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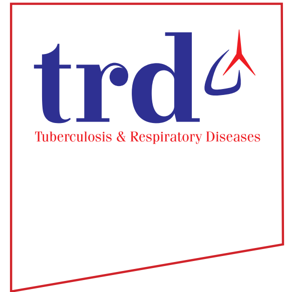

Mutations in Streptomycin Resistance Genes and Their Relationship to Streptomycin Resistance and Lineage of *Mycobacterium tuberculosis* Thai Isolates

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Background: Streptomycin (SM) is recommended by the World Health Organization (WHO) as a part of standard regimens for retreating multidrug-resistant tuberculosis (MDR-TB) cases. The incidence of MDR-TB in retreatment cases was 19% in Thailand. To date, information on SM resistance (SMR) gene mutations correlated to the SMR of *Mycobacterium tuberculosis* Thai isolates is limited. In this study, the mutations in *rpsL*, *rrs*, *gidB,* and *whiB7* were investigated and their association to SMR and the lineage of *M. tuberculosis* were explored.
Methods: The lineages of 287 *M. tuberculosis* collected from 2007 to 2011 were identified by spoligotyping. Drug susceptibility profiles were evaluated by the absolute concentration method. Mutations in SMR genes of 46 SM-resistant and 55 SM-susceptible isolates were examined by DNA sequencing.
Results: Three *rpsL* (Lys43Arg, Lys88Arg, and Lys88Thr) and two *gidB* (Trp45Ter and Gly69Asp) mutations were present exclusively in the SM resistant *M. tuberculosis*. Lys43Arg *rpsL* was the most predominant SMR mutations (69.6%) and prevailed among Beijing isolates (p<0.001). No SMR-related mutation in was found *rrs*. The combination of *rpsL* and *gidB* mutations provided 76.1% sensitivity for detecting SMR in *M. tuberculosis* Thai isolates. *whiB7* was not responsible for SMR in SM resistant isolates lacking *rpsL* and *rrs* mutations. The significance of the three *gidB* mutations, 276A>C, 615A>G, and 330G>T, as lineage signatures for Beijing and EAI were underscored. This study identified 423G>A *gidB* as a novel sub-lineage marker for EAI6-BGD1.
Conclusion: Our study suggested that the majority of SMR in *M. tuberculosis* Thai isolates were responsible by *rpsL* and *gidB* polymorphisms constantly providing the novel lineage specific makers.

Keywords: *Mycobacterium tuberculosis*; Streptomycin; Drug Resistance, Microbial; Mutation; *rpsL*; *rrs*; *gidB*; *whiB7*

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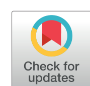

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Introduction

Streptomycin (SM) is the first anti-tuberculous drug that combated efficiently to tuberculosis (TB). Owing to the high rates of resistance due to mono-therapy and toxic side effects, the usage of SM as the first option for TB has been gradually declined. To date, it is recommended by World Health Organization (WHO) in standard regimens for the retreatment and multidrug-resistant tuberculosis (MDR-TB) cases[1].

SM binding to bacterial ribosomal protein S12 and 16S rRNA encoded by *rpsL* and *rrs*, respectively, which locate in the decoding center of 30S ribosomal subunit interrupts the protein translation fidelity, thereby inhibiting protein synthesis and resulting in bacterial death[2]. Consequently, *rpsL* and

