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## Infection of Multiple *Mycobacterium tuberculosis* Strains among Tuberculosis/Human Immunodeficiency Virus Co-infected Patients: A Molecular Study in Myanmar

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## Abstract

**Background:** Appearance of *Mycobacterium tuberculosis* (MTB) in the sputum of a tuberculosis (TB)/human immunodeficiency virus (HIV) co-infected patient under treatment may indicate either failure or new infection. This study aims to evaluate whether TB treatment failure among TB/HIV co-infected patients is a real failure.

**Methods:** A prospective cohort study was conducted among 566 TB/HIV co-infected patients who started TB treatment in 12 townships in the upper Myanmar. Among the 566 participants, 16 (2.8%) resulted in treatment failure. We performed a molecular study using mycobacterial interspersed repetitive-unit-variable number of tandem repeat (MIRU-VNTR) genotyping for them. The MIRU-VNTR profiles were analyzed using the web server, MIRU-VNTRplus. All data were entered into EpiData version 3.1 and analyzed using R version 3.4.3.

**Results:** Among 16 failure patients, seven had incomplete laboratory results. Of the nine remaining patients, nobody had exactly the same MIRU-VNTR pattern between the initial and final isolates. Four patients had persistent East-African Indian (EAI) lineages and one each had persistent Beijing lineage, changing from EAI to Beijing, from Beijing to EAI, NEW-1 to Beijing, and NEW-1 to X strains. Female patients have significantly larger genetic difference between MTB of the paired isolates than male patients (*t*-test, P = 0.04).

Conflicts of interest

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There are no conflicts of interest.

**Conclusion:** Thus, in our study patients, infection of multiple MTB strains is a possible cause of TB treatment failure. Explanation for the association between gender and distance of genotypes from the initial to subsequent MTB infection needs further studies.

#### Keywords

Infection of multiple *Mycobacterium tuberculosis* strains; tuberculosis treatment failure; tuberculosis/human immunodeficiency virus co-infection

## Introduction

Tuberculosis (TB) treatment outcome is poor among human immunodeficiency virus (HIV)positive TB patients compared with HIV-negative TB patients.<sup>[1–8]</sup> Moreover, people living with HIV (PLHIV) are at higher risk of recurrence after successful previous TB treatment. <sup>[9–11]</sup> Many molecular studies reported that recurrent TB among PLHIV was mostly due to exogenous reinfection of *Mycobacterium tuberculosis* (MTB),<sup>[12–17]</sup> especially those who reside in TB-endemic countries.<sup>[18–20]</sup> However, the evidence to support this idea among TB/HIV co-infected patients who have treatment failure is not solid.

There is a need to conduct such assessment in Myanmar where TB/HIV is a high burden. The objectives of this study therefore were to evaluate whether TB treatment failure among TB/HIV co-infected patients was a real failure and to assess the associated factors.

## Methods

#### Study design, setting, and participants

A prospective cohort study was conducted in 12 townships in the upper Myanmar. TB/HIV co-infected patients aged 15 years were diagnosed according to the WHO and National guidelines.<sup>[21,22]</sup> An individual with rifampicin resistance by Xpert MTB/RIF assay was excluded due to the need of long period of treatment. Five hundred and sixty-six newly registered TB/HIV co-infected patients at the TB clinics during the study period were included. They were followed up bacteriologically until the end of TB treatment.

#### Sample collection and molecular typing

Two sputum samples from each patient were collected before starting TB treatment and sent to the Upper Myanmar TB Reference Laboratory in Mandalay. Those samples were inoculated onto Mycobacteria Growth Indicator Tube (MGIT) by standard procedures.<sup>[23]</sup> The isolates were frozen in 7H9 broth plus glycerol to preserve for further analysis. After getting TB treatment outcomes, sputum samples were collected and inoculated into MGIT again if the sputum smear result was positive. The initial and final isolates of the same patient were confirmed by having the same identification number, name, gender, and address. The DNA of eleven paired (initial and final) culture isolates were extracted as described by van Helden *et al.*<sup>[24]</sup> Those extracted DNAs were sent to perform mycobacterial interspersed repetitive-unit-variable number of tandem repeat (MIRU-VNTR) genotyping at the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. The copy numbers of DNA tandem repeat of 24 MIRU-VNTR loci were

identified using 24 sets of standard polymerase chain reaction (PCR) primers.<sup>[25,26]</sup> Determination of the PCR product sizes was done by agarose gel electrophoresis. Any paired samples with more than two MIRU-VNTR loci failed in genotyping were not included in the statistical analysis.

