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1   **Genotypic Characterization of Multi-drug-Resistant *Mycobacterium tuberculosis* isolates in**

2   **Myanmar**

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22

23   Runnning Head: Multi-drug-resistant *M. tuberculosis* in Myanmar

24

Abstract

The number of multi-drug-resistant tuberculosis (MDR-TB) cases is rising worldwide. As a countermeasure against this situation, the implementation of rapid molecular tests to identify MDR-TB would be effective. To develop such tests, information on the frequency and distribution of mutations associating with phenotypic drug resistance in *Mycobacterium tuberculosis* is required in each country. During 2010, the common mutations in the *rpoB*, *katG* and *inhA* of 178 phenotypically MDR *M. tuberculosis* isolates collected by the National Tuberculosis Control Program (NTP) in Myanmar were investigated by DNA sequencing. Mutations affecting the 81-bp rifampicin (RIF) resistance-determining region (RRDR) of the *rpoB* were identified in 127 of 178 isolates (71.3%). Two of the most frequently affected codons were 531 and 526, with percentages of 48.3% and 14.0% respectively. For isoniazid (INH) resistance, 114 of 178 MDR-TB isolates (64.0%) had mutations in the *katG* in which a mutation-conferring amino acid substitution at codon 315 from Ser to Thr was the most common. Mutations in the *inhA* regulatory region were also detected in 20 (11.2%) isolates, with the majority at position -15. Distinct mutation rate and pattern from surrounding countries might suggest that MDR-TB has developed and spread domestically in Myanmar.

1. Introduction

In 2013, there were an estimated 9.0 million new cases and 1.5 million deaths from tuberculosis (TB), including TB-suffering HIV-positive people, globally [1]. TB is the second leading cause of death among infectious diseases worldwide. The increasing spread of multi-drug-resistant TB (MDR-TB), i.e. resistant to more than two drugs including isoniazid (INH) and rifampicin (RIF), along with the recent emergence of extensively drug-resistant TB (XDR-TB), which has exhibited an additional resistance to fluoroquinolone (FQ) and to at least one of the three injectable second-line drugs, possess a significant threat to tuberculosis control. The lack of adequate treatment, often due to irregular drug supply, inappropriate regimens or poor patient compliance, is associated with the emergence of problematic *M. tuberculosis* strains [1-2]. In 2010, there were an estimated 650,000 cases of MDR-TB among the world's 12.0 million prevalent cases of TB [1].

MDR-TB cases, including those new and previously treated in 2002, were reported to be 4.2% and 18.4%, respectively, in Yangon, Myanmar [3-5]. The national drug resistant TB survey in 2002 also revealed those in the whole country to be 4.0% and 15.5%, respectively [6]. Hence, rapid identification of MDR-TB is crucial for proper treatment to avoid additional resistance development. In this context, the molecular characterization of drug resistance by identifying mutations in associated genes is applicable for developing potentially rapid molecular drug susceptibility tests as an alternative to conventional methods.

A convergence of data from different countries has indicated that resistance to RIF in 78% – 100% 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, or RIF resistance-determining region (RRDR) [7-21]. In contrast, INH resistance is mediated by mutations in several genes, most frequently within *katG*, which encodes a catalase-peroxidase that transforms INH into its active form, and in the regulatory region of *inh*A, which encodes a putative enzyme involved in mycolic acid biosynthesis. Mutations

67   in the *inh*A regulatory region result in the overexpression of *inh*A and INH resistance via a titration

68   mechanism [7, 14-24].

69   The present study aims to determine the prevalence of resistance-associated mutations in

70   three specific genes (*rpo*B, *kat*G and the *inh*A regulatory region) of MDR-TB isolates in Myanmar

71   and compare the frequency of different mutations with those in isolates circulating in neighboring

72   countries.

