for rapid drug susceptibility testing compared to time consuming conventional methods.[10,11] It has shown that association of rifampicin (RIF) resistance with mutations within the RIF resistance-determining region (RRDR) varies from 78 to 100% of cases[7,12–14] in different countries. On the contrary, *M. tuberculosis* acquires isoniazid (INH) resistance through mutations in several genes including the *katG* and *inhA* regulatory region. Mutations in *katG* induced resistance from 50 to 95% of INH resistance strains.[7,15,16] Mutations associated with up-regulation of the expression of *inhA* encoding a long-chain enoyl-acyl carrier protein reductase contributes to INH resistance via a titration mechanism in approximately 10–30% of strains.[7] Association of mutation with drug resistance differs in countries studied. Thus, it is important to study molecular mechanism of drug resistance in each country. Nevertheless, very limited information is available on molecular mechanism of drug resistance associated with MDR stains of *M. tuberculosis* in Bangladesh. Hence, we conducted this study to analyze mutations of *rpoB* and *katG* genes and the *inh*A regulatory region of MDR strains of *M. tuberculosis* of this country.

2. Materials and methods

2.1. Bacterial strains

M. tuberculosis strains were isolated from sputum samples at the Tuberculosis Laboratory of ICDDR,B from June 2001 to December 2010. Outdoor of the Shyamoli Chest Disease Clinic and the National Institute of the Diseases of Chest and Hospital (NIDCH) at Dhaka were the catchment areas for non-hospitalized TB patients (outpatient). Indoor wards of the NIDCH were the catchment area of hospitalized TB patients (inpatients).

2.2. Isolation of Mycobacterium and drug susceptibility testing (DST)

Isolation of *Mycobacterium* strains and DST were performed following the method already described.[17] Briefly; 5 ml sputum sample was collected from the TB suspects and sputum sample was decontaminated following the standard Petroff's method.[18] One loop-full (10 μl) of decontaminated sputum samples was inoculated on two Löwenstein-Jensen (L-J) slants. Then, well isolated characteristic colonies of *Mycobacterium* was taken from surface of the L-J slants for DST with respect to streptomycin (4 mg/L), INH (0.2 mg/L), RIF (40 mg/L) and ethambutol (2 mg/L) following proportion method of Canetti et al.[19] In each lot of DST, one strain of H37Rv was used as sensitive and known MDR strain was used as resistant control strains. DST result of a lot was considered valid when the sensitivity patterns of the control strains were consistent with previous sensitivity patterns.

2.3. Preparation of DNA

One loop full of *Mycobacterium* culture was scraped from the surface of the drug containing L-J slants and re-suspended in 500 μl sterile distilled water in a micro centrifuge tubes and the bacterial cells were lysed by boiling for 20 min. The cells were centrifuged (10,000× *g* for 10 min) and the supernatant was transferred into another micro centrifuge tube to use as template DNA.

2.4. Identification of strains

For the identification of MDR strains, an in-house multiplex PCR technique was used. This technique targeted three genetic regions (*cfp32*, RD9 and RD12) as described previously.[20]

2.5. Standard PCR for sequencing of rpoB and katG genes and inhA regulatory region

The target template DNA was amplified using the primer pairs listed in Table 1. PCR reactions were performed in a 20 μl mixture consisting of 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 μM of each primer, 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 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. Desired PCR product was confirmed by agarose gel electrophoresis. PCR products were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., CA, USA) in 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).[21]

3. Results

3.1. Bacterial strains

All the isolates in a collection of 218 MDR mycobacterial strains (161 from inpatients and 57 from outpatients) were identified by in-house multiplex PCR as *M. tuberculosis* because of their carriage of RD9 and RD12.

3.2. Mutations in the rpoB gene

To elucidate the molecular mechanism of RIF resistance, *rpoB* gene of MDR strains were sequenced. Mutated codons, amino acid and nucleotide substitution of these strains are shown in Table 2. Mutations at *rpoB* gene were detected in 95% (207 out of 218) of RIF resistant strains with majority having single mutations at codon 531 (57.3%). This was followed by mutation in codon 526 (20.6%) and 516 (7.3%). Low frequency mutations were detected at codons 513, 530 and 533. Deletion, insertion and double mutations were detected in a very few strains. Eleven (5.0%) phenotypic MDR strains were without known mutation in *rpoB* gene. Mutation conferring Asp to Val amino acid substitution at codon 516 (Asp516Val) was found only in strains from inpatient and mutation giving His526Arg was found only in strains from outpatients.

3.3. Mutations in the katG gene and inhA regulatory regions

For the molecular characterization of INH resistance, the nucleotide sequences of *katG* gene and *inhA* regulatory region were sequenced. Mutated codons, amino acid and nucleotide substitutions of these strains are shown in Table 3. Out of 218 strains, 206 (94.5%) had mutations in *katG* coding region or *inhA* regulatory region or both with the vast majority having the commonly

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

Locus	Primer name	Nucleotide sequence (5′–3′)	Target region	Product size (bp)
rpoB	TB rpoB S	CAGGACGTGGAGGCGATCAC	1519–1599*	278
	TB rpoB AS	GAGCCGATCAGACCGATGTTGG		
katG	TB katG S	ATGGCCATGAACGACGTCGAAAC	823–1140	392
	TB katG AS	CGCAGCGAGAGGTCAGTGGCCAG		
inhA	TB inhA S	TCACACCGACAAACGTCACGAGC	−40 to −1	231
	TB inhA AS	AGCCAGCCGCTGTGCGATCGCCA		

* Corresponding *Escherichia coli* numbering was used for rpoB.

Table 2
Distribution of mutations in the rpoB RRDR of 218 MDR- isolates from Bangladesh.

Mutated codon	Amino acid change		Nucleotide change		No. (%) of isolates		
	From	To	From	To	Inpatients (n = 161)	Outpatients (n = 57)	Total (n = 218)
513	Gln	Pro	CAA	CCA	1 (0.6)	1 (1.8)	2 (0.9)
		Lys		AAA	1 (0.6)	–	1 (0.5)
		Leu		CTA	1 (0.6)	–	1 (0.5)
516	Asp	Val	GAC	GTC	12 (7.5)	–	12 (5.5)
		Tyr		TAC	3 (1.8)	1 (1.9)	4 (1.8)
526	His	Tyr	CAC	TAC	13 (8.1)	4 (7.0)	17 (7.8)
		Asp		GAC	9 (5.6)	4 (7.0)	13 (6.0)
		Arg		CGC	–	6 (10.5)	6 (2.8)
		Leu		CTC	2 (1.2)	3 (5.3)	5 (2.3)
		Cys[a]		TGC	1 (0.6)	1 (1.8)	2 (0.9)
		Asn		AAC	1 (0.6)	–	1 (0.5)
		Gly[a]		GGC	–	1 (1.8)	1 (0.5)
530	Leu	Val	CTG	GTG	1 (0.6)	–	1 (0.5)
531	Ser	Leu	TCG	TTG	90 (55.9)	24 (42.1)	114 (52.3)
		Trp		TGG	9 (5.6)	1 (1.8)	10 (4.6)
		Phe[a]		TTT	1 (0.6)	–	1 (0.5)
533	Leu	Pro	CTG	CCG	2 (1.2)	3 (5.3)	5 (2.3)
510/526	Gln/His	His/Tyr	CAG/CAC	CAT/TAC	1 (0.6)	–	1 (0.5)
511/516	Leu/Asp	Arg/Tyr	CTG/GAC	CGG/TAC	1 (0.6)	–	1 (0.5)
513/526	Gln/His	Lys/Asp	GAC/CAC	GTC/CCG	1 (0.6)	–	1 (0.5)
516/517	Asp/Gln	Val/Pro	GAC/CAG	GTC/CCG	–	1 (1.8)	1 (0.5)
516/526	Asp/His	Glu/Asn	GAC/CAC	GAA/AAC	2 (1.2)	–	2 (0.9)
516/531	Asp/Ser	Tyr/Leu	GAC/TCG	TAC/TTG	1 (0.6)	–	1 (0.5)
526/533	His/Leu	Gln/Pro	CAC/CTG	CAA/CCG	1 (0.6)	–	1 (0.5)
514	Phe ins		TTC ins		1 (0.6)	–	1 (0.5)
514-516	Del		Del		1 (0.6)	–	1 (0.5)
516-517	Del		Del		1 (0.6)	–	1 (0.5)
Wild type[b]	None		None		4 (2.5)	7 (12.3)	11 (5.0)

[a]Double mutation in a codon.
[b]No mutations in the sequenced region.

described mutation in *katG* coding region conferring Ser315Thr amino acid substitution (n = 178; 83.9%). Seven and one strains respectively had Ser to Asn and Ser to Ile amino acid substitution at the same position. Tyr275Pro, Ala291Pro, Trp300Gly, Trp300Cys amino acid substitutions were observed in each one of INH-resistant strains. Three strains showed double mutations in two separate codons: Thr275Ala and Ser315Thr. One strain showed double mutations conferring Ser315Thr and Asp329Gly substitution. One strain showed frame shift mutation as a result of insertion of two base pairs after codon 300. In addition, simultaneous six amino acid deletions from 333 to 338 and Tyr339Asp amino acid substitution were noted. Thirty-nine (17.9%) INH-resistant strains

Table 3
Distribution of mutations in KatG gene and the inhA promoter region of 218 MDR- isolates from Bangladesh.

