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Phenotypic and Genotypic Analysis of Anti-Tuberculosis Drug Resistance in *Mycobacterium tuberculosis* Isolates in Myanmar

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Background: Tuberculosis (TB) is one of the most serious health problems in Myanmar. Because TB drug resistance is associated with genetic mutation(s) relevant to responses to each drug, genotypic methods for detecting these mutations have been proposed to overcome the limitations of classic phenotypic drug susceptibility testing (DST). We explored the current estimates of drug-resistant TB and evaluated the usefulness of genotypic DST in Myanmar.

Methods: We determined the drug susceptibility of *Mycobacterium tuberculosis* isolated from sputum smear-positive patients with newly diagnosed pulmonary TB at two main TB centers in Myanmar during 2013 by using conventional phenotypic DST and the GenoType MTBDR*plus* assay (Hain Lifescience, Germany). Discrepant results were confirmed by sequencing the genes relevant to each type of resistance (*rpoB* for rifampicin; *katG* and *inhA* for isoniazid).

Results: Of 191 isolates, phenotypic DST showed that 27.7% (n=53) were resistant to at least one first-line drug and 20.9% (n=40) were resistant to two or more, including 18.3% (n=35) multidrug-resistant TB (MDR-TB) strains. Monoresistant strains accounted for 6.8% (n=13) of the samples. Genotypic assay of 189 isolates showed 17.5% (n=33) MDR-TB and 5.3% (n=10) isoniazid-monoresistant strains. Genotypic susceptibility results were 99.5% (n=188) concordant and agreed almost perfectly with phenotypic DST (kappa=0.99; 95% confidence interval 0.96-1.01).

Conclusions: The results highlight the burden of TB drug resistance and prove the usefulness of the genotypic DST in Myanmar.

Key Words: *Mycobacterium tuberculosis*, Drug, Resistance, Genotype, Myanmar

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INTRODUCTION

Tuberculosis (TB) is a major global health problem and the foremost cause of death from a single infectious agent, *Mycobacterium tuberculosis*. Nearly nine million new cases of TB and 1.4 million deaths occurred worldwide in 2011. With the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, TB has become an even greater threat [1]. Myanmar is one of the 22 countries with high-burden TB and among 27 countries with high-burden MDR-TB. An estimated 180,000

new TB cases occurred in Myanmar in 2011, and the proportion of MDR-TB was 4.2% among these cases and 10.0% among previously treated cases according to the second nationwide drug-resistant TB survey (2007-2008) [1].

The identification of resistant strains through drug susceptibility testing (DST) and subsequent appropriate treatment might be the most effective strategy for controlling the spread of drug-resistant TB. The phenotypic DST widely used in developing countries is a proportion method that uses fixed concentrations of first-line TB drugs: isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and streptomycin (SM). This method is simple and cost-effective; however, the results require a long time to obtain and can be unreliable in some diagnostic examination settings.

Because TB drug resistance is associated with mutation(s) in the genes relevant to responses to each drug, genotypic methods for detecting mutations in these genes have been proposed to overcome the limitations of phenotypic DST [2]. A promising genotypic DST, the line-probe assay (LiPA), was endorsed by WHO in its 2008 policy statement for use with smear-positive pulmonary specimens [3]. Commercially available LiPAs for the rapid detection of INH and RIF resistance are based on selected numbers of commonly reported mutations. One LiPA, GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany), was designed for simultaneous detection of the most important *rpoB* mutations, which confer RIF resistance, and *katG* and *inhA* mutations, which confer high-level INH resistance [4].

We used both phenotype-based conventional DST and a commercially available genotypic DST (GenoType MTBDR*plus*) to investigate TB drug resistance in patients with newly diagnosed pulmonary TB in Myanmar to obtain more accurate information on drug resistance, evaluate the usefulness of GenoType MTBDR*plus*, and characterize genetic alterations specific to TB in Myanmar.

METHODS

1. Patient enrollment and sample collection

A cross-sectional descriptive study was carried out at the Latha TB Diagnostic Centre (Yangon) and Mandalay Regional TB Centre (Mandalay) in Myanmar from January to August 2013. The WHO case definition was applied, in which a new TB case was defined as a sputum- smear positive patient who had never received treatment for TB or who had taken TB drugs for less than one month [1]. Two sputum samples were collected from each patient after obtaining written informed consent. The following definition was applied to determine smear-positive TB: one or more initial sputum smear examinations (direct smear microscopy) positive for acid-fast bacilli (AFB) or one AFB-positive sputum examination plus radiographic abnormalities consistent with active pulmonary TB as determined by a clinician [1]. Demographic characteristics were recorded in the pro forma when patients were enrolled.