73

74   **2. Materials and Methods**

75   **2.1. Isolates**

76   A total of 178 MDR-TB clinical isolates, each corresponding to an individual TB patient, were

77   randomly selected from the nation wide collection of the National TB Control Programme (NTP),

78   Myanmar, during 2010. Drug susceptibility tests (DST) were carried out on Löwenstein-Jensen

79   medium by the conventional proportional method at critical drug concentrations of 0.2, 2, 4, 40

80   µg/ml of INH, ethambutol (EMB), streptomycin (STR) and RIF, respectively [9]. Laboratory

81   capacity and quality assurance were controlled according to the supranational reference laboratory

82   of tuberculosis in Bangkok and the Foundation for Innovative New Diagnostics (FIND).

83   **2.2. DNA extraction**

84   DNA was prepared for PCR using an EXTRAGEN MB DNA extraction kit (Tosoh Corporation,

85   Tokyo, Japan), according to the manufacturer's instruction.

86   **2.3. Sequencing of *rpoB, katG* encoding regions and *inh*A regulatory region**

87   PCR was conducted with 20 µl of a mixture consisting of 0.25 mM of each dNTP, 0.5 M of betaine,

88   0.5 µM of each primer [16], 1 U of GoTaq DNA Polymerase (Promega, WI, USA), GoTaq buffer

89   and 1 µl of DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories,

90   CA, USA) as follows: pre-denaturation at 96 °C for 60 s; 35 cycles of denaturation at 96 °C for 10 s;

91   renaturation at 55 °C for 10 s; and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5

92   min. The PCR products were separated by 1% agarose gel electrophoresis. DNA fragments of

interest were recovered from the agarose gel and used for sequencing according to the manufacturer's protocol with primers TB *rpoB* S, TB *katG* S and TB *inhA* S for *rpoB*, *katG* and *inhA*, respectively, a Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., CA, USA) and an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp., CA, USA). Resulting sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) [25].

2.4. Comparison of the frequencies of the drug-resistance associating mutations

Fisher's exact test was used to compare the drug-resistance associating mutations in this study with those in previous publications from surrounding countries. A two-tailed p-value less than 0.05 was considered statistically significant.

2.5. Ethical approval

Ethical approval is not required for the study as only clinical isolates of *M. tuberculosis* already stored at National TB Control Programme were analyzed.

3. RESULTS

3.1. Drug susceptibility patterns

Among 178 MDR-TB clinical isolates, two isolates were resistant to only INH and RIF, 66 isolates showed additional STR resistance and the remaining 110 isolates were resistant to four first-line anti-TB drugs (Table 1).

3.2. Mutations in the *rpo*B gene

Mutations in the RRDR of the *rpoB* gene were identified in 127 isolates (Table 2). A single nucleotide alteration in codon 531, resulting in an amino acid substitution from Ser to Leu, was most prevalent and observed in 84 isolates (47.2%). The second most affected codon was 526, which was found in 25 isolates (14.0%) and showed six types of amino acid substitutions. Eight isolates had a mutation in codon 533, six had a mutation in codon 516, two had a mutation in codon 513 and one each in codons 510 and 517, respectively. Two of the isolates that had a mutation in codons 526, 516

119 and 533, also had an additional non-synonymous mutation in the RRDR (Table 2). No mutations

120 were detected in 51 RIF-resistant isolates.

121 **3.3. Mutations in the *kat*G encoding region and *inh*A regulatory region**

122 Out of 178 MDR isolates, 114 (64.0%) had an amino acid substitution in KatG, with the vast

123 majority being Ser to Thr substitutions at the codon 315 (Table 3). Three isolates had a Ser to Asn

124 substitution and a fourth had a Ser to Ile substitution at the same codon. Amino acid substitutions in

125 KatG other than codon 315 were found in only one isolate: a Gly to Arg substitution at codon 285.

126 Mutations in the *inhA* regulatory region were observed in 20 (11.2%) MDR isolates, 18 of which had

127 a C to T mutation at position -15, one had a G to T change at position -17 and one had a C to T

128 mutation at position -15 in combination with a T to C mutation at position -8 (Table 3).

