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Original article

Genotypic characterization of multi-drug-resistant *Mycobacterium tuberculosis* isolates in Myanmar

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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.

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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 *Mycobacterium 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.

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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 inhA 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). R*esulting* 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 rpoB 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 katG encoding region and inhA 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 Tables 4 and 5, respectively, and the results of Fisher's exact test were shown in Tables 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]),

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

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