2. Study patients

During the study period, 212 sputum samples from newly diagnosed pulmonary TB patients were subjected to culture. The mean age of the patients was 39±16.4 yr, and 128 (60.4%) were males. After excluding 21 contaminated or unculturable samples, 191 *M. tuberculosis* isolates comprising 142 (74.3%) from the Yangon region and 49 (25.7%) from the Mandalay region were subjected to phenotypic and genotypic DST.

3. Isolation of *M. tuberculosis* and phenotypic DST

Isolation of *M. tuberculosis* from sputum samples and DST with conventional culture was performed at the National TB Reference Laboratory (NTRL; Yangon, Myanmar) and Upper Myanmar TB Laboratory (Mandalay, Myanmar). *M. tuberculosis* was isolated from sputum samples according to the WHO method [5]. Sputum samples were decontaminated with N-acetyl-L-cysteine sodium hydroxide. After centrifugation at a speed of 3,000-3,500*g* for 15 min, the pellet was suspended in 1 mL 1×phosphate-buffered saline, inoculated on two Lowenstein-Jensen (L-J) media slants, and incubated at 37°C for 6-8 weeks depending on the time required for the organisms to become evident. Mycobacterial growth was monitored every week. The *M. tuberculosis* isolates were identified according to growth rate and colony morphology. The Capilia TB test (Tauns, Numazu, Japan), an immunochromatographic assay that uses a monoclonal antibody to detect MPB64 antigen, was used to differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria.

Phenotypic DST was carried out on confirmed *M. tuberculosis* isolates. The test was performed on L-J media containing INH (0.2 μg/mL), RIF (40 μg/mL), SM (4 μg/mL), and EMB (2 μg/mL) according to the WHO-recommended proportional method for all primary isolates [6]. Inocula were cultured in a 37°C incubator for 6 weeks, and the results were interpreted as susceptible or resistant. The standard criterion of the proportion method for classifying a strain as resistant was the ratio of the number of colonies obtained on drug-containing medium to the number of colonies obtained on drug-free medium (growth of ≥ 1% of colonies). Any-drug resistance was defined as resistance to one or more first-line drugs. Monoresistance was defined as

resistance to only one of the four drugs.

4. Genotypic detection of INH and RIF resistance

Genotypic detection of INH and RIF resistance was carried out at Pusan National University Yangsan Hospital (Yangsan, Korea). The GenoType MTBDR*plus*, a reverse hybridization line probe assay, was performed according to the manufacturer's recommendations. Multiplex PCR was used to amplify the genes responsible for drug resistance, including *katG*, *inhA*, and *rpoB*, and the resulting biotin-labeled amplicons were hybridized to DNA probes that affixed to the membrane. DNA probes for each gene consisted of wild-type (WT) and mutant (MUT) samples. Resistance to INH and RIF was defined as either (1) missing WT probe signal with MUT probe signal, or (2) missing WT probe signal.

5. Confirmation of discrepant results between phenotypic and genotypic DSTs

The genotypic and phenotypic DST results were compared. Further examinations were performed with additional phenotypic DST or DNA sequencing (*rpoB* for RIF and *katG* and *inhA* for INH) when the results disagreed. Each discordant gene region was amplified with PCR, and direct sequencing of PCR products was carried out by Genotech (Daejeon, Korea). The sequencing results were analyzed with the CLC Main Workbench (CLC bio, Aarhus, Denmark) at the International Tuberculosis Research Center (Changwon, Korea).

6. Statistical analysis

The results were analyzed with the SPSS statistical software package, version 16 (SPSS Inc., Chicago, IL, USA). Drug-susceptible and drug-resistant cases were documented as the percentages of the total study population, and the drug-resistance pattern in newly diagnosed patients was determined. The resistance results of phenotypic and genotypic DST were tested for kappa agreement [7].

7. Ethics approval

This study was approved by the Ethics Review Committee, Department of Medical Research, Yangon, Myanmar.

RESULTS

1. Drug-resistance patterns of *M. tuberculosis* isolates from newly diagnosed patients

Of the 191 *M. tuberculosis* isolates, 27.7% (n=53) were resistant to at least one first-line drug, and 40 (20.9%) were resistant to two or more, including 35 (18.3%) classified as MDR-TB strains. Monoresistant strains accounted for 6.8% (n=13), including 2.1% (n=4) INH-monoresistant strains and 4.7% (n=9) SM-monoresistant strains. The total resistance rates were as follows: INH (44; 23.0%), RIF (35; 18.3%), EMB (15; 7.9%), and SM (41; 21.5%).

2. Gene mutation patterns in drug-resistant strains

Among the 191 tested strains, two had a PCR inhibitor and were excluded from genotypic analysis. Genotypic assay of the remaining 189 samples showed 17.5% (n=33) MDR-TB strains and 5.3% (n=10) INH-monoresistant strains. Among the 33 RIF-resistant isolates, the S531L mutation, which is the most prevalent, was present in 63.6% (n=21). Of the 43 INH-resistant strains, 93.0% (n=40) had a mutation at the S315T1 (AGC→ACC) codon of *katG* (Table 1).