129 **3.4. Frequencies of drug-resistance associating mutations**

130 Frequencies of RIF- and INH-resistance associating mutations among the isolates in this study were

131 compered with those among isolates from surrounding countries and Myanmar as presented in Table

132 4 and 5, respectively, and the results of Fisher's exact test were shown in Table 6 and 7. The frequency

133 of RIF-resistant isolates having mutations in RRDR was significantly lower than those in the studies

134 from India [12], South China ([14]), Thailand ([11]), Bangladesh ([17]) and Myanmar (isolates from

135 Yangon and Mandalay in 2013[26]) than those in our study. Mutation causing amino acid

136 substitution at the position of codon 531 in *rpoB* is most frequently observed worldwide. The

137 frequency of mutations at this position in this study was significantly lower than that in the studies

138 from Myanmar in 2013 [26]. The frequency of INH-resistant isolates having mutations in *katG* at

139 codon 315 or in *inhA* promoter regionn at the position of -15 was significantly lower than those in

140 the studies from Thailand [22], Bangladesh [17] and Myanmar in 2013 [26]. In addition, the

141 frequencies of mutations in *katG* at codon 315 was significantly lower than those in the studies from

142 Bangladesh ([17]) and Myanmar in 2013 [26] than that in our study. In strong contrast, no

143 significant difference was observed on both RIF- and INH resistance associating mutations between

144 our study and the previous study from Myanmar on the isolates from Yangon in 2009.

6

4. Discussion

Anti-tuberculosis drug resistance poses a significant threat to human health. It is usually developed due to the alteration of drug targets by mutations in *M. tuberculosis* chromosomal genes [7]. A large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported in different countries. One such study from populations in Myanmar [20] analyzed 29 MDR and 67 INH mono-resistant isolates. The study showed that 86.2% (25/29) of RIF resistant isolates had mutations in the RRDR of the *rpoB* gene, while 63.5% (61/96) and 2.1% (2/96) of INH resistant isolates had mutations at codon 315 of *katG* and a mutation in the regulatory region of *inhA*, respectively. Although the analysis seemed to be conclusive, the correlation rates between resistance and mutations in these genes were lower than those reported in countries Myanmar shares borders with: Thailand [11, 22], India [12, 23, 24], Southern China [14] and Bangladesh [17] (Tables 4 and 5). To strengthen the conclusions, the number of MDR isolates needed to be increased. Hence, we conducted molecular analysis of *rpoB* and *katG* encoding and *inhA* regulatory regions of 178 MDR isolates from Myanmar.

Consistent with previous studies that showed a large part of RIF-resistant *M. tuberculosis* isolates had mutations within the RRDR, in our study 71.3% of phenotypically RIF-resistant isolates carried mutations in the RRDR. This number is similar to that reported in the Philippines (69.7%) [8], Vietnam (74.3%) [15] and Korea (78.0%) [21], but lower than those reported in Thailand (96.1 %) [11], India (96.7 %) [12], Brazil (96.3 %) [13], South China (95.0 %) [14], Nepal (97.3 %) [16], Bangladesh (92.7 %) [17] where Thailand, India, South China and Bangladesh are sharing border with Myanmar. The present result was also lower than that of studies conducted in Yangon with samples collected in 2002 (86.2%) [20] and in Yangon and Mandalay in 2013 (100 %) [26]. The difference in the sample collection period might have affected the mutation detection rate. Further, differences in the number of isolates analyzed in the previous study and ours (29, 33 and 178, respectively) might also be the reason of different mutation detection rate. We believe the

discrepancy between the DST and the sequencing results in 51 isolates could be due to the coexistence of presence of wild type and resistant isolates in the initial culture [2], a mutation outside of the core region of the *rpo*B gene [27, 28], the alteration of cell wall permeability or metabolism of antibiotics [29, 30] or the problems in the quality control of drug susceptibility test. Therefore, the presence of isolates lacking mutations should also be considered when using commercially available gene diagnostic tools such as GenoType MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) and Xpert MTB/RIF (Cepheid, Sunnyvale, CA), or designing new diagnostic tools for the detection of RIF- resistant TB.