#### Data analysis

Analysis of the MIRU-VNTR profiles using the web server, MIRU-VNTRplus (http:// www.miru-vntrplus.org/), enabled a comparison of profiles by generating Neighbour Joining (NJ) tree and identification of clusters of strains of MTB.<sup>[27]</sup> Isolates that had identical type or a single mismatch among 24 MIRU-VNTR loci were considered clonally related. Under the constraints of small sample size, statistical analysis was carried out to explore possible factors associated with the genotype changes. All data were entered into Epidata version 3.1 (http://www.epidata.dk/) and analyzed using R version 3.4.2 (https://cran.r-project.org/). Statistical significance was tested by *t*-test because of using continuous variable. P < 0.05with 95% confidence interval was set as significance. The results would be a foundation for further in-depth studies.

The study was approved by the Ethical Review Committee of the Prince of Songkla University, Thailand, and Department of Medical Research, Myanmar.

## Results

#### Patient characteristics

Data were collected from October 2016 to February 2018. A total of 566 TB/HIV patients were identified. At the end of the 5<sup>th</sup> month of TB treatment, 16 patients (2.8%) were notified as treatment failure according to the WHO's definition.<sup>[28]</sup> Five of them had no complete laboratory results. Two other had at least more than two MIRU-VNTR loci without genotyping results. Finally, nine patients had data available for analysis. These five male and four female patients had an average age of  $37.1 \pm 4.9$  years, which was not significantly different from the five male and two female excluded patients. Among the included nine patients, five were jobless and among four who had job, one female patient had experience in sex work.

#### Genotyping results and associated factors

Table 1 shows the MIRU-VNTR genotyping results of the initial and final isolates of nine patients. All patients had discordant fingerprints indicating that they have a new strain of MTB. Their paired isolates were different in 2-17 loci (median = 8) as mentioned in the last column of Table 1.

Table 2 describes the mean and standard deviation of the number of un-identical loci by various independent variables of the patients. The only statistically significant variable associated with the number of unidentical loci was gender. In male patients, the initial and final isolates had an average of 5.6 un-identical loci compared to the average of 12.5 loci in the female (*t*-test, P = 0.04).

#### Determination of Mycobacterium tuberculosis strain diversity

The MIRU-VNTR profiles were determined for 18 isolates and compared with profiles present in the MIRU-VNTRplus database by generating trees using the NJ algorithm. Among 18 strains from nine patients, ten formed a cluster with the most closely related reference strain belonging to the East-African Indian (EAI), five to the Beijing, two to the NEW-1, and one to the X lineage. Both X and NEW-1 belong to the Euro-American lineage. Figure 1 links isolates from the same patients with green solid lines for male patients and red dotted lines for female patients. Male patients were more likely to have the isolate pairs closer genetically than female patients did.

Table 3 describes the distribution of nine patients by their initial and final strains according to gender. The initial and final strains of all the five male patients ( $M_1 M_3$ ,  $M_4$ ,  $M_5$ , and  $M_6$ ) belonged to the same lineages, mostly EAI. Even though the initial and final strains of a patient ( $M_6$ ) converted from New-1 to X subfamily, both were Euro-American strains. In contrast, only one ( $F_2$ ) of the four female patients had the same Beijing genotyping of both strains. Other female patients ( $F_7$ ,  $F_8$ , and  $F_9$ ) had different strains.

### Discussion

None of our patients with MTB in their initial and final sputum had genetically unchanged MTB strains. Female patients were more likely to have greater genetic distances between the initial and final strains of MTB.