Mutated locus	Amino acid change		Nucleotide change		No. (%) of isolates		
	From	To	From	To	Inpatients (n = 161)	Outpatients (n = 57)	Total (n = 218)
KatG 275	Tyr	Pro	ACC	CCC	–	1 (1.8)	1 (0.5)
KatG 291	Ala	Pro	GCT	CCT	1 (0.6)	–	1 (0.5)
KatG 300	Trp	Gly	TGG	GGG	1 (0.6)	–	1 (0.5)
		Cys		TGC	1 (0.6)	–	1 (0.5)
KatG 315	Ser	Thr	AGC	ACC	100 (62.1)	36 (63.2)	136 (62.4)
		Asn		AAC	5 (3.1)	2 (3.5)	7 (3.2)
		Ile		ATC	–	1 (1.8)	1 (0.5)
KatG 275/315	Thr/Ser	Ala/Thr	ACC/AGC	GCC/ACC	3 (1.9)	–	3 (1.4)
KatG 315/329	Ser/Asp	Thr/Gly	ACC/GAC	GCC/GCC	1 (0.6)	–	1 (0.5)
KatG 300	frame shift		GC ins		1 (0.6)	–	1 (0.5)
KatG 333–338/339	Leu-Glu-Ile-Leu-Try-Gly/Tyr	Del*/Asn	CTCGAGATCCTGTACGGC/TAC	del/GAC	1 (0.6)	–	1 (0.5)
Deletion†	Deletion		Deletion		1 (0.6)	–	1 (0.5)
inhA −15	NA§		C	T	10 (6.2)	5 (8.8)	15 (6.9)
KatG 315/inhA −8[d]	Ser/NA	Thr/NA	AGC/T	ACC/A	1 (0.6)	–	1 (0.5)
				ACC/C	1 (0.6)	–	1 (0.5)
KatG 315/inhA −15[d]	Ser/NA	Thr/NA	AGC/C	ACC/T	16 (9.9)	5 (8.8)	21 (9.6)
KatG 315/inhA −34[d]	Ser/NA	Thr/NA	AGC/C	ACC/T	–	1 (1.8)	1 (0.5)
KatG 315/inhA −47[d]	Ser/NA	Thr/NA	AGC/G	ACC/A	10 (6.2)	2 (3.5)	12 (5.5)
Wild type**	None		None		8 (5.0)	4 (7.0)	12 (5.5)

[d]Mutations identified in both loci.
* Deletion.
† No amplification.
§ Not applicable.
** No mutations in sequenced regions of katG and inhA promoter.

showed mutation at *inhA* regulatory region. Thirty six (16.5%) of them had a C to T mutation at −15 in *inhA* regulatory region (*inhA* C −15 T). Among these strains, 21 had additional mutation at *katG* 315. One each had additional concurrent mutations at *katG* Ser315Thr/*inhA* T −8 A, *katG* Ser315Thr/*inhA* T −8 C and *katG* Ser315Thr/*inhA* C −34 T. Twelve (5.5%) had a G to A at position −47 of the *inhA* regulatory region in association with the *katG* Ser315Thr substitution. And twelve (5.5%) phenotypic MDR strains were without mutation in both *katG* gene and *inhA* regulatory regions.

4. Discussion

Recent reports indicate that MDR-TB is increasing in Bangladesh[5,6] but there is scarcity of data on molecular mechanism of drug resistance. Studies on the RIF resistance in several countries demonstrated that majority of resistant strains had mutation at *rpoB* gene.[7,12,13] And the mutation percentage varied from 78 to 100% worldwide.[1,14] In this study, *rpoB* mutation was detected in 95% (207 out of 218) of MDR strains from Bangladesh (Table 2), which is consistent with the data obtained in majority of countries studied. The most frequently observed amino acid substitution occured in Bangladesh strains at codon 531 of the *rpoB* gene (57.4%) and was similar to those reported from Nepal (57.8%),[22] Morocco (59.6%),[23] Singapore (54.9%),[24] Thailand (58.5%)[25] and Brazil (56.1%).[26] However, significantly lower percentage of *rpoB* mutation at codon 531 was reported from Vietnam (39.2%),[27] Hungary (34.5%)[28] and Poland (32.4%).[29] Surprisingly low percentage was detected in strains isolated from Eastern China (3.6%).[30] On the contrary, high percentage of mutation at codon 531 was reported from Germany (75.7%),[31] Kazakhstan (82.7%)[24] and Spain (72.3%).[32] Second highest mutation at codon 526 (22.9%) was observed in Bangladesh as has been reported from many countries. There are some clinical strains of *M. tuberculosis*, which are phenotypically resistant to RIF but lack of any detectable mutation at *rpoB* gene. Eleven strains (5%) from Bangladesh had this characteristic. These types of discrepancy may happen as a result of the mixture of wild type and resistant strains (10), mutation outside the *rpoB* core region,[33,34] altered permeability of cell wall and metabolism of antibiotics.[7,35]

Unlike RIF, NIH resistance is associated with mutations in several genes such as *katG*, *inhA*, *kasA*, *ahpC* and *oxyR*.[7] Besides, mutations in genes like *furA*, *iniA*, *iniB* and *iniC* are responsible for INH resistance in a very limited number of strains of *M. tuberculosis*.[17] Majority of INH resistant strains carried mutations at codon 315 of the *katG* gene and 20–35% strains contained mutation at the *inhA* regulatory region.[7,14] In the present study, point mutations at *katG* gene and *inhA* regulatory region were 83.9 and 16.5%, respectively (Table 3). The amino acid substitution at *katG* Ser315Thr has been reported to be favorable for the bacteria because this change damages the activation process of INH with retaining 30–40% of the catalase–peroxidase activity essential for virulence.[36] Mutations at codon 315 of the *katG* gene similar to our finding was reported in other studies from Nepal (82.2%),[22] Southern Turkey (76.7%)[37] and Ghana (80.1%).[38] In contrast, higher percentage of mutation was reported from Russia (90.9%)[39] and Kazakhstan (98.4%),[40] and lower percentage was reported from United States (32.1%),[41] Canada (45.5%)[42] and Spain (60.8%).[32] In general, a higher percentage of this type of substitution mutation was observed in high TB burden country like Bangladesh, compared to intermediate or low TB prevalent countries.

Up-regulation mutations in the regulatory region of *inhA* (preceding the *mabA-inhA* operon) results in the over expression of *InhA*, thereby contributes to the acquisition of INH resistance via a titration mechanism.[7,14] Several studies have demonstrated about 10–34% of INH-resistant cases with mutations in the *inhA* regulatory region16, 43. In our study, 17.9% (39 out of 218) of INH resistant strains carried mutations in *inhA* regulatory region with the majority (16.5%) had mutation at position −15 (36 out of 218). Percentage of mutation at −15 in *inhA* regulatory region in this study is comparable with studies from Southern Turkey (16.7%),[37] Russia (13.9%)[39] and Ghana (13.3%).[38] High percentage of *inhA* mutation was reported from United States (46.4%),[41] Tunisia (36.1%)[44] and Canada (26.0%).[42] In contrast, none or low percentage was reported from Spain (0%),[32] Myanmar (0%)[45] and Germany (1.94%).[31]

It was noteworthy that one and twelve isolates carried novel mutations T to C at position −34 and G to A at position −47 of the *inhA* regulatory region, respectively, in association with the *katG* Ser315Thr amino acid substitution. The contribution of these novel mutations on INH resistance is not clear and necessary to be elucidated in future studies.

Substantial numbers of isolates with dual mutations in the genes associated with RIF or INH resistance in the isolates, particularly from inpatients were found (Tables 2 and 3). We have carefully reanalyzed the raw data to find that all isolates with dual mutation in *rpoB* and *katG* except for that with Asp516Tyr and Ser531Leu amino acid substitution in *rpoB* showed clear single peaks at the mutational positions indicating dual mutations. In contrast, sequence peaks at the both mutational sites in the isolate with Asp516Tyr and Ser531Leu in *rpoB* showed overlapping feature indicating the mixed population. These results indicated the low prevalence of mixed population with two different lineages in these isolates. Constant treatment at the hospital might select the strains with high-level resistance to both RIF and IHN or those with low fitness cost through acquiring secondary mutations.

