Detection of Insertion Sequence IS*6110* Deficient *Mycobacterium tuberculosis* Strains from Myanmar

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Insertion sequence (IS) *6110* is a widely used target for detection of *Mycobacterium tuberculosis* (MTB). Molecular diagnosis of tuberculosis (TB) especially extra-pulmonary TB in Myanmar is based on detection of IS *6110* from clinical samples. However, 8-11% of MTB stains in South-East Asia do not contain this target, thus it can lead to false negative results for molecular diagnostic testing using this target. The present study was carried out to determine the occurrence of MTB complex that lacking IS*6110* in Myanmar. IS*6110* PCR assay was performed to detect IS*6110* deficient strains among 232 MTB isolates collected during 2015-2017 from adult TB patients in different regions of Myanmar. These isolates included 125 rifampicin-resistant and 107 rifampicin-sensitive strains detected by Xpert MTB/RIF. Phenotypic first-line anti-TB drug susceptibility testing and 24-loci Mycobacterial Intersperses Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) typing on IS*6110* deficient strains were also conducted. Of 232 clinical isolates, three isolates (1.3%) showed absence of IS*6110* element. All of three IS*6110* deficient MTB isolates were sensitive to rifampicin, isoniazid and ethambutol but two isolates were resistant to streptomycin. Two isolates belong to Beijing genotype and one belongs to East African-Indian (EAI) genotype. Myanmar MTB strains have low percentage of IS*6110* deficient strain and these strains. Although IS*6110* based molecular diagnosis for TB in clinical sample is a useful method, there can be chance of false negative results. Further study with larger sample size should be conducted to discover the clinical significance of IS*6110* deficient Myanmar MTB strains and to evaluate usefulness in application of IS*6110*-based diagnostic and molecular epidemiology tools in Myanmar.

Keywords: IS*6110*, *Mycobacterium tuberculosis*, PCR

INTRODUCTION

Approximately 1.7 billion people of the world's population has been infected with *Mycobacterium tuberculosis* (MTB), the causal bacterium of tuberculosis (TB) and 5-15% of them were estimated to develop TB disease during their lifetime. Myanmar is one of the high TB and multidrug-resistant TB (MDR-TB) burden countries worldwide with the incidence of 338/100,000 population in 2018.[1] Insertion sequences (IS) IS*6110* is a mobile genetic element, which is inserted in a 36-bp array Direct Repeat region (DR region: Rv2813-Rv2820c, RD207) of almost all MTB complex isolates. IS*6110* presents

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as multiple copies (usually up to 25) and because of this, it is used widely as a target for detection of MTB complex genome. MTB genome is also used in DNA finger-printing, using restriction fragment length polymorphism (RFLP) analysis, for studying MTB strains transmission[2, 3] IS*6110* based RFLP analysis is widely used in molecular epidemiology for MTB because the insertion element varies sufficiently in copy number and chromosomal location in MTB that allows strains to differentiate easily.[4, 5] There is a standardized method for comparison of IS*6110* RFLP patterns within and between laboratories. Moreover, detection of IS*6110* in clinical samples extra plumonary TB has also been used as a diagnosis method.[6, 7]

However, there were some reports that revealed presence of clinical MTB isolates which possess a single copy or lack of IS*6110* from different geographical regions of the world that can lead to false negative results. In South-East Asia 8-11% of MTB strains do not contain IS*6110* Sequence[8] especially in India where, 11% of MTB clinical isolates showed absence of IS*6110* element in their genome and prevalence of zero copy number isolates varies from 10%.[9]

A small number (2%) of IS*6110* deficient strains have been reported in Vietnam.[10] Depending on the presence of IS*6110* copy numbers, MTB strains can be classified into high copy-number (>7) and low copy-number strains. It is believed that high copy number strains are highly pathogenic (like in Beijing genotype). In contrast, low copy number strains have been associated with drug resistance and outbreak. It is not clear yet whether these two MTB strains possess different physiological or pathogenic behavior or not.[11] Recent studies on IS*6110* PCR diagnosis in Myanmar showed low detection rate from clinically probable extra-pulmonary TB cases and there is possibility IS*6110* deficient strains among the tested samples. [12, 13]

Moreover, an ongoing molecular typing study on Myanmar MTB strains indicated low copy number or absence of IS*6110* in their RFLP pattern. Thus, there is a need to detect the occurrence of *M. tuberculosis* complex that lacking IS*6110* in Myanmar and their characteristics. This information can support for the evaluation of usefulness of IS*6110* based diagnostic and molecular epidemiology tools for MTB strain from Myanmar.

MATERIALS AND METHODS

Study design and study period

A cross-sectional descriptive study was carried out from May 2018- April 2019.

Study population

Altogether 232 MTB isolates collected during 2015-2017 from adult TB patients in different regions of Myanmar included in this study. These isolates were 125 rifampicin-resistant and 107 rifampicin-sensitive strains detected by Xpert MTB/RIF in routine diagnosis program by National Tuberculosis Program.