rrs mutations confer the high or intermediate SM resistant levels in *Mycobacterium tuberculosis*[3,4]. The *rpsL* 128A>G (Lys43Arg) and single nucleotide polymorphisms (SNPs) in areas of the 530 and 912 loops of *rrs* are the most frequently found mutations in SM resistant isolates[5,6]. Furthermore, the low-level SM resistance (SMR) in *M. tuberculosis* is caused by mutations in a 7-methylguanosine (m^7G) methyltransferase (*gidB*) gene which functions for methylation of G527 in 530-loop of 16S rRNA[5]. The frequencies and types of SMR associated mutations in the above-mentioned genes are dependent on the geographic areas and *M. tuberculosis* lineages[6-8]. In addition, some *gidB* SNPs are responsible as *M. tuberculosis* lineage signatures rather than SM resistant markers[6,8]. Approximately 30% of SM resistant strains worldwide carried no mutation in those three genes and therefore, the contribution of alternative genes conferring intrinsic mechanism of SMR such as aminoglycosides efflux pump Tap (*Rv1258c*) and its transcriptional regulator, *whiB7* (*Rv3197A*), have been suggested[4,9-11].

Thailand is one of the 22 highest TB burden countries and the incidence of MDR-TB in new and retreatment cases were 2% and 19%, respectively in 2014[12]. However, to date, the information on *M. tuberculosis* mutations associated to SMR in Thailand is limited to only one study[8]. Therefore, we further expanded the investigation on the mutations of *rpsL*, *rrs*, *gidB*, and *whiB7* in *M. tuberculosis* isolates collected from the different time and region of Thailand, and associated with their SMR and lineages.

Materials and Methods

1. *M. tuberculosis* isolates, drug susceptibility testing, and spoligotyping

A total of 287 *M. tuberculosis* isolates collected during 2007 to 2011 were obtained from Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand. Their drug susceptibility testing (DST) was tested for four first-line anti-TB drugs using absolute concentration method on Löwenstein-Jensen medium with following concentrations, 0.2 and 1 μg/mL for isoniazid (INH), 40 μg/mL, 4 μg/mL, and 2 μg/mL for rifampicin, SM, and ethambutol, respectively[13]. Among those, 46 isolates were found SM resistant. These and other 55 SM susceptible isolates in the same collection were further analyzed for mutational profiles. Their lineage and strain type (clade) were identified by standard spoligotyping protocol and TB lineage online tool[14,15]. Details of anti-TB drug susceptibility phenotypes and lineage differentiation of 101 isolates were shown in Table 1. The pan anti-TB drug–susceptible *M. tuberculosis* H37Rv (ATCC 27294) strain was used as a reference in all assays.

2. Amplification and sequencing of *rpsL*, *rrs*, *gidB*, and *whiB7*

Genomic DNA was extracted from 101 *M. tuberculosis* clinical isolates by the cetyltrimethyl ammonium bromide method as previously described[16]. Oligonucleotide primers used for polymerase chain reaction (PCR) amplification and sequencing of *rpsL*, *rrs*, *gidB*, and *whiB7* were based on previous studies or newly designed in the present study (Table 2)[17-21]. Amplifications were carried out using *Pfu* DNA polymerase (Bioneer Corp., Daegeon, Korea). Sequencing was done in both forward and reverse directions using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) in an ABI 3730XL DNA analyzer (Life Technologies, Applied Biosystems). DNA sequencing data were analyzed and assembled using Sequence Scanner v2.0 (Applied Biosystems) and DNAMAN (version 4.15, Lynnon BioSoft, Quebec, Canada) software. Mutations were identified by comparing to the wild type (WT) *M. tuberculosis* H37Rv reference sequence (accession No. AL123456.3) (Table 3)[22].

For the 12 SM resistant isolates lacking *rpsL* and *rrs* mutations (Table 3), the 810-bp sequence containing the *whiB7* open reading frame (ORF) and its promoter region were further amplified and sequenced (Table 2).