We, then, compared the mutation rate in both RIF and INH resistant strains from inpatients and outpatient to correlate with nosocomial infection and specific mutations. Isolates without any mutation in RRDR was higher in outpatients than in inpatients (12.3% vs 2.5%). In contrast, no significant difference was observed between strains from inpatients and outpatients with respect to INH resistance. Mutations outside RRDR or other mechanism giving RIF resistance might contribute to higher transmission between outpatients. The precise comparison of mutations between strains isolated from inpatients and outpatients did not give any clear difference. However, more than 10% (n = 6) of strains from outpatients had His to Arg substitution at codon 526 in *rpoB* of RIF resistant strains compared to none in inpatient strains. Strains with this mutation might have high means of transmission.

Bangladesh is surrounded by India and Myanmar in three and one borders, respectively. There are in and out migration of people among these countries. This situation favors transmission of pathogen among people of these three countries. Data on the

Table 4
Frequency of the mutations in rpoB RRDR in RIF-resistant clinical isolates in Myanmar, India and Bangladesh.

Mutated codon(s)	% Mutations in different geographic regions*			
	North India (reference 46; n = 93)	India† (reference 43; n = 149)	Myanmar (reference 45; n = 29)	This study (n = 218)
511	9.7	1.3	–	0.5
513	–	0.7	–	2.3
516	20.5	11.5	3.4	9.6
526	20.4	22	17.2	22.9
531	38.7	59	55.2	57.8
533	–	4	3.4	2.8
Others	23.7	3.3	20.6	6.8

* Including isolates having mutations at multiple codons.
† North India (n = 110) and South India (n = 39).

Table 5
Frequency of the mutations in KatG 315 or/and inhA promoter region −15 in INH-resistant clinical isolates in Myanmar, India and Bangladesh.

Locus	% Mutations in different geographic regions*			
	North India (reference 48; $n = 121$)	South India (reference 476; $n = 70$)	Myanmar (reference 40; $n = 96$)	This study ($n = 218$)
katG 315	55.4	65.7	63.5	83.9
InhA −15	25.7	11.4	–	16.5
Others†	27.3	23.9	36.5	14.2

* Including isolates having mutations at both loci.
† Including other mutations and no mutations.

mutation at codon 531 RIF resistant strains showed similar trend to India[43] and Myanmar[45] and distinct from North India[46] (Table 4). In contrast, the occurrence of *katG* codon 315 alterations among Bangladesh strains (83.9%) was higher than those reported in South India,[47] Myanmar[45] and North India[48] (Table 5). *M. tuberculosis* strains with similar type of mutations found suggested the possible transmission among the people of these three countries. Genotypes from clinical isolates consisting of drug-susceptible and resistant phenotypes have been published from these three countries. Spoligotype analysis of clinical isolates in urban Bangladesh[49] revealed 22.9, 33.3 and 16.7% of the isolates to be East African Indian (EAI), Beijing and Central Asian (CAS) type, respectively. In contrast, distribution of these three genotypes was 25.0, 15.5 and 7.1%, respectively in rural Bangladesh.[50] Distinct distributions of Spoligotypes were observed in Myanmar[51] (EAI: 41.8%, Beijing: 32.7%, CAS: 0%), northern India[52] (EAI: 10.5%, Beijing: 8.6%, CAS; 36.2%) and southern India[53] (EAI: 84.4%, Beijing: 1.9%, CAS; 3.9%). Difference in strain types in the five regions in three countries might reflect the differences in proportions of certain mutations associated with either RIF- or INH-resistance. Further analysis of genotypes focusing on MDR strains will confirm this.

In conclusion, this study provides valuable information of mutations at *rpo*B and *katG* genes and *inh*A regulatory regions of clinical isolates of *M. tuberculosis* from Bangladesh. It expands our current knowledge and understanding of the molecular mechanisms of drug resistance and also assists in developing low cost tailor-based molecular tools for rapid susceptibility testing of *Mycobacterium* in Bangladesh. Such methods could be appropriate choice for early detection of resistance and to initiate early treatment to stop further transmission of TB.

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References

1. Raviglione MC, Snider Jr DE, Kochi A. Global epidemiology of tuberculosis: Mortality and morbidity of a worldwide epidemic. *JAMA* 1995;**273**:220–6.
2. World Health Organization. *Global tuberculosis control*, http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf; 2011.
3. Nathanson E, Nunn P, Uplekar M, Floyd K, Jaramillo E, Lönnroth K, Weil D, Raviglione M. MDR tuberculosis-critical steps for prevention and control. *N Engl J Med* 2010;**363**:1050–8.
4. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDRTB): 2010 global report on surveillance and response. http://whqlibdoc.who.int/publications/2010/9789241599191_eng.
5. van Deun A, Aung KJ, Chowdhury S, Saha S, Pankaj A, Ashraf A, Rigouts L, Fissette K, Portaels F. Drug susceptibility of *Mycobacterium tuberculosis* in a rural area of Bangladesh and its relevance to the national treatment regiments. *Int J Tuberc Lung Dis* 1999;**3**:143–8.
6. Zaman K, Rahim Z, Yunus M, Arifeen S, Baqui A, Sack D, Hossain S, Banu S, Islam MA, Ahmed J, Breiman R, Black R. Drug resistance of *Mycobacterium tuberculosis* in rural and urban areas in Bangladesh. *Scand J Infect Dis* 2005;**37**:21–6.
7. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: update. *Tuber Lung Dis* 1998;**79**:3–29.
8. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry 3rd CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998;**393**:537–44.
9. Lewis K. Multidrug resistance pumps in bacteria: variations on a theme. *Trend Biochem Sci* 1994;**19**:119–23.
10. Nettleman MD. Multidrug-resistant tuberculosis: news from the front. *JAMA* 2005;**293**:2788–90.
11. World Health Organization. Molecular line probe assays for rapid screening of patients at risk of multidrug resistant tuberculosis (MDR-TB). Policy statement. http://www.who.int/tb/dots/laboratory/line_probe_assays/en/index.html.
12. Sajduda A, Brzostek A, Poplawska M, Augustynowicz-Kopec E, Zwolska Z, Niemann S, Dziadek J, Hillemann D. Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. *J Clin Microbiol* 2004;**42**:2425–31.
13. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;**341**:647–50.
14. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 2009;**13**:1320–30.
15. Hazbón MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, León CI, Bose M, Chaves F, Murray M, Eisenach KD, Sifuentes-Osornio J, Cave MD, Ponce de León A, Alland D. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2006;**50**:2640–9.
16. Kiepiela P, Bishop KS, Smith AN, Roux L, York DF. 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. *Tuber Lung Dis* 2000;**80**:47–56.
17. Zhang M, Yue J, Yang YP, Zhang HM, Lei JQ, Jin RL, Zhang XL, Wang HH. Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. *J Clin Microbiol* 2005;**43**:5477–82.
18. Petroff SA. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and faeces. *J Exp Med* 1915;**21**:38–42.
19. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N, Šmelev NA. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull WHO* 1969;**41**:21–43.
20. Nakajima C, Rahim Z, Fukushima Y, Sugawara I, van der Zanden AG, Tamaru A, Suzuki Y. Identification of *Mycobacterium tuberculosis* clinical isolates in Bangladesh by a species distinguishable multiplex PCR. *BMC Infect Dis* 2010;**10**:118.
21. Hall A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;**41**:95–8.

22. Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey BD, Maharjan B, Suzuki Y. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. *Antimicrob Agents Chemother* 2012;**56**:2831–6.
23. Kourout M, Chaoui I, Sabouni R, Lahlou O, El Mzibri M, Jordaan A, Victor TC, Akrim M, El Aouad R. Molecular characterisation of rifampicin-resistant *Mycobacterium tuberculosis* strains from Morocco. *Int J Tuberc Lung Dis* 2009;**13**:1440–2.
24. Lee AS, Lim IH, Tang LL, Wong SY. High frequency of mutations in the *rpoB* gene in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Singapore. *J Clin Microbiol* 2005;**43**:2026–7.
25. Prammananan T, Cheunoy W, Taechamahapun D, Yorsangsukkamol J, Phunpruch S, Phdarat P, Leechawengwong M, Chaiprasert A. Distribution of *rpoB* mutations among multidrug-resistant *Mycobacterium tuberculosis* (MDRTB) strains from Thailand and development of a rapid method for mutation detection. *Clin Microbiol Infect* 2008;**14**:446–53.
26. Valim AR, Rossetti ML, Ribeiro MO, Zaha A. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from Brazil. *J Clin Microbiol* 2000;**38**:3119–22.
27. Minh NN, Van Bac N, Son NT, Lien VT, Ha CH, Cuong NH, Mai CT, Le TH. Molecular characteristics of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Vietnam. *J Clin Microbiol* 2012;**50**:598–601.
28. Bártfai Z, Somoskövi A, Ködmön C, Szabó N, Puskás E, Kosztolányi L, Faragó E, Mester J, Parsons LM, Salfinger M. Molecular characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. *J Clin Microbiol* 2001;**39**:3736–9.
29. Paluch-Oles J, Kozioł-Montewka M, Magrys A. Mutations in the rpoB gene of rifampin-resistant *Mycobacterium tuberculosis* isolates from Eastern Poland. *New Microbiol* 2009;**32**:147–52.
30. Sheng J, Li J, Sheng G, Yu H, Huang H, Cao H, Lu Y, Deng X. Characterization of *rpoB* mutations associated with rifampin resistance in *Mycobacterium tuberculosis* from eastern China. *J Appl Microbiol* 2008;**105**:904–11.
31. Hillemann D, Kubica T, Rüsch-Gerdes S, Niemann S. Disequilibrium in distribution of resistance mutations among *Mycobacterium tuberculosis* Beijing and non-Beijing strains isolated from patients in Germany. *Antimicrob Agents Chemother* 2005;**49**:1229–31.
32. Torres MJ, Criado A, Gónzalez N, Palomares JC, Aznar J. Rifampin and isoniazid resistance associated mutations in *Mycobacterium tuberculosis* clinical isolates in Seville, Spain. *Int J Tuberc Lung Dis* 2002;**6**:160–3.
33. Heep M, Brandstätter B, Rieger U, Lehn N, Richter E, Rüsch-Gerdes S, Niemann S. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2001;**39**:107–10.
34. Hirano K, Abe C, Takahashi M. 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* 1999;**37**:2663–6.
35. Hui J, Gordon N, Kajioka R. Permeability barrier to rifampin in mycobacteria. *Antimicrob Agents Chemother* 1977;**11**:773–9.
36. Rouse DA, DeVito JA, Li Z, Byer H, Morris SL. Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. *Mol Microbiol* 1996;**22**:583–92.
37. Aslan G, Tezcan S, Serin MS, Emekdas G. Genotypic analysis of isoniazid and rifampin resistance in drug-resistant clinical *Mycobacterium tuberculosis* complex isolates in southern Turkey. *Jpn J Infect Dis* 2008;**61**:255–60.
38. Homolka S, Meyer CG, Hillemann D, Owusu-Dabo E, Adjei O, Horstmann RD, Browne EN, Chinbuah A, Osei I, Gyapong J, Kubica T, Ruesch-Gerdes S, Niemann S. Unequal distribution of resistance-conferring mutations among *Mycobacterium tuberculosis* and *Mycobacterium africanum* strains from Ghana. *Int J Med Microbiol* 2010;**300**:489–95.
39. Afanas'ev MV, Ikryannikova LN, Il'ina EN, Sidorenko SV, Kuz'min AV, Larionova EE, Smirnova TG, Chernousova LN, Kamaev EY, Skorniakov SN, Kinsht VN, Cherednichenko AG, Govorun VM. Molecular characteristics of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Russian Federation. *J Antimicrob Chemother* 2007;**59**:1057–64.
40. Kozhamkulov U, Akhmetova A, Rakhimova S, Belova E, Alenova A, Bismilda V, Chingissova L, Ismailov S, Ramanculov E, Momynaliev K. Molecular characterization of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Kazakhstan. *Jpn J Infect Dis* 2011;**64**:253–5.
41. Guo H, Seet Q, Denkin S, Parsons L, Zhang Y. Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. *J Med Microbiol* 2006;**55**:1527–31.
42. Bolotin S, Alexander DC, Chedore P, Drews SJ, Jamieson F. Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Ontario, Canada. *J Antimicrob Chemother* 2009;**64**:263–6.
43. Suresh N, Singh UB, Arora J, Pant H, Seth P, Sola C, Rastogi N, Samantaray JC, Pande JN. *rpo*B gene sequencing and spoligotyping of multidrug-resistant *Mycobacterium tuberculosis* isolates from India. *Infect Genet Evol* 2006;**6**:474–83.
44. Soudani A, Hadjfredj S, Zribi M, Messaadi F, Messaoud T, Masmoudi A, Zribi M, Fendri C. Genotypic and phenotypic characteristics of tunisian isoniazid-resistant *Mycobacterium tuberculosis* strains. *J Microbiol* 2011;**49**:413–7.
45. Valvatne H, Syre H, Kross M, Stavrum R, Ti T, Phyu S, Grewal HM. Isoniazid and rifampicin resistance-associated mutations in *Mycobacterium tuberculosis* isolates from Yangon, Myanmar: implications for rapid molecular testing. *Antimicrob Agents Chemother* 2009;**64**:694–701.
46. Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N, Prachee, Banerjee S, Savithri GR, Alam M, Pathak N, Amin A, Hanief M, Katoch VM, Sharma SK, Hasnain SE. Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in north India. *Antimicrob Agents Chemother* 2002;**46**:443–50.
47. Nusrath Unissa A, Selvakumar N, Narayanan S, Narayanan PR. Molecular analysis of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from India. *Int J Antimicrob Agents* 2008;**31**:71–5.
48. Mathuria JP, Nath G, Samaria JK, Anupurba S. Molecular characterization of INH-resistant *Mycobacterium tuberculosis* isolates by PCR-RFLP and multiplex-PCR in north India. *Infect Genet Evol* 2009;**9**:1352–5.
49. Banu S, Gordon SV, Palmer S, Islam R, Ahmed S, Khan Alam KM, Cole ST, Roland Brosch R. Genotypic analysis of *Mycobacterium tuberculosis* in Bangladesh and prevalence of the Beijing strain. *J Clin Microbiol* 2004;**42**:674–82.
50. Banu S, Uddin MKM, Islam MR, Zaman K, Ahmed T, Talukder AH, Rahman MT, Rahim Z, Akter N, Khatun R, Brosch R, Endtz HP. Molecular epidemiology of tuberculosis in rural Matlab, Bangladesh. *Int J Tuberc Lung Dis* 2012;**16**:319–26.
51. Phyu S, Jureen R, Ti T, Dahle UR, Grewal HMS. Heterogeneity of *Mycobacterium tuberculosis* isolates in Yangon, Myanmar. *J Clin Microbiol* 2003;**41**:4907–8.
52. Singh UB, Suresh N, Bhanu NV, Arora J, Pant H, Sinha S, Aggarwal RC, Singh S, Pande JN, Sola C, Rastogi N, Seth P. Predominant tuberculosis Spoligotypes, Delhi, India. *Emerg Infect Dis* 2004;**10**:1138–42.
53. Narayanan S, Gagneux S, Hari L, Tsolaki AG, Rajasekhar S, Narayanan PR, Small PM, Holmes S, DeRiemer K. Genomic interrogation of ancestral *Mycobacterium tuberculosis* from south India. *Infect Genet Evol* 2008;**8**:474–83.

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

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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

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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 (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.

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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.

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Short communication

Sensitivities of ciprofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates to fluoroquinolones: role of mutant DNA gyrase subunits in drug resistance

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ABSTRACT

Minimum inhibitory concentrations of sitafloxacin, gatifloxacin, moxifloxacin, sparfloxacin, levofloxacin and ciprofloxacin against 59 ciprofloxacin-resistant clinical isolates of *Mycobacterium tuberculosis* from Japan were determined. The isolates were most susceptible to sitafloxacin and gatifloxacin. To understand better the basis for drug resistance, nucleotide sequences encoding the *gyrA* and *gyrB* quinolone resistance-determining region were determined. Predicted amino acid sequences revealed distinct mutational patterns likely to be responsible for fluoroquinolone resistance. Double *gyrA* mutations as well as mutations in both *gyrA* and *gyrB* correlated with increased resistance to all fluoroquinolones.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a serious global health problem as one-third of the world's population is infected, resulting in 9 million new cases and nearly 2 million deaths in 2010 [1]. The World Health Organization (WHO) recommends a four-drug combination therapeutic strategy termed 'directly observed treatment, short-course (DOTS)' to prevent the spread of drug-resistant TB; nevertheless, an increasing number of multidrug-resistant TB (MDR-TB) isolates, resistant to more than two drugs including rifampicin and isoniazid, have arisen in some Eastern European and Western Asian countries [1]. Accordingly, broad-spectrum fluoroquinolones (FQs) have been used to treat MDR-TB [2]. Unfortunately, their increasing use has generated large numbers of FQ-resistant *M. tuberculosis* strains [1] and thus researchers have focused on elucidating the mechanism of acquired resistance.

FQs target type II DNA topoisomerases, including DNA gyrase and topoisomerase IV. DNA gyrase mediates the supercoiling of double-stranded DNA during DNA replication, and topoisomerase IV separates or deconcatenates replicated chromosomes. Amino acid substitutions in putative FQ-binding regions in DNA gyrase, topoisomerase IV, or both, confer resistance upon several bacterial species [3]. These regions are highly conserved in bacteria and are referred to as the quinolone resistance-determining regions (QRDRs) [3]. *Mycobacterium tuberculosis* lacks topoisomerase IV [4], thus leaving DNA gyrase as the apparent sole target of FQs.

Single missense mutations in *gyrA* have been associated with FQ resistance [5–9], and strains carrying two missense mutations in both *gyrA* and *gyrB* generally exhibit higher-level resistance [8,9]. These findings suggest that amino acid substitutions in GyrA and GyrB impart FQ resistance to *M. tuberculosis*.