Isolation of M. tuberculosis from stock culture isolates

The colonies from stock culture isolates were taken using a disposable loop and put in a conical tuber with glass beads and vortexed for 2 minutes to disrupt colony clumps and was stand 15 min to allow the undisrupted colony clumps to settle. Supernatant was taken and adjusted turbidity to McFarland of *M. tuberculosis* were cultured on Lowenstein Jenson (LJ) median BSL2+ laboratory, Advanced Molecular Research Centre, Department of Medical Research according to the following standard procedures.[14]

DNA extraction of MTB isolates

Three to four colonies from solid culture were suspended in 500 µl molecular grade water and mixed using vortex. The DNA was extracted from colonies at 90°C for 30 minutes shaking 5 minutes at 15 minutes interval in mixing block. The supernatant containing DNA was transferred into fresh tube and placed at 4°C overnight and then stored at -20°C.

Dilution of DNA concentration

DNA stored in freezer was taken out to the room temperature. DNA concentration was measured by spectrophotometer and sample was diluted to 10 ng/µl concentration for further molecular testing.

IS6110 PCR

The extracted DNAs were subjected to amplify the target segment IS*6110*. Control MTB strain H37RV DNA were used for positive control. Primers used for amplification were as follow:

IS*6110* primers[8]

IS*6110* primers	Sequence
INS1	CGT GAG GGC ATC GAG GTG GC
INS2	GCG TAG GCG TCG GTG ACA AA

PCR reaction (1 reaction) mixture was as follow:

DDW	36.8 µl
10x buffer	5 µl
dNTP	4 µl
10 uMFw primer	1 µl
10 uMRv primer	1 µl
HotstarTaq polymerase	0.2 µl
sample DNA	2 µl
Total	50 µl

Thermal cycle condition was as follow: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds with 35 rounds of repetitive cycles for denaturation, annealing, extension followed by final extension at 72°C for 5 minutes. Presence of IS*6110* segment (245bp) was observed by gel electrophoresis on 1% agarose gel under Ultra Violet light.[12]

First line anti-TB phenotypic drug susceptibility (DST) test

Proportion method was used for drug susceptibility testing. Drug resistance was determined when more than 1% colony growth at 28 or 42 days of incubation at 37°C on LJ medium. The growth was compared to that of drug free control LJ medium at the following drug concentration: isoniazid (INH) 0.2µg/ml, rifampicin (RMP) 40 µg /ml, streptomycin (SM) 4 µg/ml and ethambutol (EMB) 2 µg/ml.

Interpretation of DST test for first line anti-TB

On the 28th day of inoculation, first reading was made. On the 42nd day of inoculation, second reading was made which provided the definitive result. Colonies were counted and recorded in both drug free containing L-J slopes. Quantity of growth was recorded as follow:

Numbers of colony on slope	Record
500 colonies (confluent)	4+
201-500 colonies (almost confluent)	3+
101-200 colonies	2+
50-100 colonies	1+
<50 colonies	Actual number of colony count
Non	0

MIRU-VNTR typing

Mycobacterial genomic DNA was extracted from MTB growth on LJ medium. Two to three loopful of colony for each isolate was suspended in 300µl aliquot of distilled water. The sample was heated at 99°C for 20 minutes with shaking at 5 minutes intervals. The supernatant was stored at -20ºC until used. Mycobacteria strain typing was per-formed using 24-loci MIRU-VNTR method employed 15 pairs of primers and additional 9 primer pairs for MIRU-VNTR typing (Supply, 2005). Hotstar Taq kit (Qigen, Hilden, Germany) was used for PCR mixture which was subjected to the thermo cycling conditions (conducted in Veriti Thermal Cycler; Applied Biosystems, Foster City, CA): 95ºC for 15 minutes; 40 cycles of 95ºC for 1 minute, 59ºC for 1 minute and 72ºC for 1.5 minutes; with a final heating at 72ºC for 10 minutes.

The amplicons were electrophoresed in 3% agarose gel at 50 volts for 2 hours and were visualized using Geldoc imager (Bio-Rad, Hercules, CA). The sizes of amplicon was determined by comparing with 100 plus base pair DNA ladder (Bioneer, Daejeon, Korea). The sizes of repeat sequences were converted to allelic numbers according to Supply

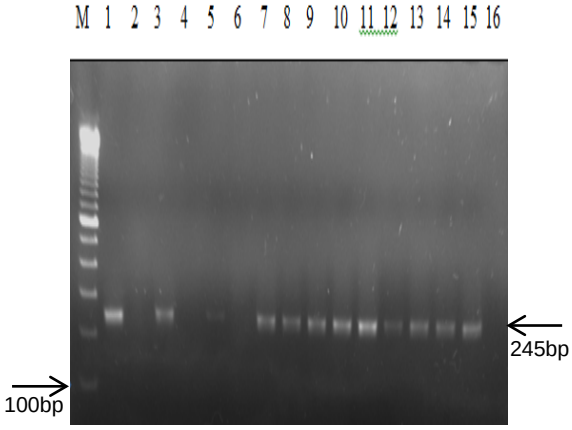
(2005) and the patterns analyzed using MIRU-VNTR *plus*.[15-17]

Ethical consideration

The Ethics Review Committee, Department of Medical Research, approved this study.