The most frequently mutated codon in the RRDR in our study was codon 531 (48.3%), which had a rate higher than those reported in clinical isolates from Poland (32.4 %) [10], North India (38.7%) [18] and Vietnam (39.2%) [15], but lower than those reported in Brazil (56.1%) [13], Bangladesh (57.4%) [17], Southern China (58.5%) [14], Thailand (58.5%) [11], Nepal (58.7%) [16], India (59.0%) [12] and Morocco (59.6%) [9]. A much higher percentage of mutations at codon 531 was reported in Spain (72.3%) [19]. The second most frequently mutated codon was observed at codon 526 (14.0%) in Myanmar, which also has been reported in many countries [11, 13-18]. The sample collection site, Yangon, is a southern city in Myanmar and near to neighboring country Thailand (Fig. 1). The distinct mutation pattern and mutation detection rate (Table 4) in isolates from the sorrounding countries might dipict the low influence of neighboring countries.

Previous studies indicate that INH resistance is mediated by mutations in several genes, most commonly *kat*G, particularly at codon 315, and the regulatory region of *inh*A [7, 14-24]. Similarly, we found that 64.0% and 11.2% of the phenotypically INH-resistant clinical isolates had point mutations in *kat*G and the *inh*A regulatory region, respectively. Furthermore, 49 INH-resistant isolates had no resistant–associated alterations in the two targets. This indicates that the resistance in these isolates could be due to mutations outside of the sequenced area or in other genes such as *oxyR-ahpC*, *kasA* or *ndh* [7].

The amino acid substitution from Ser to Thr at residue 315 of KatG (KatG-Ser315Thr) has been predicted to be favored by *M. tuberculosis*, as this amino acid substitution appeared to reduce the activation of INH without abolishing the catalase-peroxidase activity of KatG, which has been recognized as a virulence factor [30]. However, the prevalence of KatG-Ser315Thr in clinical isolates around the world varies, especially with regard to the prevalence of TB. The present study documented the prevalence of KatG-Ser315Thr in 61.2% of INH-resistant isolates, which was quite similar to that of a previous study on the isolates collected from Yangon in 2002. The occurrence of KatG315 alteration was similar to those reported in India [12, 18] and South China [14] (Table 5), but not as high as those reported in isolates from Northeastern Russia (93.6%) and Yangon and Mandalay in Myanmar in 2013 (93.0 %) [26]. In general, a higher prevalence of this substitution has been observed in regions with a high incidence of TB compared with regions where the prevalence of TB is intermediate or low. And furthermore, this substitution was frequently seen in Beijing and MDR *M. tuberculosis* strains where strong correlation between Beijing genotype and MDR phenotype has been reported. [32]

Mutations in the regulatory region of *inhA* (preceding the *mabA-inhA* operon) that induce the overexpression of InhA have been known to contribute to INH resistance through an increased number of the target molecule [7]. Several studies from different countries have shown that about 10% – 34% of INH-resistant *M. tuberculosis* strains have mutations in the *inh*A regulatory region [7, 14-24]. We found a number within this range (11.2%). Four INH-resistant isolates carrying the KatG-Ser315Thr substitution also had a -15 C to T mutation in the *inh*A regulatory region. Additionally, one isolate possessed nucleotide substitutions -8 T to C and -15 C to T. Previous studies have shown that both inhA -8 T to C and -15 C to T are associated with a low-level resistance to INH [7]. It is possible that double mutations might cause an increased resistance to IHN. Data showed the frequency of KatG-Ser315Thr and *inhA* -15 C to T mutations are different between Myanmar and its neighboring countries (Table 5). These data together with those of RIF resistance-associated mutations may depict a distinct emergence and evolution of MDR-TB in

Myanmar caused by recent political isolation. However, detailed comparison of genotyping data obtained by such as restriction fragment length polymorphism analysis or multiple-locus variable-number tandem repeat analysis is needed to conclude this.

