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

Khin Saw Aye[1, †], Chie Nakajima[2, 3, †], Tomoyuki Yamaguchi[2], Min Min Win[1], Mu Mu Shwe[1], Aye Aye Win[1], Thandar Lwin[4], Wint Wint Nyunt[4], Ti Ti[4] and Yasuhiko Suzuki[2, 3 *]

[1] Immunology Research Division, Department of Medical Research (Lower Myanmar), Yangon, Myanmar

[2] Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

[3] Hokkaido University The Global Station for Zoonosis Control, Sapparo, Japan.

[4] National TB Control Programme, Department of Health, Yangon, Myanmar

Keywords: rifampicin, isoniazid, *Mycobacterium tuberculosis*, resistance, Myanmar

*Corresponding author: Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20-Nishi 10, Kita-ku. Sapporo 001-0020, Japan.
Phone: +81-11-706-9503; Fax: +81-11-706-7310
E-mail: suzuki@czc.hokudai.ac.jp

[†]K. S. A. and C. N. contributed equally to this work.

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

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

in the *inhA* regulatory region result in the overexpression of *inh*A and INH resistance via a titration mechanism [7, 14-24].

The present study aims to determine the prevalence of resistance-associated mutations in three specific genes (*rpo*B, *kat*G and the *inh*A regulatory region) of MDR-TB isolates in Myanmar and compare the frequency of different mutations with those in isolates circulating in neighboring countries.

2. Materials and Methods

2.1. Isolates

A total of 178 MDR-TB clinical isolates, each corresponding to an individual TB patient, were randomly selected from the nation wide collection of the National TB Control Programme (NTP), Myanmar, during 2010. Drug susceptibility tests (DST) were carried out on Löwenstein-Jensen medium by the conventional proportional method at critical drug concentrations of 0.2, 2, 4, 40 µg/ml of INH, ethambutol (EMB), streptomycin (STR) and RIF, respectively [9]. Laboratory capacity and quality assurance were controlled according to the supranational reference laboratory of tuberculosis in Bangkok and the Foundation for Innovative New Diagnostics (FIND).

2.2. DNA extraction

DNA was prepared for PCR using an EXTRAGEN MB DNA extraction kit (Tosoh Corporation, Tokyo, Japan), according to the manufacturer's instruction.

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

PCR was conducted with 20 µl of a mixture consisting of 0.25 mM of each dNTP, 0.5 M of betaine, 0.5 µM of each primer [16], 1 U of GoTaq DNA Polymerase (Promega, WI, USA), GoTaq buffer and 1 µl of DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories, CA, USA) as follows: pre-denaturation at 96 °C for 60 s; 35 cycles of denaturation at 96 °C for 10 s; renaturation at 55 °C for 10 s; and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 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

and 533, also had an additional non-synonymous mutation in the RRDR (Table 2). No mutations were detected in 51 RIF-resistant isolates.

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

Out of 178 MDR isolates, 114 (64.0%) had an amino acid substitution in KatG, with the vast majority being Ser to Thr substitutions at the codon 315 (Table 3). Three isolates had a Ser to Asn substitution and a fourth had a Ser to Ile substitution at the same codon. Amino acid substitutions in KatG other than codon 315 were found in only one isolate: a Gly to Arg substitution at codon 285. Mutations in the *inhA* regulatory region were observed in 20 (11.2%) MDR isolates, 18 of which had 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 mutation at position -15 in combination with a T to C mutation at position -8 (Table 3).

3.4. Frequencies of drug-resistance associating mutations

Frequencies of RIF- and INH-resistance associating mutations among the isolates in this study were compered with those among isolates from surrounding countries and Myanmar as presented in Table 4 and 5, respectively, and the results of Fisher's exact test were shown in Table 6 and 7. The frequency of RIF-resistant isolates having mutations in RRDR was significantly lower than those in the studies from India [12], South China ([14]), Thailand ([11]), Bangladesh ([17]) and Myanmar (isolates from Yangon and Mandalay in 2013[26]) than those in our study. Mutation causing amino acid substitution at the position of codon 531 in *rpoB* is most frequently observed worldwide. The frequency of mutations at this position in this study was significantly lower than that in the studies from Myanmar in 2013 [26]. The frequency of INH-resistant isolates having mutations in *katG* at codon 315 or in *inhA* promoter regionn at the position of -15 was significantly lower than those in the studies from Thailand [22], Bangladesh [17] and Myanmar in 2013 [26]. In addition, the frequencies of mutations in *katG* at codon 315 was significantly lower than those in the studies from Bangladesh ([17]) and Myanmar in 2013 [26] than that in our study. In strong contrast, no significant difference was observed on both RIF- and INH resistance associating mutations between our study and the previous study from Myanmar on the isolates from Yangon in 2009.