RESULTS

Of 232 clinical isolates, PCR (IS*6110)* was positive in 229(98.7%) isolates and three isolates (1.3%) showed absence of IS*6110* element. Gel electrophoresis picture of PCR products was shown in Fig.1.



Arrows indicate the position of the fragments of 245 bp (diagnostic for MTB complex).

M: Marker (Gene Ruler 100bp DNA ladder), Lane 1: Positive control, Lane 2: Negative control, Lanes 3-16: PCR products of MTB isolates.

Fig. 1. Analysis of PCR amplified 245-bp fragment by 1% agarose gel electrophoresis

Table 1. First line anti- TB phenotypic DST pattern of MTB isolates (n=3)

First-line anti-TB drug resistant pattern	Number of isolates
Sensitive to all drugs (SIRE)	1
Sensitive to three (RIE) drugs	3
Streptomycin mono-resistance	2

S=Streptomycin, I=Isoniazid, R=Rifampicin, E=Ethambutol

First line anti-TB DST pattern of MTB isolates

All of three IS*6110* deficient MTB isolates were sensitive to RMP, INH and EMB but two isolates were resistant to SM (Table 1).

Distribution of genotypes of IS*6110 deficient MTB isolates*

Two isolates belong to Beijing genotype and one belongs to East African-Indian (EAI genotype.

DISCUSSION

Although previous studies reported as 8-11% of MTB strains in South-East Asia do not contain IS*6110* sequence[8, 9], the present study found low percentage, 1.3% of IS*6110* deficient strains among 232 clinical MTB isolates. The detected proportion of IS*6110* negative MTB isolates was lower than that of other Asian countries such as India and Vietnam, both of which detected no-copy strains using RFLP method only, and in which the frequency of these strains was estimated at respectively 8% and 11% of tested isolates. In the present study, we used the IS*6110* PCR and the chance of having false negative for IS*6110* PCR-based detection was controlled by optimizing PCR reactions with positive and negative controls, and repetition of negative tests.

Previous studies reported IS*6110* negative MTB was associated with ancient genotypes and anti-TB drug susceptibility. Thus, low detection of IS*6110* deficient MTB strains in the present study may be also due to Beijing genotype dominant nature of Myanmar isolates and comprising MDR-TB in half of tested isolates. Geographic variation might be the cause of different copy number in MTB. At the same time, identification method will also affect the detection of no copy strains. According to this study, it can be included that Myanmar MTB strains can have co copy of the IS*6110* element in their genome. Therefore, when the IS*6110* is used as a target sequence for diagnosis of MTB, false negative result will affect the sensitivity of the detection method. In Myanmar, pulmonary TB diagnosis may not be affected by presence of these strains because the routine pulmonary TB diagnosis was carried out by sputum smear microscopy and GeneXpert® MTB/RIF (Cepheid, CA, USA) which used a specific *rpo*B gene signature

that is present in all *M. tuberculosis* complex strains. However, for the diagnosis of extra-pulmonary TB such as TB lymphadeno-pathy, TB meningitis, TB pleural effusion, TB abdomen etc.., IS*6110* PCR was widely used to detect MTB DNA from clinical specimens. This implies a significant number of misdiagnosis and the patients may remain a source of TB transmission in the community.

For this reason, this method should be replaced by another method that targeting multiple areas on IS*6110* segment as well as well as *rpo*B gene. Addition to this, IS*1081*, hich is usually present as multiple copies in strains lack of IS*6110* should be added. Another approach should be by confirmation of IS*6110* PCR negative results by another PCR tests targeting *rpo*B and IS*1081*. Those approaches will generate a higher sensitivity and specificity for detecting no-copy strains, resulting in fewer misdiagnosis cases.

Compare to other origin, such as Americans, South-East Asians are more frequently infected with no-copy strains, which may be due to the historical geographical isolation of South-East Asian populations, or there may involve unknown host-pathogen co evolution. The most representative lineage for no-copy strains is EAI, as defined by spoligo-typing 20. As well as Lineage1 or Rim of Indian Ocean, as defined by single nucleotide polymorphisms (SNPs).[18, 19] Those strains represent the 'ancient lineage' by SNP, a lineage that show better similarity to the ancestor of all *M. tuberculosis* complex strains.

In Contrast to this, the Beijing genotype strain, which belong to the modern lineage, show high IS*6110* copy and revealed a strong association with anti-tuberculosis drug resistance. In the present study, two isolates belong to Beijing genotype and one belongs to East African-Indian (EAI) genotype. Myanmar MTB strains have lower per-centage of IS*6110* deficient strain compare to neighboring country, India and these strains. Although IS*6110* based molecular diagnosis for TB in clinical sample is a useful method, there can be a chance of false negative results.

Further study with larger sample size should be conducted to discover the clinical significance of IS*6110* deficient Myanmar MTB strains and to evaluate usefulness in application of IS*6110*-based diagnostic and molecular epidemiology tools in Myanmar.

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