3. Mismatched amplification mutation assay PCR for detection of mutation at *rrs* nucleotide position 16

Due to our *rrs* sequencing data could not reveal the first 39 nucleotides of the coding sequence, mismatched amplification mutation assay PCR (MAMA-PCR) was developed to detect the recently identified 16T>C (16u>c for RNA) mutation of *rrs* sequence (Table 2). MAMA-PCR primers were designed in this study to differentially detect T to C nucleotide alteration at nucleotide position 16 of *rrs* (previously annotated as nucleotide position 15)[23,24]. As shown in Table 2, the mutant allele-specific forward primer (*rrs*-16 mt) contained mutated nucleotide 'C' instead of WT 'T' at the 3'-end. The *rrs*-RM primer was used as a shared reverse primer. The amplification of nucleotide position 16 mutant allele present exclusively in reactions containing *rrs*-16 mt primer but not *rrs*-16 wt and *vice versa* for the WT allele. The genomic DNA of H37Rv was used as a control for WT allele amplification. Mutant allele control, 16T>C *rrs* was constructed by overlap extension PCR (data not shown).

4. Verification of Beijing isolates by RD105 deletion-targeted multiplex PCR

In order to verify the spoligotyping results, RD105 deletion-targeted multiplex PCR (DTM-PCR) was performed for all Beijing (East Asian) isolates (n=57) and representatives of non-Beijing isolates (n=18) as previously described[21]. The

Table 1. Drug resistant profile, LSP-based lineages, and SpolDB4 family of 101 *Mycobacterium tuberculosis* clinical isolates

Drug resistant profile	LSP-based lineages	SpolDB4 family
SMR (46)		
SM mono-resistance (20)	East Asian (15)	Beijing (15)
	Indo-Oceanic (4)	EAI5 (1)*
		EAI2-Nonthaburi (1)
		Manu1 (1)
		Unknown (1)
	Euro-American (1)	T2 (1)
SM+INH (16)	East Asian (13)	Beijing (13)
	Indo-Oceanic (3)	EAI2-Nonthaburi (3)*
SM+RIF (1)	East Asian (1)	Beijing (1)
SM+MDR (9)	East Asian (3)	Beijing (3)
	Indo-Oceanic (4)	EAI5 (2)
		EAI1-SOM (1)*
		Manu 1 (1)
	Euro-American (2)	T1 (1)
		T3-ETH (1)
SM susceptible (55)		
Susceptible to INH, RIF, SM, and EMB (20)	East Asian (9)	Beijing (9)
	Indo-Oceanic (8)	EAI5 (1)*
		EAI1-SOM (2)*
		EAI2-Manila (2)*
		EAI2-Nonthaburi (2)*
		Manu3 (1)
	Euro-American (3)	T1 (1)
		H3 (2)
INH mono-resistance (18)	East Asian (7)	Beijing (7)
	Indo-Oceanic (9)	EAI5 (1)*
		EAI1-SOM (1)*
		EAI2-Manila (3)*
		EAI6-BGD1 (4)*
	Euro-American (2)	T3-OSA (1)
		H3 (1)
RIF mono-resistance (7)	East Asian (3)	Beijing (3)
	Indo-Oceanic (3)	EAI1-SOM (1)*
		EAI2-Nonthaburi (1)*
		Manu_ancestor (1)
	Unknown (1)	Unknown (1)
MDR (10)	East Asian (6)	Beijing (6)
	Indo-Oceanic (3)	EAI5 (1)*
		EAI2-Nonthaburi (1)*
		EAI6-BGD1 (1)*
	Euro-American (1)	H3 (1)

Number of isolates are indicated in parentheses.
*Indo-Oceanic (EAI) isolates with *gidB* 330T>G and 615A>G polymorphisms.
LSP: large sequence polymorphism; SMR: streptomycin resistance; SM: streptomycin; INH: isoniazid; RIF: rifampicin; MDR: multidrug-resistant; EMB: ethambutol.