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].

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

210 Mutations in the regulatory region of *inhA* (preceding the *mabA-inhA* operon) that induce the
211 overexpression of InhA have been known to contribute to INH resistance through an increased
212 number of the target molecule [7]. Several studies from different countries have shown that about
213 10% – 34% of INH-resistant *M. tuberculosis* strains have mutations in the *inh*A regulatory region [7,
214 14-24]. We found a number within this range (11.2%). Four INH-resistant isolates carrying the
215 KatG-Ser315Thr substitution also had a -15 C to T mutation in the *inh*A regulatory region.
216 Additionally, one isolate possessed nucleotide substitutions -8 T to C and -15 C to T. Previous
217 studies have shown that both inhA -8 T to C and -15 C to T are associated with a low-level
218 resistance to INH [7]. It is possible that double mutations might cause an increased resistance to
219 IHN. Data showed the frequency of KatG-Ser315Thr and *inhA* -15 C to T mutations are different
220 between Myanmar and its neighboring countries (Table 5). These data together with those of RIF
221 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.

ACKNOWLEDGEMENTS

We would like to acknowledge the Board of Directors, DMR (LM) for its encouragement of this study. We also thank the National TB Controlled Program, DOH for its collaboration. This work was supported in part by J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT), in part by JSPS KAKENHI Grant Number 24406021 and 24580443, and in part by the grant from MEXT for the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University; by the Global COE Program, the Establishment of International Collaboration Centers

247 for Zoonosis Control, Hokkaido University from MEXT, and in part by a grant from the U.S.-Japan
248 Cooperative Medical Science Programs.

249

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

References

[1] World Health Organization. Global Tuberculosis Report. http://www.who.int/tb/publications/global_report/gtbr14_main_text.pdf?ua=1; 2014.

[2] Nathanson E, Nunn P, Uplekar M, Floyd K, Jaramillo E, Lönnroth K, et al. MDR tuberculosis-critical steps for prevention and control. N Engl J Med. 2010;363:1050-8.

[3] Phyu S, Ti T, Jureen R, Hmun T, Myint H, Htun A, et al. Drug-resistant *Mycobacterium tuberculosis* among new tuberculosis patients, Yangon, Myanmar. Emerg Infect Dis. 2003;9:274-6.

[4] Phyu S, Lwin T, Ti T, Maung W, Mar WW, Shein SS, et al. Drug-resistant tuberculosis in Yangon, Myanmar. *Scand J Infect Dis.* 2005;37:846-51.

[5] Aung WW, Ti T, Than KK, Thida M, Nyein MM, Htun YY, et al. Study of drug resistant cases among new pulmonary tuberculosis patients attending a tuberculosis center, Yangon, Myanmar. Southeast Asian J Trop Med Public Health. 2007;38:104-10.

[6] Ti T. National anti-tuberculosis drug resistance survey, 2002, in Myanmar. Int J Tuberc Lung Dis. 2006;10:1111-6.

[7] Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009;13:1320-30.

[8] Agdamag DMD, Kageyama S, Solante R, Espantaleon AS, Sangco JC, Suzuki Y. 2003. Characterization of clinical isolates of *Mycobacterium tuberculosis* resistant to drugs and detection of *rpoB* mutation in multidrug-resistant tuberculosis in the Philippines. Int J Tuberc Lung Dis. 2003;7:1104–8.

[9] Kourout M, Chaoui I, Sabouni R, Lahlou O, El Mzibri M, Jordaan A, et al. Molecular characterisation of rifampicin-resistant *Mycobacterium tuberculosis* strains from Morocco. Int J Tuberc Lung Dis. 2009;13:1440-2.

