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Multidrug-resistant *Mycobacterium tuberculosis* Strains in Myanmar Patients

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Myanmar is one of the high MDR-TB burden countries globally. The aim of this study was to provide genetic information of MDR-TB strains from Myanmar. A total of 139 high-positive sputum samples (i.e., AFB grade 2+ or 3+) from MDR-TB suspected cases which were referred to National TB Reference Laboratory, Yangon during 2012-2013 were included. GenoType MTBDR*plus* assay was used to detect resistance to rifampicin and isoniazid, and results were compared with phenotypic Lowenstein-Jensen proportion method at National TB Reference Laboratory, Yangon. Genotypes were also identified by spoligotyping method. The most common rifampicin resistance mutation was S531L (67.5%) in the *rpoB* gene and the most prevalent isoniazid resistance mutation was S315T (90.4%) in the *katG* gene. There was high agreement (kappa=0.934) between the MTBDR*plus* assay and phenotypic susceptibility results in detecting MDR-TB. The *inhA* C15T mutation was more likely to occur in isoniazid mono-resistant cases than in MDR-TB cases (p=0.038). Beijing genotype was predominantly identified in 76.4% of strains (55/72). Strains belonging to Beijing genotypes are significantly associated with MDR-TB (p=0.001) as well as resistance to isoniazid, rifampicin, streptomycin and ethambutol (all p<0.05). The *katG* S315T mutation was more likely to develop in strains of Beijing genotype (p=0.009). This study provides relevant data which can be applied for the development of new and better tools (diagnostics, therapeutics, vaccines) for effective measures in the control of drug-resistant tuberculosis in the country. It also gives information on the general anti-TB drug resistance status in Myanmar patients.

Key words: Multidrug resistant, Tuberculosis, Myanmar, Genotype, Mutation

INTRODUCTION

Tuberculosis (TB) ranks as the second leading cause of death from an infectious agent around the world, killing 1.3 million people every year.[1] Drug resistance has been a cause of major concern for TB control in both developed and developing countries. Multidrug-resistant TB (MDR-TB), i.e., resistant to both isoniazid and rifampicin, does not respond to the standard six-month treatment with first-line anti-TB drugs and can take up to two years or more to treat with less potent, more toxic and expensive second-line drugs.[2] Myanmar is among the 27 MDR-TB high burden countries worldwide. A nationwide drug resistance survey in 2012-2013 showed a MDR-TB prevalence of 5.0% among newly diagnosed and 27.1% among previously treated cases in Myanmar.

Rapid detection of drug-resistant strains of *Mycobacterium tuberculosis* is definitely necessary in order to control the spread and transmission of these strains. Diagnosis of

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drug-resistant TB relies on establishment of the drug susceptibility of *M. tuberculosis* strains, which is either assessed phenotypically or genotypically. Phenotypic or conventional methods based on culture are the gold standard or reference method; however they take several weeks to months to yield results. Several genotypic assays such as the line probe assay (LPA) GenoType MTBDR*plus* and Xpert MTB/RIF diagnostic test have been developed for rapid detection of MDR-TB, based on the frequent genetic mutations associated with anti-TB drugs.[3]

Almost all isolates of *M. tuberculosis* resistant to rifampicin (RIF) have a mutation in the *rpoB* gene encoding the β-subunit of RNA polymerase. Several isoniazid (INH)-resistant organisms have mutations in the *katG* gene encoding catalase-peroxidase and is found in more than 80% of INH-resistant strains. Some INH-resistant organisms also have mutations in the regulatory region of *inhA* locus, exhibiting low-level resistance to INH.[4]

Among the available commercial LPAs, the GenoType MTBDR*plus* assay can identify the presence of *M. tuberculosis* complex as well as its mutations in the *rpoB* gene for RIF resistance, the *katG* and *inhA* genes for INH resistance directly from smear-positive sputum within one day.[5] The assay readability rates are significantly higher in sputum specimens with acid fast bacilli (AFB) graded 2+ than in specimens graded 1+.[6]

Unfortunately, these genotypic or molecular assays only identify the most frequent resistance mutations for a limited number of antibiotics. There is wide variation in circulating *M. tuberculosis* drug-resistant strains across the world and the genetic diversity within the organism has practical concerns for molecular methods for drug susceptibility testing (DST).[7] Hence, prior knowledge about the nature and frequency of resistance-conferring mutations is critical to develop more accurate, region-specific molecular diagnostic methods.