Table 2. Primers for PCR amplification and DNA sequencing co-ordinated according to *Mycobacterim tuberculosis* H37Rv (AL123456.3)

Target	Primer name	Primer sequence (5' → 3')	PCR product (bp)	Nucleotide position	Ta (°C)	Reference
PCR amplification and sequencing						
rpsL	S13	GGCCGACAAACAGAACGT	504	781,514–782,017	50	Meier et al.[17]
	S16	GTTCACCAACTGGGTGAC				
rrs	285	GAGAGTTTGATCCTGGCTCAG	1,589	1,471,855–1,473,443	64	Springer et al.[18]
	rrs-1R	ACAGACAAGAACCCCTCACG				Via et al.[19]
	264*	TGCACACAGGCCACAAGGGA				Springer et al.[18]
gidB	gidB3	GAACGGAAGATCGTCCAC	977	4,407,459–4,408,435	58	Villellas et al.[11]
	gidB4	CGATAGTTGAAGCCTGGC				
whiB7	F UTR whiB7	GCTGGTTCGCGGTCGGACCT	810	3,568,321–3,569,130	60	Sowajassatakul et al.[20]
	R-whiB7	AGGAGCTGATCCCGGGTTTC				This study
MAMA-PCR						
rrs	*rrs*-16 wt	TGGGTTTTGTTTGGAGAG**TT**	233	1,471,842–1,472,074	62	This study
	rrs-16 mt	TGGGTTTTGTTTGGAGAG**CC**			60	This study
	rrs-RM	CATCCCACACCGCTAAAG				This study
DTM-PCR						
RD105	P1	GGAGTCGTTGAGGGTGTTCATCAGCTCAGTC	4,253	78,876–83,128 (P1-P2)	68	Chen et al.[21]
	P2	CGCCAAGGCCGCATAGTCACGGTCG				
	P3	GGTTGCCCACTGGTCGATATGGTGGACTT	1,495	78,876–80,370 (P1-P3)		

*Primer used for sequencing only.
PCR: polymerase chain reaction; Ta: annealing temperature; MAMA-PCR: mismatched amplification mutation assay PCR; DTM-PCR: deletion-targeted multiplex PCR.

Table 3. Mutations found in ***rpsL*, *gidB*, and *whiB7*** of 101 ***Mycobacterium tuberculosis*** clinical isolates

Resistance pattern (No. of isolates)	No. of isolates with LSP-based lineages				Mutations in nucleotides position (amino acid position)		
	East Asian	Indo-Oceanic	Euro-American	Unknown	*rpsL*	*gidB**	*whiB7*[†]
SMR (46)							
SM+MDR or INH or RIF resistance (26)	15	1[‡]	-	-	128A>G (Lys43Arg)	276A>C (Glu92Asp), 615A>G	ND
	-	-	1	-	128A>G (Lys43Arg)	–[§]	ND
	1	-	-	-	263A>G (Lys88Arg)	276A>C (Glu92Asp), 615A>G	ND
	-	-	1	-	263A>C (Lys88Thr)	206G>A (Gly69Asp)[‖]	ND
	1	-	-	-	–[§]	276A>C (Glu92Asp), 615A>G	242G>A (Arg81His), 246_247insTT (Arg83fs)
	-	3	-	-	–[§]	330G>T, 615A>G	188delG (Gly64fs)
	-	1[‡]	-	-	–[§]	276A>C (Glu92Asp), 615A>G	–[§]
	-	1	-	-	–[§]	330G>T, 615A>G	–[§]
	-	1	-	-	–[§]	330G>T[¶], 615A>G	–[§]
SM mono-resistance (20)	14	-	-	-	128A>G (Lys43Arg)	276A>C (Glu92Asp), 615A>G	ND
	-	1	-	-	128A>G (Lys43Arg)[¶]	276A>C (Glu92Asp)[¶], 330G>T[¶], 615A>G	ND
	-	-	1	-	–[§]	134G>A (Trp45Ter)[‖]	–[§]
	1	-	-	-	–[§]	276A>C (Glu92Asp), 615A>G	–[§]
	-	1	-	-	–[§]	330G>T, 615A>G	–[§]
	-	1	-	-	–[§]	615A>G	–[§]
	-	1	-	-	–[§]	–[§]	–[§]
SM susceptible (55)							
MDR or INH or RIF resistance (35)	15	-	-	-	–[§]	276A>C (Glu92Asp), 615A>G	ND
	-	5	-	-	–[§]	330G>T, 423G>A, 615A>G	ND
	1[‡]	9	-	1	–[§]	330G>T, 615A>G	ND
	-	1	-	-	–[§]	615A>G	ND
	-	-	3	-	–[§]	–[§]	ND
Susceptible to INH, RIF, SM and EMB (20)	2	-	-	-	128A>G (Lys43Arg)	276A>C (Glu92Asp), 615A>G	ND
	6	-	-	-	–[§]	276A>C (Glu92Asp), 615A>G	ND
	1[‡]	7**	-	-	–[§]	330G>T, 615A>G	ND
	-	1	3	-	–[§]	–[§]	ND
Total No. of isolates	57	34	9	1			