Interestingly, the rate and mode of mutations vary with respect to geographical origin [5–9]; however, similar studies have not, to our knowledge, been conducted in Japan. Japan suffers from the unhappy distinction of nearly 30% of its MDR-TB being extensively drug-resistant TB (XDR-TB) [10], which, in addition to being resistant to rifampicin and isoniazid, is also resistant to any FQ and any injectable agent. Therefore, the aim of the present study was to compare the antimicrobial activities of FQs against Japanese ciprofloxacin (CIP)-resistant clinical isolates and to identify QRDR mutations imparting FQ resistance.

2. Materials and methods

2.1. Bacterial strains

The 59 *M. tuberculosis* clinical isolates studied came from 11 hospitals in Japan and were resistant to ≥4 μg/mL CIP using the

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proportion method on Ogawa medium (2% phosphate) [20 g of KH_2PO_4, 5.0 g of sodium glutamate, 1.0 g of magnesium citrate, 30 g of starch, 2.0 g of sodium pyruvate, 40 mL of glycerol, 40 mL of malachite green (2% solution), 2000 mL of homogenised whole egg and 1000 mL of distilled water]. *Mycobacterium tuberculosis* H37Rv was used as a quinolone-susceptible strain.

2.2. Drugs

Sitafloxacin (STFX) and levofloxacin (LVX) were provided by Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan). Gatifloxacin (GFLX) and sparfloxacin (SPFX) were from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and Kyorin Pharmaceutical Inc. (Tokyo, Japan), respectively. Moxifloxacin (MXF) and CIP were from Bayer Yakuhin, Ltd. (Osaka, Japan). Drugs were dissolved in 0.1 M NaOH at 50 mg/mL (except for MXF, which was dissolved in distilled water to 10 μg/mL) and used as stock solutions.

2.3. Minimum inhibitory concentration (MIC) determination

Isolates were cultured in Middlebrook 7H9 medium (Becton Dickinson, Franklin Lakes, NJ) containing 0.05% Tween 80, 10% oleic–albumin–dextrose–catalase (OADC) and 0.2% glycerol at 37 °C for 7 days and then diluted with the same medium to an optical density at 540 nm of 0.2. Middlebrook 7H11 agar (Becton Dickinson) plates (15 mL volume) without or with drugs diluted serially to yield final concentrations from 0.39–12.5, 0.78–6.25, 0.78–12.5, 1.56–12.5, 3.13–25 and 6.25–50 μg/mL, respectively, for STFX, GFLX, MXF, SPFX, LVX and CIP were inoculated with 0.1 mL of 100-fold-diluted bacterial suspensions described above. Bacterial growth was assessed after 3 weeks at 37 °C under 5% CO_2. The MIC was defined as the lowest concentration that inhibited visible growth.

2.4. Sequence analysis

Chromosomal DNA was extracted from a colony by mechanical disruption [11]. DNA fragments encoding GyrA and GyrB QRDRs were amplified and were sequenced using the primer pairs TBgyrA1N (5′-AGCGCAGCTACATCGACTATGCG-3′)/TBgyrA2N (5′-CTTCGGTGTACCTCATCGCCGCC-3′) and TBgyrB1N (5′-TCGGCGCAAGCCCGTATCGCGGC-3′)/TBgyrB2N (5′-CATCAGCACGATCTTGTGGTAGC-3′). Reaction mixtures (50 μL) contained 1.25 U of LA *Taq*™ DNA polymerase (Takara Bio Inc., Shiga, Japan), LA PCR buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.5 mM of each primer and 10 ng of DNA. Polymerase chain reaction (PCR) was carried out using a Takara PCR Thermal Cycler PERSONAL (Takara Bio Inc.) for 35 cycles of denaturation for 5 s at 98 °C, annealing for 10 s at 55 °C and extension for 30 s at 72 °C; and final extension for 3 min at 72 °C. PCR products were separated by 1% agarose gel electrophoresis in TAE buffer [40 mM Tris–acetate, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.3] extracted from an excised gel block into supernatant by centrifugation at 20 000 × *g* for 5 min and were directly sequenced using a BigDye® Terminator v.1.1 Cycle Sequencing Kit and a Model 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

3. Results

3.1. Characterisation of Mycobacterium tuberculosis isolates

MICs to six FQs for all 59 CIP-resistant strains were determined (Table 1). Most of the CIP-resistant isolates (52/59) were inhibited by STFX and GFLX at 3.13 μg/mL. MXF and SPFX inhibited 35/59 and 32/59 strains, respectively, at the same concentration, in contrast to LVX that only inhibited 12/59 strains. These tests verified that all strains were CIP-resistant.

3.2. Quinolone resistance-determining region mutations

Sequence analysis found eight single-point and six double-point mutational patterns (Table 1). All isolates harboured QRDR mutations. Substitution at amino acid 94 was most common (39/59) followed by amino acid 90 (17/59).

3.3. Minimum inhibitory concentration as a function of single GyrA quinolone resistance-determining region mutations

Table 2 summarises the drug susceptibility assays of GyrA and GyrB mutants. A strain with Asp → Val substitution at amino acid 94 (Asp94Val) exhibited susceptibility to each drug with the exception of LVX and CIP, both with MIC_{50} values (MICs inhibiting >50% of tested strains) of 6.25 μg/mL. Four strains with a Ser91Pro substitution exhibiting low CIP resistance were significantly inhibited by the five other FQs. Strains with Ala90Val or Asp94Ala substitution showed slightly diminished susceptibility to CIP and LVX but were susceptible to the other FQs. In contrast, MIC_{50} values for the Asp94Tyr mutant were 0.78, 1.56, 3.13, 3.13, 6.25 and 12.5 μg/mL, respectively, for STFX, GFLX, MXF, SPFX, LVX and CIP. The Asp94Asn mutant was less susceptible than the aforementioned mutants, but more so than the Asp94Gly and Gly88Cys mutants, the latter of which was the least susceptible. In addition, variation in MICs for strains with the same mutation was found.

3.4. Minimum inhibitory concentration as a function of multiple quinolone resistance-determining region mutations

Strains with double mutations tended to exhibit lower susceptibility than the corresponding single mutant. An isolate harbouring both Ala90Val and Asp94Ala amino acid substitutions (isolate ID55) (Table 1) exhibited lower susceptibility to CIP than single Ala90Val or Asp94Ala mutants. Similarly, Ala90Val + Ser91Pro and Ala90Val + Asp94Val mutants exhibited lower susceptibility to CIP than single mutants at each position (Table 2). Two *gyrB* mutations linked to a *gyrA* mutation were identified as follows: GyrA-Ala90Val + GyrB-Thr539Asn and GyrA-Asp94Gly + GyrB-Ser486Phe, of which the former *gyrB* mutation was associated with reduced susceptibility and the latter was not (Table 2).

4. Discussion

Treating MDR-TB has come to rely increasingly on FQs, as emphasised by the WHO recommendation [1] that each MDR clinical isolate registered should be routinely tested for ofloxacin (OFX) or CIP susceptibility. Because FQs are widely used for treating other infectious diseases, their efficacy for treating TB may be compromised. To counteract this possibility, it is hoped that CIP-resistant strains will be cured with newer FQs [2]. To our knowledge, the present study is the first to investigate drug sensitivities of CIP-resistant clinical isolates from patients in Japan, a country with a high rate of XDR-TB amongst MDR-TB [9]. Drug susceptibility patterns have been previously analysed in several geographic locations (Table 3). In Hong Kong, STFX, GFLX and MXF MIC_{50} values were lower than those of OFX, LVX and SPFX ($n = 32$) [6]. A study in Italy ($n = 17$) reported median MICs and the results can be summarised as OFX > CIP and LVX > SPFX > GFLX and MXF [7]. MXF and GFLX were shown to be more active than OFX in Belgium ($n = 22$) [8]. A similar effectiveness of CIP, OFX, LVX and MXF was demonstrated in Taiwan ($n = 6$) [9]. All the data showed similarity and the main conclusion drawn from these studies and the present study is that STFX,

Table 1
Minimum inhibitory concentrations (MICs) of six quinolones and amino acid substitutions in GyrA and GyrB for ciprofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates in Japan.