Conclusion

This study provides valuable information on the mutations found in the *rpo*B, *kat*G encoding and *inh*A regulatory regions in clinical isolates of *M. tuberculosis* in Myanmar. The results expand our current knowledge of the molecular mechanisms of drug resistance and also assist in improving current molecular-based techniques for the diagnosis of MDR tuberculosis fit to Myanmar. These improvements promise a more rapid detection compared with those achieved by methods based solely on the cultures of the isolates. Myanmar is a country bordered by China (northeast), India (northwest), Laos (mideast), Bangladesh (midwest) and Thailand (southeast) (Fig. 1). However, the mutation detection rate and the pattern of mutations are thought not to be affected by the surrounding countries. The political situation in Myanmar, which severely limits the movement of people, might be causing a distinct development and spread of multi-drug-resistant tuberculosis. To confirm this possibility, further information by molecular typing of MDR-TB strains circulating within Myanmar is necessary.

250   **Conflict of interest statements:** None of the authors have competing interests.

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343   **Figure ledgend**

344   Fig. 1. Myanmar and surrounding countries

345   Myanmar is a country bordered by China, India, Laos, Bangladesh and Thailand at northeast,

346   northwest, mideast, midwest and southeast, respectively.

347

Table 1. Drug-resistance patterns of MDR *M. tuberculosis* isolates.

Drug resistance pattern	Number of isolates
INH + REF + STR + EMB	110
INH + REF + STR	66
INH + REF	2

348

349

Table 2. Distribution of mutations in the *rpoB* RRDR of 178 MDR- isolates from Myanmar

Affected codon (s)	Nucleotide change		Amino acid change		Number	ratio (%)
	From	To	From	To		
510	CAG	CCG	Gln	Arg	1	0.6
513	CAA	CCA	Gln	Pro	2	1.1
516	GAC	GTC	Asp	Val	3	1.7
		TAC		Tyr	1	0.6
517	CAG	CCG	Gln	Pro	1	0.6
526	CAC	TAC	His	Tyr	5	2.8
		GAC		Asp	9	5.1
		CGC		Arg	5	2.8
		CTC		Leu	4	2.2
531	TCG	TTG	Ser	Leu	84	47.2
		TGG		Trp	1	0.6
		TTT		Phe	1	0.6
533	CTG	CCG	Leu	Pro	6	3.4
516/518	GAC/AAC	TAC/GAC	Asp/Asn	Tyr/Asp	1	0.6
516/533	GAC/CTG	GGC/CCG	Asp/Leu	Gly/Pro	1	0.6
526/527	CAC/AAG	CCC/CAG	His/Lys	Pro/Gln	1	0.6
526/533	CAC/CTG	CAA/CCG	His/Leu	Gln/Pro	1	0.6
none	none		none		51	28.7

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Table 3. Distribution of mutations in KatG gene and the inhA regulatory region of 178 MDR- isolates from Myanmar

Kat G					inhA regulatory region			Number of isolates	Ratio (%)
Affected codon	Amino acid change		Nucleotide change		mutated position	Nucleotide change			
	From	To	From	To		From	To		
285	Gly	Arg	GGC	CGC	-15	C	T	1	0.6
315	Ser	Thr	AGC	ACC	none	none		105	59.0
		Asn		AAC	none	none		3	1.7
		Ile		ATC	none	none		1	0.6
		Thr		ACC	-15	C	T	4	2.2
none	none		none		-15	C	T	13	7.3
none	none		none		-8/-15	T/C	C/T	1	0.6
none	none		none		-17	G	T	1	0.6
none	none		none		none	none		49	27.5