Among different methods of TB strain typing or genotyping currently available, spoligotyping in particular, is simple, highly reproducible and used as a first-line screening technique. It greatly enhances the understanding of the dissemination of *M. tuberculosis* by using international databases to compare isolates from widespread geographic areas.[8]

The present study was done to provide genetic information of the circulating MDR-TB strains from Myanmar cases using GenoType MTBDR*plus* assay and phenotypic DST as well as spoligotyping method for strain differentiation.

MATERIALS AND METHODS

Patient selection

Sputum samples were collected from TB patients at risk of MDR-TB around Yangon Region and from some states and regions in Myanmar who were referred to National Tuberculosis Reference Laboratory (NTRL), Yangon during 2012-2013 for MDR-TB screening. Retreatment cases including Category II failure, Category I failure, relapse and return after default, close contacts of MDR-TB patients who develop active TB and all HIV-infected cases at the start of anti-TB treatment are recommended to do so since they are at risk of MDR-TB.[2] A total of 139 MDR-TB suspected patients whose sputum smear examination report was AFB 2+ or 3+ were chosen for the study.

GenoType MTBDRplus assay

The MTBDR*plus* assay version 1.0 (HAIN Lifescience, Germany) was performed on two sputum samples from each patient having AFB grading 2+ or 3+ according to the manufacturer's specifications at NTRL, Yangon. This DNA strip assay is based on multiplex polymerase chain reaction (PCR) technology in combination with reverse hybridization. A 500-ml portion of the NALC-NaOH-decontaminated sputum sediment was used for DNA extraction that included heating, sonification, and centri-

fugation. The amplification of genes responsible for drug resistance, including *rpoB*, *katG* and *inhA* was done. Hybridization was performed using a TwinCubator. The absence of at least one of the wild-type bands (WT) or the presence of bands indicating a mutation (MUT) implies that the sample tested is resistant to the particular antibiotic (RIF and/or INH) tested.[5] All tests were performed before the culture and DST results were available.

Isolation of M. tuberculosis and phenotypic DST

The remaining NALC-NaOH decontaminated sputum samples were inoculated on Lowenstein-Jensen (L-J) media and incubated at 37°C for 6-8 weeks at NTRL, Yangon. The isolates were identified by colony morphology, acid-fastness and positive TB Ag MPT 64 Rapid assay with no growth on L-J medium containing paranitrobenzoic acid. Susceptibilities to INH, RIF, streptomycin (SM) and ethambutol (EMB) were determined by the proportion method on L-J media containing INH (0.2 μg/ml), RIF (40 μg/ml), SM (4 μg/ml), and EMB (2 μg/ml). Resistance was defined as 1% or more growth for those drugs.[9]

DNA extraction from isolates on L-J media for spoligotyping

The DNAs from the isolates grown on drug free L-J media were extracted by modified thermolysis method. A loopful of heat-killed mycobacterial colonies grown on L-J medium was suspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.5) and subjected to three cycles of boiling and freezing for 20 minutes at -20ºC. The solution was centrifuged at 13,000 rpm for 5 minutes to obtain the supernatant. All the supernatant was used as the sample DNA solution.[10]

Spoligotyping

Spoligotyping of 72 out of 139 *M. tuberculosis* isolates was performed at Immunology Research Division, Department of Medical Research using microspoligoarrays according to the procedure by Kamerbeek and co-workers[11] with slight modifications[12]. The spoligotyping method is based on the polymorphism at one particular genomic region, the so-called direct repeat (DR) locus. Each DR is interspersed by a non-repetitive spacer sequence of 35 to 41 base-pairs. The entire DR locus was amplified by a hot start PCR using two inversely oriented primers complementary to the sequence of short DRs. The amplified DNA was hybridized to a set of 43 spacer oligonucleotides covalently bound to a membrane which were derived from spacer sequences of the laboratory strain *M. tuberculosis* H37Rv and *M. bovis* BCG. The resulting hybridization patterns are strain specific because of the strain-dependent presence or absence of spacer sequences in different isolates. The spoligotype was defined according to the SpolDB4 spoligotyping database.[8]

Ethical consideration

Ethical issues concerning informed consent, confidentiality and benefits were fully given to the subjects. This study was approved by the Research and Ethical Committee, University of Medicine 1, Yangon, Myanmar.

RESULTS

According to GenoType MTBDR*plus* assay, 110(79.1%) out of 139 patients were MDR-TB cases and 29(20.9%) cases were not multidrug-resistant. However, reference phenoltypic DST detected MDR-TB in 111(79.9%) of patients and non-MDR TB in 28(20.1%) cases. Agreement or concordance rate (kappa value) for detecting MDR-TB was 0.934 (95% confidence interval).