3. Comparison of phenotypic and genotypic TB drug susceptibility patterns

After isolates with discordant phenotypic and genotypic DST results were confirmed with additional phenotypic DST or the se-

Table 1. Distribution of mutation patterns in *rpoB*, *katG*, and *inhA* among drug-resistant *Mycobacterium tuberculosis* isolates

Drug resistance	N and proportion of resistant gene	Mutation type	N (%) in MDR strains (n=33)	N (%) in INH monoresistant strains (n=10)
RIF (n=33)	*rpoB* (33, 100%)	L511P	1 (3.0)	-
		D516V	2 (6.1)	-
		H526Y	9 (27.3)	-
		S531L	21 (63.6)	-
INH (n=43†)	*katG* (40, 93.0%)	S315T1*	31 (72.1)	9 (20.9)
	inhA (3, 7.0%)	C15T	2 (4.7)	1 (2.3)

*S315T1 means AGC→ACC/Ser→Thr exchange; †includes 33 INH-resistant isolates that were also resistant to RIF, composing 33 MDR isolates.
Abbreviations: INH, isoniazid; MDR, multidrug resistant; RIF, rifampicin.

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Table 2. Comparison of phenotypic and genotypic drug susceptibility tests

Drug susceptibility test	Type of drug resistance	N of isolates	Phenotypic DST		
			Susceptible	Resistant	
Genotypic DST	INH	Susceptible	145	1*	Kappa = 0.99 (95% CI 0.96-1.01)
		Resistant	0	43	
	RIF	Susceptible	156	0	
		Resistant	0	33	

*isolate no. 1998 in Table 3.
Abbreviations: DST, drug susceptibility testing; INH, isoniazid; RIF, rifampicin; CI, confidence interval.

Table 3. Confirmation of 17 discordant *Mycobacterium tuberculosis* isolates via additional phenotypic drug susceptibility test and DNA sequencing

Isolate No.	First phenotypic DST		Genotypic DST (GenoType MTBDR*plus* assay)		Second phenotypic DST		DNA sequencing		Final interpretation of results
	RIF	INH	RIF	INH	RIF	INH	RIF (*rpoB*)	INH (*katG*/*inhA*)	
381	R	R	S	S	-	-	NM	NM	Concordant*
606	S	S	S	R	S	R	-	-	Concordant*
674	R	R	S	S	-	-	NM	NM	Concordant*
677	R	R	S	S	-	-	NM	NM	Concordant*
779	R	R	S	S	-	-	NM	NM	Concordant*
1417	R	R	S	R	-	-	NM	*katG*: 315AGC to ACC	Concordant*
1419	R	S	S	S	S	S	NM	NM	Concordant*
1542	S	S	S	R	S	R	NM	*inhA*: -15 C to T	Concordant*
1864	S	S	R	R	R	R	531TCG to TTG	*katG*:315AGC to ACC	Concordant*
1865	S	S	R	R	S	R	531TCG to TTG	*katG*:315AGC to ACC	Concordant†
1868	S	R	S	S			NM	NM	Concordant*
1998	S	R	S	S	S	R	NM	NM	Discordant‡
2215	S	S	R	S	R	R	531TCG to TTG	*inhA*: -15 C to T	Concordant§
U3	S	S	R	R	R	R	-	-	Concordant*
U5	S	S	R	R	R	R	531TCG to TTG	*KatG*-315AGC to ACC	Concordant*
U9	S	R	S	S	S	S	-	-	Concordant*
U10	S	S	S	R	S	R	-	-	Concordant*

*first DST error; †both first and second DST error; ‡mutation other than those in target genes might be involved; §GenoType MTBDR*plus* assay test error.
Abbreviations: DST, drug susceptibility testing; INH, isoniazid; NM, no mutation; R, resistant; RIF, rifampicin; S, susceptible.

quencing of discordant genes, genotypic DST results were 99.5% (188/189) concordant with the phenotypic DST results. One isolate, which was resistant to INH on phenotypic DST and susceptible to INH in the GenoType MTBDR*plus* assay, was confirmed as resistant without any mutation in the target genes (*katG* or *inhA*). The genotypic DST had almost perfect agreement with the phenotypic DST (kappa=0.99; 95% confidence interval [CI] 0.96-1.01); Table 2). The 17 discordant phenotype and genotype results are listed in Table 3.