Exogenous reinfection of TB has also been observed in many other studies in TB-endemic areas.<sup>[9,12,13,15–17,29]</sup> This could have a few possible explanations. First, the patient could initially simultaneously harbor multiple strains of MTB before starting the treatment.<sup>[30,31]</sup> Bacterial isolation may pick up only one but different strain at a time. Otherwise, the patients could have been re-infected by a new strain of MTB during the treatment course as PLHIV are highly susceptible to TB infection.<sup>[1]</sup>

Female patients were more likely to have two different MTB strains with greater genetic distances than male patients.

This concurred with a study in Sydney where two re-infected patients were both female.<sup>[18]</sup> In our study, a female patient was a sex worker. Having high number of sex partners may have the chance to get infection of different MTB strains. However, with limited information on sexual behaviors of the participants and the smallness of our sample size, this conjecture needs to be proved by further studies.

The Beijing lineage is the most predominant MTB strain in many Asian countries.<sup>[32]</sup> Some studies reported that the EAI, one of the ancient MTB lineages, is prevalent in South East Asian countries.<sup>[33–35]</sup> The genetic diversity of MTB in Myanmar is mainly driven by both the EAI and the Beijing subfamilies.<sup>[36,37]</sup> In this study, most patients have the EAI lineage followed by the Beijing, which concurred with those results.

## Conclusion

Infection of multiple MTB strains could be a cause of apparent treatment failure in our study population. The association between gender and distance of genotypes from the initial to subsequent MTB infection was a result of our preliminary study. More data are needed to get a comprehensive conclusion.

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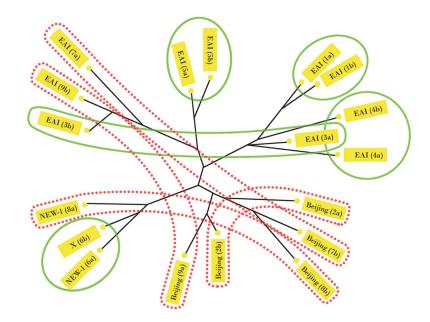
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#### Figure 1:

Neighbour-Joining tree (displayed as radial tree) showing the extent of difference between initial and final strains of the same patient (five males and four females). The strains of male patients are linked in green straight lines and those of female patients in red dotted lines

Mycobacterial interspersed repetitive-unit-variable number of tandem repeat analysis of 18 isolates from nine patients

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Me     Me<	*a	Gender											Number	Number of alleles in MIRU-VNTR loci	MIRU-VN	TR loci											$\mathbf{Loci}^{\dagger}$
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MIRU=Mycobacterial interspersed repetitive unit; ETR=Exact tandem repeat; VNTR=Variable number of tandem repeat

#### Table 2:

Mean and standard deviation of number of un-identical loci by patient characteristics

Variables	Un-id	entical loci	<i>P</i> *
	n (%)	Mean±SD	
Integrated models			
Partially	5 (56)	$8.4{\pm}5.8$	0.88
Fully	4 (44)	9±5.3	
Gender			
Male	5 (56)	5.6±4.1	0.04
Female	4 (44)	12.5±3.9	
Age group (years)			
36	5 (56)	11.4±4.4	0.07
Above 36	4 (44)	5.2±4.3	
Marital status			
Married	5 (56)	9.8±4.7	0.5
Others	4 (44)	7.2±6.2	
Education			
Primary and below	5 (56)	9.4±4.7	0.67
Middle and above	4 (44)	7.8±6.4	
Occupation			
No	5 (56)	7.4±5.4	0.45
Yes	4 (44)	10.2±5.3	
Monthly family income			
Below average	5 (56)	7.4±5.4	0.44
Above average	4 (44)	10.2±5.3	
Art			
No	6 (67)	10.3±5.5	0.19
Yes	3 (33)	5.3±3.1	

\* t-test, 7 degree of freedom. SD=Standard deviation

#### Table 3:

Distribution of nine patients by their initial and final strains according to gender

Initial strains		Final strain	ns		Total
	Beijing	EAI	NEW-1	Х	
Beijing	$F_2$	F <sub>9</sub>			2
EAI	F <sub>7</sub>	$M_1M_3M_4M_5$			5
NEW-1	$F_8$			$M_6$	2
X					0
Total	3	5	0	1	

M=Male; F=Female; Subscript number=Patient's ID; EAI: East-African Indian