Drug resistance patterns of four major first-line anti-TB drugs

According to phenotypic L-J proportion method, the present study found the most resistance to INH in 117(84.2%) cases

followed by RIF in 116(83.5%), SM in 110(79.1%) and EMB in 52(37.4%) cases. The drug resistance patterns are shown in Table 1. Among them, MDR-TB was found in 111(79.9%) cases.

Table 1. Distribution of anti-TB drug resistance according to phenotypic DST results

Drug resistance patterns	No. of cases	(%)
Resistance to only one anti-TB drug		
INH	1	0.7
RIF	4	2.9
SM	0	0
EMB	0	0
Resistance to any two anti-TB drugs		
NH+RIF	6	4.3
INH+SM	5	3.6
INH+EMB	0	0
RIF+SM	1	0.7
Resistance to any three anti-TB drugs		
INH+RIF+SM	53	38.1
INH+RIF+EMB	1	0.7
INH+SM+EMB	0	0
Resistance to all four anti-TB drugs		
INH+RIF+SM+EMB	51	36.7
MDR-TB (at least INH+RIF)	**111**	**79.9**

INH=Isoniazid, RIF=Rifampicin, SM=Streptomycin, EMB=Ethambutol

Table 2. Disagreements between molecular and phenotypic DST results

Research ID	Molecular DST (GenoType MTBDR*plus*)					Phenotypic DST	
	RIF	INH	*rpoB*	*katG*	*inhA*	RIF	INH
007	R	R	Δwt8, S531L	WT	Δwt1, C15T	R	S
008	R	S	Δwt8, S531L	WT	WT	R	R
010	R	S	Δwt7, H526Y	WT	WT	S	S
016	S	S	WT	WT	WT	S	R
018	S	S	WT	WT	WT	S	R
025	R	S	Δwt3, 4,7;H526D	WT	WT	R	R

RIF=Rifampicin, INH=Isoniazid, R=Resistant
S=Sensitive, DST=Drug susceptibility testing
WT=All WT probes are present
Δwt=Missing WT probe(s)

Gene mutation patterns in all M. tuberculosis strains

The line probe assay (LPA) GenoType MTBDR*plus* identified MDR-TB in 110 strains, RIF mono-resistance in 7 strains and INH mono-resistance in 4 strains.

For detection of RIF resistance, *rpoB* S531L mutation was found to occur most commonly with 67.5% (79/117) of all RIF-resistant strains identified by GenoType MTBDR*plus* assay having the mutation. Other mutations occurred at *rpoB* D516V and H526D (2.6%, 3/117 and 3.4%, 4/117, respectively, of all RIF-resistant strains).

Of all INH-resistant strains, 90.4% (103/114) had a S315T mutation in the *katG* gene, and 8.8% (10/114) had a C15T mutation in the *inhA* gene. Only one strain had mutations in both *katG* and *inhA* genes. There was a significant difference in *inhA* C15T mutation between INH-monoresistant cases and MDR-TB cases (50%, 2/4 *vs.* 7.3%, 8/110; p=0.038). Thus, *inhA* C15T mutation was more likely to occur in INH-monoresistant cases compared to MDR-TB cases. Six discrepant results between GenoType MTBDR*plus* assay and phenoltipic DST results are shown in Table 2.

Table 3. Distribution of tested strains belonging to the circulating lineages

Lineage or sub-lineage	No. of strains	Percentage of strains
Beijing	55	76.4
EAI	7	9.8
CAS	4	5.6
Beijing-like	2	2.7
U	2	2.7
LAM	1	1.4
KILI	1	1.4
Total	72	100

Distribution of genotypes

The resulting genotypes of the 72 strains were also divided into two groups consisting of Beijing strains (55/72; 76.4%) and non-Beijing strains which included all strains other than Beijing strains (17/72; 23.6%) (Table 3). 89.1% (49/55) of Beijing strains developed MDR while 52.9% (9/17) of non-Beijing strains were multidrug-resistant. The asso-ciation between Beijing lineage and MDR-TB was found to be significant (p=0.001).

INH resistance was found in 70.6% (12/17) of non-Beijing strains and in 90.9% (50/55) of Beijing genotype. RIF resistance was seen in 52.9% (9/17) of non-Beijing strains

and in 92.7% (51/55) of Beijing genotype. SM resistance occurred in 58.8% (10.17) of non-Beijing strains and in 89.1% (49/55) of Beijing genotype. EMB resistance developed in 11.8% (2/17) of non-Beijing strains and in 45.5% (25/55) of Beijing genotype. Thus, Beijing genotype was found to be significantly associated with resistance to INH, RIF, SM and EMB (p=0.023, <0.001, 0.005, 0.012, respectively).