DISCUSSION

Drug resistance is a key cause of treatment failure in TB, particularly when strains are resistant to the primary drugs, INH and RIF, which results in the development of MDR-TB. Managing the increasing number of MDR-TB cases is a crucial part of the STOP TB strategy and a component of all TB control programs [8]. MDR-TB strains can be transmitted in the community, replacing susceptible strains and consequently making first-line regimens inadequate for achieving high cure rates. Drug resis-

tance in new cases has been used to evaluate recent transmission and can be used as a proxy for acquired resistance in a previously treated group.

In Myanmar, the second nationwide drug resistance survey (2007-2008) showed an MDR-TB rate of 4.2% among new patients and 10.0% among previously treated patients [1]. Previous studies carried out at the Yangon Divisional TB Center, a major referral center in Myanmar, during 1994-2004 found that the MDR-TB and any-drug resistance rates were 1.25-4.2% and 15.8-35.3%, respectively [9-12].

The present study showed a high rate of MDR-TB (18.3%) and any-drug resistance rate of 27.7% among new TB cases as determined with a phenotypic method. These rates were much higher than those reported in previous nationwide and TB center-based studies. According to the 2012 Annual Report of the Myanmar National TB Program, Yangon had the highest TB case detection rate (112%), and many TB patients living in Yangon had one of the statistically significant risk factors for MDR-TB (odds ratio [OR]=3.0; 95% CI=1.5 5.8; P=0.014). In the present study, 74.3% of tested MTB isolates were from Yangon, which may account for the high drug-resistance rate [13]. INH resistance was most common. The individual anti-drug resistance pattern was similar to that reported in previous studies abroad and in Myanmar [9-12, 14].

The MDR-TB rate in Myanmar is among the highest in Southeast Asia [1], and the present study showed a high MDR-TB rate among new smear-positive pulmonary TB patients. The drug resistance results of newly diagnosed patients may reflect the primary resistance to first-line TB drugs in Myanmar, although primary resistance is difficult to determine because patients may not know or may deny that they have had previous TB treatment. The high prevalence of drug resistance in Myanmar highlights the need for a control program to improve case management and strengthen TB control strategies.

Drug resistance in *M. tuberculosis* develops as a result of random mutations in the genes responsible for resistance to each drug [15, 16]. In the case of RIF, 95-98% of resistance develops because of mutations in the 81-bp core region (RIF resistance-determining region or hot spot) of the β subunit of the RNA polymerase gene (*rpoB*), which results in peptide alterations at codon 531 or codon 526. The most common mutations are S531L and H526Y [14, 15]. The molecular basis of INH resistance is more complex and is associated with genes such as *katG*, *inhA*, *ahpC*, and *oxyR* [17]. Approximately 50-95% of INH-resistant strains contain mutations in codon 315 of *katG*, whereas 20-35% contain mutations in the *inhA* regulatory region [4].

In the present study, among the 33 RIF-resistant isolates found with genotypic DST, the S531L mutation was the most common at 63.6% (n=21). The H526Y mutation was the second most common, accounting for 27.3% (n=9) of MDR strains.

Of the 43 INH-resistant strains, 93.02% (n=40; 31 MDR strains and nine INH-monoresistant strains) had a mutation in the S315T1 region of *katG*, and only 6.97% (n=3; two MDR strains and one monoresistant strain) had a mutation in the C15T region of *inhA*. This finding was consistent with the previous results [15, 16]. The high prevalence of *katG* mutations accounts for a high proportion of INH resistance in countries with high TB prevalence, presumably as a result of the transmission of these strains in a high-burden setting [18]. No other mutation in *ahpC* or *oxyR* was observed in this study, which suggests that those mutations are infrequently involved in INH resistance in Myanmar.

The overall concordance between phenotypic and genotypic DST was 99.5% (188/189) after the confirmation of these samples with additional phenotypic DST and DNA sequencing. Similar results were reported in previous studies, with values ranging from 88.9% to 100% [19-22]. When we analyzed the phenotypic and genotypic results with kappa statistics, the genotypic assay showed almost perfect agreement with phenotypic DST (kappa=0.99; 95% CI 0.96-1.01). The one discordant isolate was susceptible to INH in the GenoType MTBDR*plus* assay but resistant to INH without any mutation in the target genes. Mutation other than that in the target gene might be involved in the resistance in this isolate. The high MDR-TB rate, high accuracy of genotypic DST, and common drug resistance-mutation pattern found in the present study suggested that genotypic DST should be more widely used as a routine test to determine drug susceptibility in newly diagnosed TB patients. Genotypic DST may also lead to the rapid detection of MDR-TB cases and be invaluable in preventing the transmission of drug-resistant strains in Myanmar. In conclusion, this study highlighted the high prevalence of drug resistance among new pulmonary TB cases in Myanmar and the usefulness of genotypic DST for determining drug susceptibility in TB patients and for the rapid and accurate diagnosis of TB in Myanmar.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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