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Table 4. Frequency of the mutations in rpoB RRDR in RIF-resistant *M. tuberculosis* isolates in sourrounding countries and Myanmar

Mutated codon(s)	% Mutations in different geographic regions[a]						
	India[b] (n=149 [12])	South China (n=60 [14])	Thailand (n=153 [11])	Bangladesh (n=218 [17])	Myanmar (n = 29 [20])	Myanmar (n = 33 [26])	This study (n=178)
510	-	-	-	-	-		0.6
513	0.7	-	2.0	2.3	-		1.1
516	11.5	5.0	9.2	9.6	3.4	6.1	3.9
517	-	-	-	-	-		0.6
526	22.0	11.7	26.8	22.9	17.2	27.3	14.0
527	-	-	-	-	-		0.6
531	59.0	58.3	56.9	57.8	55.2	63.6	48.3
533	4.0	5.0	2.0	2.8	3.4		4.5
other than QRDR[c]	3.3	10.0	2.0	7.3	20.8		28.7

[a] Including isolates having mutations at multiple codons.

[b] North India (n=110) and South India (n=39).
[c] Including isolates having no mutations.

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Table 5. Frequency of the mutations in *KatG* 315 or/and *inhA* promoter region -15 in INH-resistant isolates in surrounding countries and other studies from Myanmar

Locus	% Mutations in different geographic regions[a]						
	North India (n=121 [23])	South China (n=50 [14])	Thailand (n=160 [22])	Bangladesh (n=218 [17])	Myanmar (n = 96 [20])	Myanmar (n = 43 [31])	This study (n=178)
katG 315	55.4	60.0	79.4	83.9	63.5	93.0	63.5
inhA -15	25.7	8.0	10.6	16.5	ND[c]	7.0	10.1
Others[b]	27.3	36.0	6.9	14.2	36.5	0.0	28.7

[a] Including isolates having mutations at both loci.
[b] Including other mutations and no mutations.
[c] Not detected

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Table 6. Significance of the frequency of the mutations in RIF-resistant isolates in surrounding countries and other studies in Myanmar

Mutated codon(s)[a]	two-tailed p-value						
	India[b] (n=149 [12])	South China (n=60 [14])	Thailand (n=153 [11])	Bangladesh (n=218 [17])	Myanmar (n = 29 [20])	Myanmar (n = 33 [26])	This study (n=178)
510	-	-	-	-	-	-	NA
513	1.00	-	0.67	0.47	-	-	NA
516	0.01	0.72	0.11	0.03	1.00	0.12	NA
517	-	-	-	-		-	NA
526	0.06	0.83	0.01	0.03	0.58	0.00	NA
527	-	-	-	-		-	NA
531	0.06	0.23	0.12	0.07	0.55	0.00	NA
533	1.00	1.00	0.23	0.42	1.00	-	NA
other than QRDR[c]	0.00	0.00	0.00	0.00	0.50	-	NA

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

[b] North India (n=110) and South India (n=39).
[c] Including isolates having no mutations.

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Table 7. Significance of the frequency of the mutations in INH-resistant isolates in surrounding countries and other studies from Myanmar

Locus	twe-tailed p-value						
	North India (n=121 [23])	South China (n=50 [14])	Thailand (n=160 [22])	Bangladesh (n=218 [17])	Myanmar (n = 96 [20])	Myanmar (n = 43 [26])	This study (n=178)
katG 315	0.19	0.74	0.30	0.00	1.00	0.00	NA
inhA -15	0.00	0.79	1.00	0.07	ND	0.77	NA
Others[b]	0.90	0.38	0.00	0.00	0.2195	0.00	NA

[a] Including isolates having mutations at both loci.
[b] Including other mutations and no mutations.
[c] Not detected

363

364

Khin Saw Aye et al. Fig. 1