokyo, Japan[d]

Despite the fact that Nepal is 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 isolates collected in Nepal) by DNA sequencing. Mutations affecting the 81-bp rifampin (RIF) resistance-determining region (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 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 tests to monitor drug-resistant strains in this country.

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), which is resistant to at least two drugs, including isoniazid (INH) and rifampin (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, pose 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 *Mycobacterium tuberculosis* (9, 13). In 2008, approximately 440,000 cases of MDR-TB were estimated throughout the world, and 58 nations had reported to World Health Organization (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 prevalences of MDR-TB in Nepal among new and retreatment cases were 2.9 and 11.7%, respectively (14).

Nepal is a landlocked country in Southeast 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, 88% of whom were Indians (20). However, this information does not 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 >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 upregulation mutation in the *inhA* promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism (24).

In the present study, we 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

Received 23 December 2011 Returned for modification 8 February 2012
Accepted 15 March 2012
Published ahead of print 26 March 2012
Address correspondence to Yasuhiko Suzuki, suzuki@czc.hokudai.ac.jp.

doi:10.1128/AAC.06418-11

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[a]	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*.

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 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 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 (*cfp32*, RD9, and RD12), as described previously (18).

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 3130*xl* 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) (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 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 *cfp32*, RD9, and RD12 by multiplex PCR and were classified as *M. tuberculosis* (data not shown).

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 Phe 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.

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

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

Characteristic	Resistance pattern[a]	No. of isolates
MDR	INH + RIF	7
	INH + RIF + EMB	6
	INH + RIF + STR	17
	INH + RIF + EMB + STR	79
Non-MDR	None	41
	INH	2
	STR	3
	EMB	1
	INH + STR	2

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

TABLE 3 Distribution of mutations in the *rpoB* RRDR of 109 rifampin-resistant and 49 rifampin-susceptible *M. tuberculosis* isolates from Nepal

Mutated codon(s)	Amino acid change(s)[a]	Nucleotide change(s)	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] That is, there were no mutations in the sequenced region.

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.

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 (24, 26). 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 highest TB prevalence. 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 the present study that 100% of the RIF^r isolates were MDR. Consistent with previous studies that ca. 95% of RIF-resis-

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] That is, there were no mutations in sequenced regions of *katG* and *inhA* promoter.
[b] NA, not applicable.

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]							
	Northern India ($n = 93$)	India 1[b] ($n = 149$)	India 2[c] ($n = 44$)	Southern China ($n = 60$)	Eastern China 1[d] ($n = 242$)	China[e] ($n = 72$)	Eastern 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 (27), India 1 (28), India 2 (15), southern China (4), eastern China 1 (12), China (36), and eastern China 2 (10).
[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 ($n = 30$).

tant *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^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 (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 northern India (20.5%) (27).

Phenotypically RIF^r 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^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* (6, 11, 16, 24). Accordingly, we 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 (1, 3, 8). 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 (4, 8, 10, 11, 12, 16, 22). 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*) (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 to 40% of the catalase-peroxidase activity necessary for virulence (25); 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 (10, 17). 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%) (17) but was comparable to those in Lithuania and Germany (85.7 and 88.4%, respectively) (1, 26). The occurrence of the KatG Ser315Thr alteration among Nepalese isolates was higher than that reported in India (16, 22) and in China (4, 10, 12) (Table 6).

Van Soolingen et al. (32) reported that strains with amino acid substitutions in *katG* 315 are more likely to develop resistance to

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

Locus	% Mutations in different geographic regions[a]					
	Northern India ($n = 121$)	Southern India ($n = 70$)	Southern China ($n = 50$)	Eastern China 1 ($n = 131$)	Eastern 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 (16), southern India (22), southern China (4), eastern China 1 (10), and eastern China 2 (12).
[b] Includes both other mutations and no mutations.
[c] Collected only in Shanghai ($n = 242$).

other drugs. In this respect, we 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 (6, 26, 31). Several studies from different countries have shown that ca. 10 to 34% of INH^r cases have mutations in the *inhA* promoter region (11, 34). In contrast, we 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 (20). Patients from northern 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 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.

In conclusion, we provide here valuable information on mutations occurring at *rpoB*, the *katG* gene, and the promoter region of *inhA* in Nepalese clinical isolates of *M. tuberculosis*. These findings expand our current knowledge of the molecular mechanisms of drug resistance and also assist in improving current molecular techniques for the diagnosis of MDR-TB in Nepal. Such methods promise rapid detection rates compared to those achieved by methods based solely on culture of the isolates.

ACKNOWLEDGMENTS

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 (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 the Japan Society for the Promotion of Science to Y.S. and C.N.