[10] Paluch-Oles J, Kozioł-Montewka M, Magrys A. Mutations in the *rpoB* gene of rifampin-

276 resistant *Mycobacterium tuberculosis* isolates from Eastern Poland. New Microbiol.
277 2009;32:147-52.
278 [11] Prammananan T, Cheunoy W, Taechamahapun D, Yorsangsukkamol J, Phunpruch S, Phdarat P,
279 et al. Distribution of *rpoB* mutations among multidrug-resistant *Mycobacterium tuberculosis*
280 (MDRTB) strains from Thailand and development of a rapid method for mutation detection.
281 Clin Microbiol Infect. 2008;14:446-53.
282 [12] Suresh N, Singh UB, Arora J, Pant H, Seth P, Sola C, et al. *rpoB* gene sequencing and
283 spoligotyping of multidrug-resistant *Mycobacterium tuberculosis* isolates from India. Infect
284 Genet Evol. 2006;6:474-83.
285 [13] Valim AR, Rossetti ML, Ribeiro MO, Zaha A. Mutations in the *rpoB* gene of multidrug-
286 resistant *Mycobacterium tuberculosis* isolates from Brazil. J Clin Microbiol. 2000;38:3119-22.
287 [14] Guo JH, Xiang W-L, Zhao Q-R, Luo T, Huang M, Zhang J, et al. Molecular characterization
288 of drug-resistant mycobacterium tuberculosis isolates from Sichuan Province in China. Jpn J
289 Infect Dis. 2008;61:264–8.
290 [15] Minh NN, Van Bac N, Son NT, Lien VT, Ha CH, Cuong NH, et al. Molecular characteristics of
291 rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Vietnam. J
292 Clin Microbiol. 2012;50:598-601.
293 [16] Poudel A, Nakajima C, Fukushima Y, Suzuki H, Pandey BD, Maharjan B, et al. Molecular
294 Characterization of Multidrug-Resistant *Mycobacterium tuberculosis* Isolated in Nepal.
295 Antimicrob Agents Chemother 2012;56:2831-6.
296 [17] Rahim Z, Nakajima C, Raqib R, Zaman K, Endtz HP, van der Zanden AG, et al. Molecular
297 mechanism of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* from
298 Bangladesh. Tuberculosis. 2012;92: 529-34.
299 [18] Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N, Prachee, et al. Molecular
300 characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in
301 North India. Antimicrob. Agents Chemother. 2002;46**:**443-50.

[19] 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.

[20] Valvatne H, Syre H, Kross M, Stavrum R, Ti T, Phyu S, et al. Isoniazid and rifampicin resistance-associated mutations in *Mycobacterium tuberculosis* isolates from Yangon, Myanmar: implications for rapid molecular testing. J Antimicrob Chemother. 2009;64:694–70.

[21] Yoon JH, Nam JS, Kim KJ, Choi Y, Lee H, Cho SN, et al. Molecular characterization of drug-resistant and -susceptible *Mycobacterium tuberculosis* isolated from patients with tuberculosis in Korea. Diagn Microbiol Infect Dis. 2012;72:52-61.

[22] Boonaiam S, Chaiprasert A, Prammananan T, Leechawengwongs M. Genotypic analysis of genes associated with isoniazid and ethionamide resistance in MDR-TB isolates from Thailand. Clin Microb Infect. 2010;16: 396-9.

[23] 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.

[24] 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.

[25] 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.

[26] Aung WW, Ei PW, Nyunt WW, Swe TL, Lwin T, Htwe MM, et al. Phenotypic and genotypic analysis of anti-tuberculosis drug resistance in *Mycobacterium tuberculosis* isolates in Myanmar. Ann Lab Med. 2015;35:494-9. doi: 10.3343/alm.2015.35.5.494.

[27] Heep M, Brandstätter B, Rieger U, Lehn N, Richter E, Rüsch-Gerdes S, et al. 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.

[28] 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.

[29] Hui J, Gordon N, Kajioka R: Permeability barrier to rifampin in mycobacteria. Antimicrob Agents Chemother 1977;11:773–9.

[30] Rouse DA, DeVito JA, Li Z, Byer H, Morris SL. et al. Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosi*s: effects on catalase-peroxidase activities and isoniazid resistance. Mol Microbiol 1996;22:583-92.