Isolate ID	MIC (μg/mL)						Amino acid substitution in QRDR					
							GyrA				GyrB	
	STFX	GFLX	MXF	SPFX	LVX	CIP	Gly88	Ala90	Ser91	Asp94	Ser486	Thr539
1	12.5	3.13	12.5	6.25	12.5	>50	Cys					
2	≤0.39	≤0.78	0.78	1.56	3.13	6.25		Val				
3	≤0.39	≤0.78	1.56	1.56	3.13	6.25		Val				
4	0.78	0.78	1.56	1.56	3.13	6.25		Val				
5	0.78	0.78	1.56	3.13	3.13	6.25		Val				
6	≤0.39	1.56	1.56	1.56	6.25	6.25		Val				
7	0.78	1.56	3.13	3.13	6.25	6.25		Val				
8	0.78	1.56	1.56	1.56	3.13	12.5		Val				
9	0.78	≤0.78	0.78	3.13	6.25	12.5		Val				
10	≤0.39	≤0.78	1.56	3.13	6.25	12.5		Val				
11	≤0.39	1.56	3.13	3.13	6.25	12.5		Val				
12	6.25	3.13	3.13	3.13	6.25	12.5		Val				
13	0.78	1.56	3.13	3.13	6.25	25		Val				
14	0.78	1.56	3.13	1.56	3.13	6.25			Pro			
15	1.56	1.56	3.13	1.56	3.13	6.25			Pro			
16	3.13	3.13	3.13	1.56	3.13	6.25			Pro			
17	1.56	1.56	3.13	1.56	6.25	6.25			Pro			
18	≤0.39	≤0.78	1.56	1.56	3.13	6.25				Ala		
19	0.78	≤0.78	1.56	1.56	3.13	6.25				Ala		
20	1.56	≤0.78	1.56	1.56	3.13	6.25				Ala		
21	≤0.39	1.56	1.56	3.13	3.13	12.5				Ala		
22	≤0.39	≤0.78	1.56	3.13	6.25	12.5				Ala		
23	0.78	1.56	1.56	3.13	6.25	12.5				Ala		
24	≤0.39	1.56	3.13	3.13	6.25	12.5				Ala		
25	3.13	≤0.78	3.13	3.13	6.25	12.5				Ala		
26	3.13	1.56	3.13	3.13	6.25	25				Ala		
27	1.56	3.13	6.25	6.25	12.5	25				Ala		
28	3.13	1.56	3.13	6.25	6.25	12.5				Gly		
29	1.56	3.13	6.25	6.25	12.5	12.5				Gly		
30	1.56	1.56	3.13	3.13	6.25	25				Gly		
31	3.13	1.56	3.13	3.13	6.25	25				Gly		
32	0.78	3.13	6.25	6.25	12.5	25				Gly		
33	0.78	3.13	6.25	6.25	12.5	25				Gly		
34	0.78	3.13	6.25	6.25	12.5	25				Gly		
35	1.56	3.13	6.25	6.25	12.5	25				Gly		
36	3.13	1.56	6.25	6.25	12.5	25				Gly		
37	3.13	3.13	6.25	6.25	12.5	25				Gly		
38	3.13	3.13	6.25	6.25	12.5	25				Gly		
39	6.25	3.13	12.5	6.25	12.5	25				Gly		
40	3.13	3.13	6.25	6.25	12.5	>50				Gly		
41	6.25	6.25	12.5	6.25	12.5	>50				Gly		
42	3.13	6.25	12.5	12.5	12.5	>50				Gly		
43	1.56	1.56	3.13	6.25	6.25	12.5				Asn		
44	1.56	1.56	3.13	6.25	6.25	25				Asn		
45	1.56	3.13	6.25	6.25	6.25	25				Asn		
46	3.13	6.25	12.5	12.5	25	25				Asn		
47	>12.5	6.25	12.5	12.5	25	>50				Asn		
48	≤0.39	1.56	3.13	3.13	6.25	12.5				Tyr		
49	0.78	≤0.78	3.13	3.13	6.25	12.5				Tyr		
50	1.56	3.13	6.25	3.13	12.5	12.5				Tyr		
51	0.78	1.56	3.13	6.25	6.25	25				Tyr		
52	3.13	3.13	6.25	6.25	6.25	>50				Tyr		
53	0.78	≤0.78	1.56	1.56	6.25	6.25				Val		
54	>12.5	>6.25	>12.5	>12.5	>25	>50		Val	Pro			
55	6.25	6.25	12.5	>12.5	>25	>50		Val		Ala		
56	3.13	6.25	6.25	12.5	25	25		Val		Val		
57	0.78	3.13	12.5	12.5	12.5	25		Val				Asn
58	3.13	3.13	12.5	12.5	12.5	25		Val				Asn
59	1.56	1.56	3.13	3.13	6.25	25				Gly	Phe	
No. of strains with MIC ≤3.13 μg/mL	52	52	35	32	12	0						

STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; QRDR, quinolone-resistance determining region.

GFLX and MXF can inhibit the growth of OFX- or CIP-resistant *M. tuberculosis* in vitro. As the maximum serum concentrations (C_{max}) of OFX [12], GFLX [13], STFX [14] and MFX [15] for a 100 mg dosage were determined in clinical trials to be 1.0, 1.1, 3.1 and 4.0 μg/mL, respectively, STFX, GFLX and MFX might inhibit the OFX- or CIP-resistant *M. tuberculosis* in therapeutic use.

This study demonstrated that all CIP-resistant clinical isolates harboured *gyrA* mutations, similar to the data from Belgium (96%) [8], Italy (89%) [7] and Vietnam (83%) [5]. However, a significantly lower correlation was reported by investigators in Hong Kong (58%) [6] and Taiwan (50%) [9]. Use of a relatively high CIP MIC (≥4 μg/mL) for defining resistance might be a

Table 2
Amino acid substitutions in GyrA and GyrB and associated minimum inhibitory concentrations (MICs).

Amino acid substitution in QRDR		No. of isolates	MIC (μg/mL)											
			STFX		GFLX		MXF		SPFX		LVX		CIP	
GyrA	GyrB		Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}
Asp94Val		1	0.78	0.78	≤0.78	≤0.78	1.56	1.56	1.56	1.56	6.25	6.25	6.25	6.25
Ser91Pro		4	0.78–3.13	1.56	1.56–3.13	1.56	3.13	3.13	1.56	1.56	3.13–6.25	3.13	6.25	6.25
Ala90Val		12	≤0.39–6.25	0.78	≤0.78–3.13	≤0.78	0.78–3.13	1.56	1.56–3.13	3.13	3.13–6.25	6.25	6.25–12.5	6.25
Asp94Ala		10	≤0.39–3.13	0.78	≤0.78–3.13	≤0.78	1.56–6.25	1.56	1.56–6.25	3.13	3.13–12.5	6.25	6.25–25	12.5
Asp94Tyr		5	≤0.39–3.13	0.78	≤0.78–3.13	1.56	3.13–6.25	3.13	3.13–6.25	3.13	6.25–12.5	6.25	12.5 to >50	12.5
Asp94Gly		15	0.78–6.25	3.13	1.56–6.25	3.13	3.13–12.5	6.25	3.13–12.5	6.25	6.25–12.5	12.5	12.5 to >50	25
Asp94Asn		5	1.56 to >12.5	1.56	1.56–6.25	3.13	3.13–12.5	6.25	6.25–12.5	6.25	6.25–25	6.25	12.5 to >50	25
Gly88Cys		1	12.5	12.5	3.13	3.13	12.5	12.5	6.25	6.25	12.5	12.5	>50	>50
Ala90Val + Asp94Val		1	3.13	3.13	6.25	6.25	6.25	6.25	12.5	12.5	25	25	25	25
Ala90Val + Asp94Ala		1	6.25	6.25	6.25	6.25	12.5	12.5	>12.5	>12.5	>25	>25	>50	>50
Ala90Val + Ser91Pro		1	>12.5	>12.5	>6.25	>6.25	>12.5	>12.5	>12.5	>12.5	>25	>25	>50	>50
Asp94Gly	Ser486Phe	1	1.56	1.56	1.56	1.56	3.13	3.13	3.13	3.13	6.25	6.25	25	25
Ala90Val	Thr539Asn	2	0.78–3.13	0.78	3.13	3.13	12.5	12.5	12.5	12.5	12.5	12.5	25	25

QRDR, quinolone-resistance determining region; STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; MIC_{50}, MIC inhibiting >50% of tested strains.

Table 3
Amino acid substitutions in GyrA and GyrB and MIC_{50} values (μg/mL).