*Only nucleotide position substitution is shown for synonymous mutation. [†]Twelve samples carrying no *rpsL* and *rrs* were sequenced for *whiB7* gene. [‡]Isolates with discordance between spoligotyping and *gidB* lineage markers. [§]Wild type sequence. [‖]SMR-associated *gidB* mutation. [¶]Isolate with mixed population of wild type and mutated sequences.
**One isolate carried 1025u>c *rrs* is included.
LSP: large sequence polymorphism; SMR: streptomycin resistance; SM: streptomycin; MDR: multidrug-resistant; INH: isoniazid; RIF: rifampicin; ND: not determined; EMB: ethambutol.

non-Beijing isolates carrying intact RD105 yielded approximately 1,495 bp amplicons while the Beijing isolates (including ancestral, modern, and W family of Beijing lineage) containing a 3,467 bp deletion of RD105 region yielded only 785-bp DTM-PCR product.

5. Statistical analysis

The association of identified polymorphisms to SMR and *M. tuberculosis* lineages was assessed with GraphPad Prism 6 software (Graphpad Software Inc., La Jolla, CA, USA). The p-value (<0.05) was calculated by Fisher exact test. Odds ratio (OR), 95% confidence intervals (CI), sensitivity, and specificity were also calculated.

Results

1. SMR associated genes' mutations and their relationship to SMR

The entire length of *rpsL* and *gidB*, and 1,528 bp of *rrs* were amplified and sequenced from genomic DNA of 101 *M. tuberculosis* isolates. As shown in Table 3, the majority of SM resistant isolates, 32/46 (69.6%), carried *rpsL* 128A>G transition (Lys43Arg). Among them, mixed *rpsL* WT and 128A>G sequences were found in a SM mono-resistant EAI2-Nonthaburi isolate. Other two *rpsL* missense mutations, i.e., 263A>G (Lys88Arg) and 263A>C (Lys88Thr), were identified in two SM resistant MDR isolates. Lys43Arg was the most predominant *rpsL* mutation (94.1%) among SMR isolates in this study. However, we found 2/55 SM susceptible isolates carrying Lys43Arg.

Surprisingly, none of *rrs* mutation related to SMR was present in *M. tuberculosis* isolates from our collection. Only a single *rrs* SNP, 1025u>c, was found in a SM susceptible isolate (Table 3). Further examination of the previously found 16T>C SMR related *rrs* mutation by MAMA-PCR newly developed in this study indicated that none of our isolates carried that respective mutation (Figure 1).

Six different types of *gidB* SNPs were identified. Among them, two SNPs were exclusively found in two Euro-American isolates with SMR. The 134G>A (Trp45Ter) was carried by a SM mono-resistant isolate lacking *rpsL* mutation, whereas, the 206G>A (Gly69Asp), a novel *gidB* mutation identified in this study, was found in a SM resistant MDR isolate carrying Lys88Thr *rpsL* (Table 3). Other four *gidB* SNPs as presented in Table 3 were unlikely to associate with SMR because they were identified in both SM resistant and susceptible isolates.