REFERENCES

1. **Bakonyte D, Baranauskaite A, Cicenaite J, Anaida Sosnovskaja A, Stakenas P.** 2003. Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates in Lithuania. Antimicrob. Agents Chemother. **47**:2009–2011.
2. **Canetti G, et al.** 1969. Advances in techniques oftesting mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull. World Health Organ. **41**:21–43.
3. **Caws M, et al.** 2006. Mutations prevalent among rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam. J. Clin. Microbiol. **44**:2333–2337.
4. **Guo JH, et al.** 2008. Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Sichuan Province in China. Jpn. J. Infect. Dis. **61**:264–268.
5. **Hall A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. (Oxford) **41**:95–98.
6. **Hazbon MH, et al.** 2006. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **50**:2640–2649.
7. **Heep M, et al.** 2001. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. J. Clin. Microbiol. **39**:107–110.
8. **Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S.** 2005. Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J. Clin. Microbiol. **43**:699–703.
9. **Hirano K, Abe C, Takahashi M.** 1999. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J. Clin. Microbiol. **37**:2663–2666.
10. **Hu Y, Hoffner S, Jiang W, Wang W, Xu B.** 2010. Extensive transmission of isoniazid resistant *Mycobacterium tuberculosis* and its association with increased multidrug-resistant TB in two rural counties of eastern China: a molecular epidemiological study. BMC Infect. Dis. **10**:43.
11. **Kiepiela P, Bishop KS, Smith AN, Roux L, York DF.** 2000. Genomic mutations in the *katG*, *inhA*, and *ahpC* genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. Tuberc. Lung Dis. **80**:47–56.
12. **Luo T, et al.** 2010. Selection of mutations to detect multidrug resistant *Mycobacterium tuberculosis* strains in Shanghai, China. Antimicrob. Agents Chemother. **54**:1075–1081.
13. **Mahmoudi A, Iseman MD.** 1993. Pitfalls in the care of patients with tuberculosis: common errors and their association with the acquisition of drug resistance. JAMA **270**:65–68.
14. **Malla P, et al.** 2009. Ambulatory-based standardized therapy for multidrug-resistant tuberculosis: experience from Nepal, 2005–2006. PLoS One **4**:e8313.
15. **Mani C, Selvakumar N, Narayanan S, Narayanan PR.** 2001. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. J. Clin. Microbiol. **39**:2987–2990.
16. **Mathuria JP, Nath G, Samaria JK, Anupurba S.** 2009. Molecular characterization of INH-resistant *Mycobacterium tuberculosis* isolates by PCR-RFLP and multiplex-PCR in North India. Infect. Genet. Evol. **9**:1352–1355.
17. **Mokrousov I, et al.** 2002. High prevalence of KatGSer315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. Antimicrob. Agents Chemother. **46**:1417–1424.
18. **Nakajima C, et al.** 2010. Identification of *Mycobacterium tuberculosis* clinical isolates in Bangladesh by a species distinguishable multiplex PCR. BMC Infect. Dis. **10**:118.
19. **Nathanson E, et al.** 2010. MDR tuberculosis-critical steps for prevention and control. N. Engl. J. Med. **363**:1050–1058.
20. **Nepal B.** 2007. Population mobility and spread of HIV across the Indo-Nepal border. J. Health Popul. Nutr. **25**:267–277.
21. **Nettleman MD.** 2005. Multidrug-resistant tuberculosis: news from the front. JAMA **293**:2788–2790.
22. **Nusrath UA, Selvakumar N, Narayanan S, Narayanan PR.** 2008. Molecular analysis of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from India. Int. J. Antimicrob. Agents **31**:71–75.
23. **Piatek AS, et al.** 2000. Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob. Agents Chemother. **44**:103–110.
24. **Ramaswamy S, Musser JM.** 1998. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: update. Tuberc. Lung Dis. **79**:3–29.
25. **Rouse DA, DeVito JA, Li Z, Byer H, Morris SL.** 1996. Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. Mol. Microbiol. **22**:583–592.
26. **Sajduda A, et al.** 2004. Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. J. Clin. Microbiol. **42**:2425–2431.
27. **Siddiqi N, et al.** 2002. Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. Antimicrob. Agents Chemother. **46**:443–450.
28. **Suresh N, et al.** 2006. *rpo*B gene sequencing and spoligotyping of multidrug-resistant *Mycobacterium tuberculosis* isolates from India. Infect. Genet. Evol. **6**:474–483.

29. **Suzuki Y, et al.** 1998. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J. Clin. Microbiol. **36**:1220–1225.
30. **Telenti A, et al.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet **341**:647–650.
31. **Valvatne H, et al.** 2009. Isoniazid and rifampicin resistance-associated mutations in *Mycobacterium tuberculosis* isolates from Yangon, Myanmar: implications for rapid molecular testing. Antimicrob. Agents Chemother. **64**:694–701.
32. **van Soolingen D, et al.** 2000. 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. **182**:1788–1790.
33. **World Health Organization.** 2001. Cross-border initiatives on HIV/AIDS, TB, malaria, and kala-azar: report of an intercountry meeting in Kathmandu, 6 to 9 March 2001. World Health Organization, Regional Office for South-East Asia, Geneva, Switzerland. http://whqlibdoc.who.int/searo/2001/SEA_CD_123.pdf.
34. **World Health Organization.** 2008. Molecular line probe assays for rapid screening of patients at risk of multidrug resistant tuberculosis (MDR-TB). Policy statement, 2008. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/dots/laboratory/line_probe_assays/en/index.html.
35. **World Health Organization.** 2010. Multidrug and extensively drug-resistant TB (M/XDRTB): 2010 global report on surveillance and response. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/publications/2010/9789241599191_eng.
36. **Yue J, et al.** 2003. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from China. J. Clin. Microbiol. **41**:2209–2212.