Amino acid substitution in QRDR		These data (*n* = 59)							Italy (*n* = 17) [7]							Taiwan (*n* = 6) [9]					Hong Kong (*n* = 31) [6]							Belgium (*n* = 22) [8]			
GyrA	GyrB	*N*[a]	STFX	GFLX	MXF	SPFX	LVX	CIP	*N*[a]	GFLX	MXF	SPFX	LVX	CIP	OFX	*N*[a]	MXF	LVX	CIP	OFX	*N*[a]	STFX	GFLX	MXF	SPFX	LVX	OFX	*N*[a]	GFLX	MXF	OFX
Asp94Val		1	0.78	≤0.78	1.56	1.56	6.25	6.25	0							0					0							0			
Asp89Asn		0							0							0					0							1	1	2	4
Ser91Pro		4	1.56	1.56	3.13	1.56	3.13	6.25	2	0.5	1	1	2	4	4	0					4	1	2	1	0.5	2	4	0			
Ala90Val		12	0.78	≤0.78	1.56	3.13	6.25	6.25	9	1	1	2	4	4	8	1	1	4	8	16	5	0.5	1	1	2	4	8	10	0.5	0.5	4
Asp94Ala		10	0.78	≤0.78	1.56	3.13	6.25	12.5	1	0.5	1	2	4	4	4	0					3	0.5	2	1	2	4	8	3	0.25	0.5	4
Asp94Tyr		5	0.78	1.56	3.13	3.13	6.25	12.5	0							0					5	2	2	2	4	8	16	3	1	2	8
Asp94His		0							0							0					3	1	4	2	4	8	16	0			
Asp94Gly		15	3.13	3.13	6.25	6.25	12.5	25	4	1	1	2	4	4	8	4	1	2	4	4	11	2	4	2	4	8	16	4	1	2	8
Asp94Asn		5	1.56	3.13	6.25	6.25	6.25	25	1	1	1	2	2	4	8	0					1	4	4	2	2	4	8	1	1	4	16
Gly88Cys		1	12.5	3.13	12.5	6.25	12.5	>50	0							0					0							0			
	Asn538Asp	0							0							1	1	1	4	8	0							0			
	Asn533Thr	0							0							0					0							1	0.5	0.5	1
Ala90Val + Asp94Val		1	3.13	6.25	6.25	12.5	25	25	0							0					0							0			
Ala90Val + Asp94Ala		1	6.25	6.25	12.5	>12.5	>25	>50	0							0					0							0			
Ala90Val + Asp94Asn		0							0							0					0							1	>4	>8	>32
Ala90Val + Ser91Pro		1	>12.5	>6.25	>12.5	>12.5	>25	>50	0							0					0							0			
Asp94Gly	Ser486Phe	1	1.56	1.56	3.13	3.13	6.25	25	0							0					0							0			
Ala90Val	Thr539Asn	2	0.78	3.13	12.5	12.5	12.5	25	0							0					0							0			
Asp94His	del678–9	0							0							0					0							1	1	2	4

MIC_{50}, MIC inhibiting >50% of tested strains; STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; OFX, ofloxacin.
[a] Number of isolates.

reason for the 100% correlation between CIP resistance and *gyrA* mutations. The current data regarding GyrA QRDR mutations and their influence on MIC values of FQs for isolates with a variety of mutations are generally consistent with findings by others (Table 3). Variations in MICs in strains with the same mutations were also observed, similar to studies by others. Mechanisms other than amino acid substitution in DNA gyrase subunits, such as permeability change, might cause this phenomenon. The present study uniquely contributes data on the effect of Asp94Val, Gly88Cys, Ala90Val + Asp94Val, Ala90Val + Asp94Ala and Ala90Val + Ser91Pro mutations on resistance to FQs. The Gly88Cys mutation in particular contributed to high-level resistance. Others have also reported drug sensitivity data for GyrA QRDR double mutants, namely Ala90Val + Ser91Pro, Ala90Val + Asp94Gly and Ala90Val + Asp94Asn mutants highly resistant to OFX. Note, however, that the findings reported here not only supported the former studies' conclusions but also added an insight into cross-resistance of isolates with these double mutations in *gyrA* to a wide range of FQs. Although no single *gyrB* mutations conferring drug resistance have been identified in this study, we report here that only one of the two *gyrB* mutations found in this study appeared to clearly contribute to increased resistance beyond the level conferred by the *gyrA* mutation alone.

In conclusion, these data suggest that MXF, STFX and GFLX more potently inhibit CIP-resistant *M. tuberculosis* compared with other FQs, as has been shown in previous studies. These data provide a foundation upon which to base new therapeutic strategies for more effectively treating MDR-TB. In addition, the data enhance previously available information on the contribution of amino acid substitutions in DNA gyrase to help improve gene-based methods for predicting FQ susceptibility, which enables the appropriate choice of a FQ for the treatment of MDR-TB.

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References

[1] World Health Organization. Global tuberculosis control 2010. Geneva, Switzerland: WHO; 2010. http://www.who.int/tb/publications/global_report/2010/en/index.html [accessed 2 February 2012].
[2] Moadebi S, Harder CK, Fitzgerald MJ, Elwood KR, Marra F. Fluoroquinolones for the treatment of pulmonary tuberculosis. Drugs 2007;67:2077–99.
[3] Mdluli K, Ma Z. *Mycobacterium tuberculosis* DNA gyrase as a target for drug discovery. Infect Disord Drug Targets 2007;7:159–68.
[4] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998;393:537–44.
[5] Duong DA, Nguyen TH, Nguyen TN, Dai VH, Dang TM, Vo SK, et al. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob Agents Chemother 2009;53:4835–9.
[6] Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplimer conformation analysis for rapid detection of *gyrA* mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother 2004;48:596–601.
[7] Giannoni F, Iona E, Sementilli F, Brunori L, Pardini M, Migliori GB, et al. Evaluation of a new line probe assay for rapid identification of *gyrA* mutations in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2005;49:2928–33.
[8] Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, et al. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gyrA* and *gyrB*. Antimicrob Agents Chemother 2009;53:4498–500.
[9] Wang JY, Lee LN, Lai HC, Wang SK, Jan IS, Yu CJ, et al. Fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates: associated genetic mutations and relationship to antimicrobial exposure. J Antimicrob Chemother 2007;59:860–5.
[10] Tuberculosis Research Committee (Ryoken). Drug-resistant *Mycobacterium tuberculosis* in Japan: a nationwide survey, 2002. Int J Tuberc Lung Dis 2007;11:1129–35.
[11] Suzuki Y, Katsukawa C, Inoue K, Yin Y, Tasaka H, Ueba N, et al. Mutations in *rpoB* gene of rifampicin resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. Kansenshogaku Zasshi 1995;69:413–9.
[12] Ichihara N. Phase I study on DL-8280 (OFX). Chemotherapy 1984;32:118–49.
[13] Nakashima M, Uematsu T, Kosuge T, Uemura K, Hakusui H, Tanaka M. Pharmacokinetics and tolerance of DU-6859a, a new fluoroquinolone, after single and multiple oral doses in healthy volunteers. Antimicrob Agents Chemother 1995;39:170–4.
[14] Nakashima M, Uematsu T, Kosuge K, Kusajima H, Ooie T, Matsuda Y, et al. Single- and multiple-dose pharmacokinetics of AM-1155, a new 6-fluoro-8-methoxy quinolone, in humans. Antimicrob Agents Chemother 1995;39:2635–40.
[15] Ohnishi N, Toyoki T, Yoshikawa K, Hashizume K, Tanigawa T, Komori T, et al. Safety, pharmacokinetics and influence on the intestinal flora of BAY 12-8039 (moxifloxacin hydrochloride) after oral administration in healthy male subjects. Jpn Pharmacol Ther 2005;33:1029–45.

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Regional report

The NUITM-KEMRI P3 Laboratory in Kenya: Establishment, Features, Operation and Maintenance

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Abstract: A biocontainment facility is a core component in any research setting due to the services it renders towards comprehensive biosafety observance. The NUITM-KEMRI P3 facility was set up in 2007 and has been actively in use since 2010 by researchers from this and other institutions. A number of hazardous agents have been handled in the laboratory among them MDR-TB and yellow fever viruses. The laboratory has the general physical and operational features of a P3 laboratory in addition to a number of unique features, among them the water-air filtration system, the eco-mode operation feature and automation of the pressure system that make the facility more efficient. It is equipped with biosafety and emergency response equipments alongside common laboratory equipments, maintained regularly using daily, monthly and yearly routines. Security and safety is strictly observed within the facility, enhanced by restricted entry, strict documentation and use of safety symbols. Training is also engrained within the operation of the laboratory and is undertaken and evaluated annually. Though the laboratory is in the process of obtaining accreditation, it is fully certified courtesy of the manufactures' and constructed within specified standards.
Key words: P3 Laboratory, Kenya

INTRODUCTION

A P3 laboratory is a containment facility that enables the isolation and manipulation of dangerous biological materials for various research purposes. P3 laboratories are subjected to BSL-3 standards, which permit the handling of biological agents that can be transmitted through aerosols. The establishment and usage of P3 laboratory and facilities can be traced back to the mid-20th century, with the emergence of laboratory–acquired infections attributed to unsecured laboratory operations [1–3]. These findings stimulated the World Health Organization (WHO) to prepare designs and compile biosafety rules and strategies into a biosafety manual for purposes of providing practical guidance on biosafety techniques and procedures.

In the manual, microorganisms are classified into risk groups in ascending order of pathogenicity and stipulate four main levels of biological safety that classifies laboratories into P1, P2, and P3 and P4 depending on their level of their biosafety systems. Countries were also required to develop codes of practice for the safe handling of pathogenic agents and institute basic concepts of biological safety relevant for their geographical borders, a mandate that several countries have complied with today. While P4 laboratories are not as popular, the number of P3 facilities around the world have steadily increased, especially in developing countries owing to the increasing complexity of public health problems and the need to manage and contain incidences of laboratory acquired infections. In Kenya, there are five P3 facilities; four at KEMRI's collaborative centers and one at the International Livestock Research Institute (ILRI). Among the five facilities, our research unit hosts

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one of the most active P3 laboratories in Kenya alongside existing BSL-2 facilities.