There was a significant difference in *katG* S315T mutation of INH-resistant cases between Beijing and non-Beijing strains (92% 46/50 *vs.* 58.3% 7/12, p=0.009). Thus, *katG* S315T mutation was more likely to develop in strains of Beijing genotype.

DISCUSSION

The MDR-TB prevalence (79.1% by molecular assay and 79.9% by phenotypic method) in the present study was relatively higher than the national average in 2012-2013 survey since it recruited only MDR-TB suspected patients who were at high risk of developing multidrug resistance having AFB high-positive sputum and the majority was retreatment cases. High MDR-TB rate among previously treated cases highlights limitations in specific case management, indicating the need for reassessment in that area.

The high agreement between GenoType MTBDR*plus* test directly on smear high-positive sputum samples and phenotypic DST for detecting MDR-TB in the present study correlates well with a meta-analysis on this molecular assay[13] and a recent study in Myanmar.[14] This finding proved the assay as a useful rapid tool in detecting MDR-TB in sputum specimens with higher AFB grades in a setting of Myanmar.

Six discrepant results between phenotypic DST and GenoType MTBDR*plus* assay were detected in the present study. The occurrence of specimens initially identified as sensitive using molecular assay from which phenotypically resistant strains were grown could be clarified by the presence of mutations not detected by the molecular assay. The occurrence of phenotypically sensitive strains initially identified as resistant using molecular assay on sputum specimens could also be due to the presence of strains presenting hetero-resistance i.e., simultaneous presence of resistant and sensitive bacilli in sputum specimens[6] or a result of some technically incorrect DST results that might occur at any laboratory. Hetero-resistance is more likely to occur in areas of high TB burden and drug resistance where a patient could be infected by two or more TB strains with different patterns of resistance.

In agreement with prior local studies[14, 15] and other international studies,[5, 6] the most common *rpoB* gene mutations (67.5%) were S531L mutations in this study. It could be assumed that almost all of the circulating RIF-resistant strains in Myanmar had mutations in a hot spot region (81-bp) of *rpoB* gene as in most of the countries around the world.

Similarly, the most frequent mutation (90.4%) was *katG* S315T mutations and it was also consistent with previous studies on local TB isolates.[14, 15] The proportion of *inhA* mutations (8.8%) in the present study was also comparable with those in local studies;[14, 15] however, it was lower when compared to studies from other countries.[5, 6] It could be assumed that mutations in *katG* gene are prevalent in the circulating INH-resistant strains in Myanmar as in other countries; although *inhA* mutations are not increasing.[5, 6] However, mutation rates in the *inhA* promoter region for INH resistance were significantly higher in mono-resistant strains than in MDR strains.

In this study, Beijing and Beijing-like strains accounted for approximately 80% of total isolates, indicating their high rate of distribution among MDR-TB suspected patients in Myanmar. A domination of Beijing and EAI genotype strains among TB isolates from Myanmar was in line with previous findings in other studies.[16-18]

Gradual rise in the prevalence of Beijing genotype in *M. tuberculosis* strains from Myanmar was also observed.[16-18] Alarmingly increasing number of Beijing strains in Myanmar reflects the threat of TB in the community since this strain has been reported to be associated with recent TB transmission[19] and thus, it is of great concern for TB control in the country.

CAS and LAM lineages observed in the present study have also been detected in previous local strains from Myanmar.[18] However, strains from KILI family, which have not been previously described in Myanmar strains, are not phylogeographically specific for Asia continent.[8] They might possibly be spread from its origin to any part of the world in this era of international travel and migration.

Significant associations between Beijing genotype, MDR-TB and first-line anti-TB drugs are in agreement with the observations made by others in local settings.[17, 18] A significantly higher proportion of the *katG* S315T mutation in Beijing genotype strains compared to non-Beijing strains was also observed in the present study. A better understanding of these associations can help to identify groups of patients who would be more likely to have MDR-TB and thus prioritize the use of rapid diagnostic methods and initiate treatment earlier.

Conclusion

Analysis of drug resistance gene mutations and their associated factors in MDR-TB strains from Myanmar patients could contribute to better diagnosis and appropriate treatment of drug-resistant TB in the country. Genotypic differentiation of *M. tuberculosis* strains plays an important role in understanding the epidemiology of prevalent strains in Myanmar.

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