[31] Afanas'ev MV, Ikryannikova LN, Il'ina EN, Sidorenko SV, Kuz'min AV, Larionova EE, et al. Molecular characteristics of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Russian Federation. J Antimicrob Chemother 2007;59:1057-64. PMID: 17442757

[32] Borgdorff MW, van Soolingen D: The re-emergence of tuberculosis: what have we learnt from molecular epidemiology? Clin Microbiol Rev. 2013;26:342-60. doi: 10.1128/CMR.00087-12.

Figure ledgend

Fig. 1. Myanmar and surrounding countries

Myanmar is a country bordered by China, India, Laos, Bangladesh and Thailand at northeast, northwest, mideast, midwest and southeast, respectively.

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

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

353

354

355

356

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.

357

358

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

359

360

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).

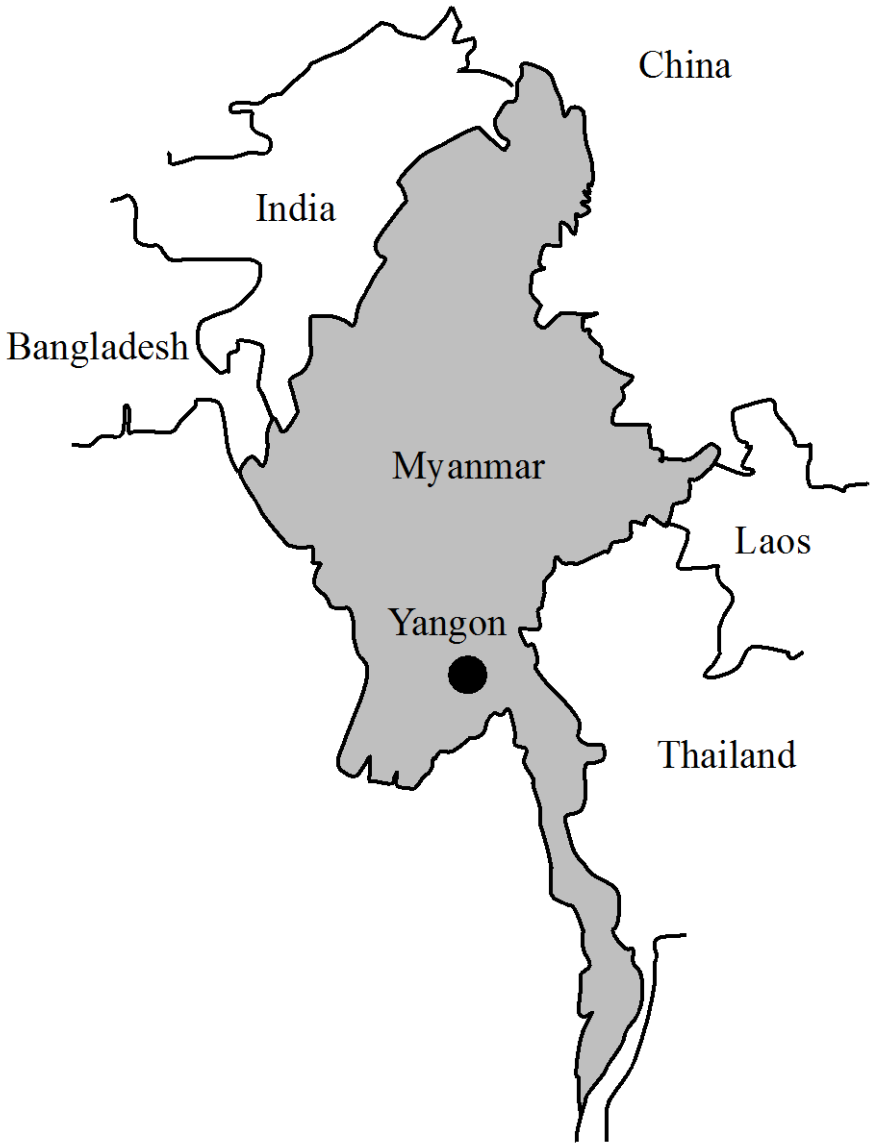
[c] Including isolates having no mutations.

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

Khin Saw Aye et al. Fig. 1



365

366

367