ESTABLISHMENT OF THE NUITM-KEMRI P3 LABORATORY

Nagasaki University, Institute of Tropical Medicine Kenya research station was established in 2005 in collaboration with KEMRI (NUITM-KEMRI) as a center for research in the area of tropical medicine with a core objective of undertaking research towards the prevention and management of tropical and emerging infectious diseases alongside building biomedical research capacity in Kenya. Currently, the station has diversified its research activities into more specialized areas including entomology, virology, TB, the eradication of Malaria and research in diarrheal diseases. In addition to these, the station is preparing to embark on a number of new research projects featuring fishery and engineering science research.

The P3 laboratory was set up in 2007 within the building that houses the Center for Microbiology Research (CMR), and it intended to support clinical, teaching and research activities involving dangerous biological agents (Fig. 1). The facility was imported from the NK system in Japan with approval from the NUITM committee but not without obvious difficulties, especially obtaining permits from relevant authorities. This was the first time a university attempted to export such a facility and, as might be expected, difficulties were encountered in obtaining an export permit from Japan's Ministry of economy, trade and industry and from Kenyan end, an import request from Ministry of finance for tax and duty exemptions. Certification was provided on the condition that the equipment was intended to use in enhancing biosafety and not as a biosecurity threat.

Since its establishment, the lab has played a significant role in improving research experiences and outcomes by enabling research activities involving highly contagious biological materials. A number of studies have been successfully completed or are on-going in our facility by researchers from this and other institutions. Among these is the Remox project, a collaborative clinical trial that sought to establish the efficiency of a moxifloxacin-containing treatment regimen in the management of drug-sensitive tuberculosis. The JICA-JSPS Arbovirus project and JICA-JST SATREPS project were also carried out in the P3 lab due to apparent pathogenicity of arboviruses. In addition to these, our staff regularly uses the lab to process BSL-3 agents for on-going projects, especially septicemia, TB and virological studies.

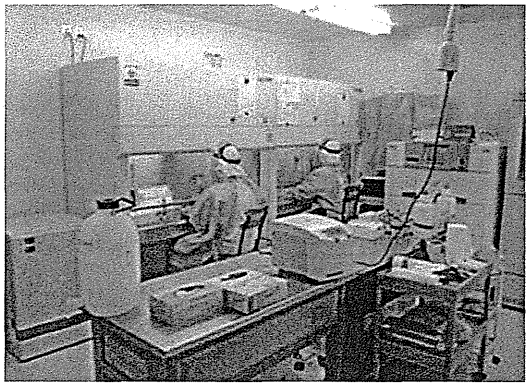

Fig. 1. Researchers in NUITM P3 lab

Our P3 laboratory is located at the furthest end of the CMR building for it to be physically separated from areas of unrestricted traffic. It is an airtight facility, complete with its own water and dust proof wall. The flooring material is seamless and resistant to damage by laboratory reagents and cleaning detergents. The walls and the ceiling are finished with noninflammable materials (polyurethane) that is easily cleaned, resistant to corrosion and devoid of cracks and disjointed junctions. In addition to normal physical features and structures, our facility is fully equipped to handle a variety of biological assays. Major functional areas of the laboratory are fully computerized, a feature that makes the laboratory more efficient and easy to maintain.

FEATURES OF THE P3 LABORATORY

A) Physical features- General features

a) Door interlock- this is a double door system that functions in maintaining negative pressure and the air conditioning system. The Main door (that opens into the ante-room) and the P3 room door cannot be opened at the same time. The doors are self-closing and lockable. The pass-box door also has a similar mechanism.

b) Air conditioning system- the P3 lab operates at a particular temperature range as extreme temperatures can be a source of discomfort for users and interfere with the normal operation of equipment or experiments. The air conditioning system therefore helps in maintaining optimum temperatures.

c) Ventilation system- an aeration system that oversees steady supply of fresh air into the facility and the removal of circulated air. It is structured in a manner that maintains the directional flow of air and the negative pressure system necessary for the functioning of a P3 lab. The exhaust system is supported by dampers that pump air out while regulating its flow while inlets have inverters that control the rate of air inflow. A unique feature of our system is the automated double damper system at each exhaust route, which is able

to automatically switch to a reserve damper in case of failure of the default system.

d) Glass windows- These are large screens, located on two separate locations around the lab. They allow people outside the P3 lab to view the lab, communicate or observe its general condition without necessarily having to get in. the screens also serve as an emergency exit as they are provided with an hammer from within the lab that can be used to break the window, creating an evacuation route in the event of an accident.

e) Interphones- these are communication gadgets located in the P3 and ante-room that are also connected to the rest of the telephone network within the institution. Interphones allow communication between the two rooms and to or from other offices and labs within the institution.

f) Pass-box- This is a large window-like structure that opens to the cell culture room. It acts as a link between the two rooms, providing an entry and exit route for samples, small equipment, and waste materials. It has double interlocking glass doors with UV lights.

g) Generator- This is a Denyo (DCA-13ESKT) generator of 10.5 KVA in power capacity backup system specific for the P3 laboratory that switches on automatically in case of failure of the main generator. It covers the air conditioning system, freezers, incubators, ventilation system and the negative pressure system. The generator is connected to the current stabilizer that also serves other sources for power stabilization in order to protect equipment from damage by fluctuating electric currents.

B) Physical features-Special features

a) Water-air filtration system (Fig. 2-A and 2-B)- this is one of the unique features of our facility. Normally, air for a P3 facility is drawn from the outside environment and passes through a normal air filtration system. However, since the environment at our location is quite dusty, such a system would require weekly pre-filter cleaning and regular changing of the intermediate filter which would significantly increase maintenance costs. Our institution therefore improvised a water-air filtration system which capitalizes on the use of water to trap fine particles.

Water runs down a filter, made of a special fibrous material, wetting its filaments while providing a medium for the initial purification of air before it is drawn into the main air filtration system. The structure is connected to a water supply system, basically a water storage tank, arranged in a manner that enables the water to be recycled. This system has significantly reduced maintenance costs since intermediate filters can now be used for a longer duration. As shown in Fig. 3, before the installation of the filtration system, the manometer reading reached to 150 Pa within 6–9 weeks, at which point the intermediate filter would need to be changed. Following the installation of the system in March, 2011, it has been extended to more than 13 weeks, hence less frequent intermediate-filter change-over is needed, translating into reduced maintenance costs.

C) Operation features- General features

a) Run mode- run mode is the normal operation mode activated during the day, when the facility is in use or when being prepared for use. It is activated using a manual switch button on the display of the control panel. At run mode, the laboratory operates optimally with maximum power consumption.

b) Directional air flow- this refers to the directed flow of air in and out of the P3 facility. Purified air flows in through the inlet system into the ante-room and the P3 through ceiling ducts. Circulated air on the other hand is exhausted through an exhaust ceiling duct and the biosafety cabinet Class II B2 exhaust route, all of which are fitted with

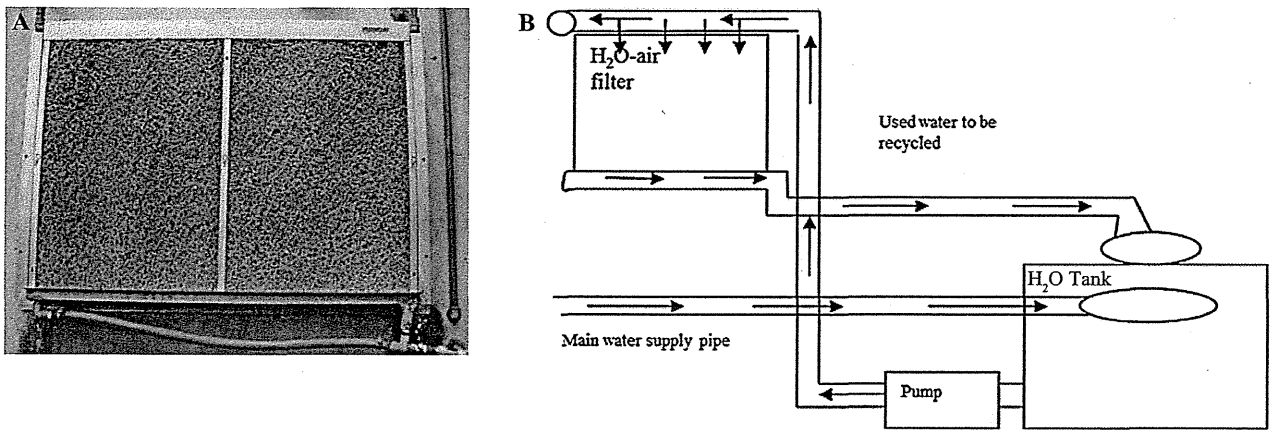

Fig. 2. A: Water-air filtration system, B: Filter's water supply scheme