The nucleotide sequence of the entire *whiB7* ORF and its promoter region were further analyzed in 12 SM resistant isolates (26%) lacking of SMR-associated *rpsL* and *rrs* mutations. Two SNPs in the ORF region, i.e. 242G>A (Arg81His) and 246_247insTT (Arg83fs), were found in a SM/INH resistant East Asian isolate (Tables 1, 3). A frameshift mutation (188delG) was present in three Indo-Oceanic (EAI2-Nonthaburi) isolates (Tables 1, 3). BLAST analysis revealed that their deduced amino acid sequences were identical to 'disrupted' efflux pump activator from EAI2-Manila lineage *M. tuberculosis*[25]. Thus, 188delG mutation was unlikely to relate to SMR.

Among three SMR-associated genes, *rpsL* mutations were highly associated to SMR (73.9%) (p<0.001). The combined detection of three *rpsL* and two *gidB* mutations provided 76.1% sensitivity and 96.4% specificity for identification of SMR in Thai *M. tuberculosis* isolates. Similar to another Thai study[8], SMR was strongly associated to East Asian (Beijing lineage) (32/46 isolates, 69.6%) (OR, 2.7; 95% CI, 1.2–6.2; p=0.017) (Table 3). Moreover, *rpsL* mutations were predominant among SM resistant Beijing isolates (93.8%) (OR, 37.5; 95% CI, 5.9–236.6; p<0.001) while their presence was significantly lower among non-Beijing lineages (28.6%). However, in this study, no significant correlation between SMR or SMR-associated mutations and MDR phenotypes was found (p=0.859 and p=0.112, respectively). Furthermore, there was no relationship between the MDR isolates and lineage of *M. tuberculosis* in our collection (data not shown).

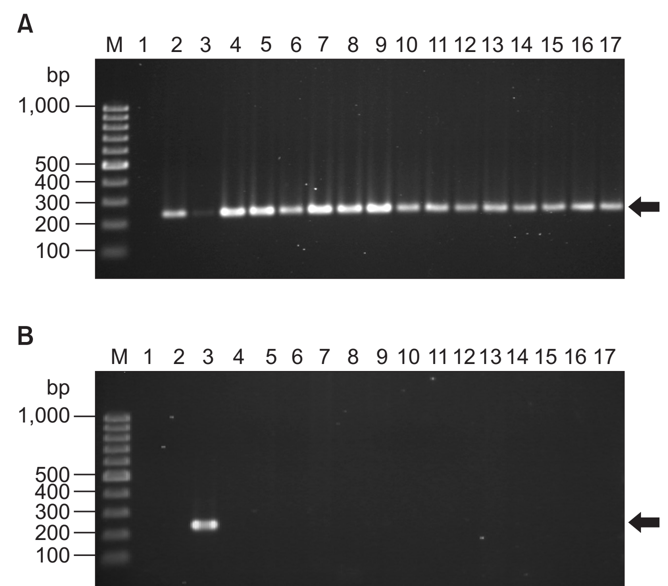

Figure 1. Representative mismatched amplification mutation assay polymerase chain reaction amplicons for detection of 16u>c *rrs* mutation in 14 *Mycobacterium tuberculosis* isolates. (A) The 233-bp wild type *rrs* allele amplicons obtained from *rrs*-16 wt and *rrs*-RM primers (arrow). (B) The 233 bp-16u>c *rrs* mutant allele amplicons obtained from *rrs*-16 mt and *rrs*-RM primers (arrow). Numbers on the left are DNA sizes (bp). Lane M: GeneRuler 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania); lane 1: no template control; lane 2: *rrs* wild type allele control (H37Rv); lane 3: *rrs*-16u>c mutant allele control; lane 4–17: clinical *M. tuberculosis* isolates.

2. Polymorphisms of *gidB* and *whiB7* as phylogenetic signatures for *M. tuberculosis* lineage

Regardless of SMR, 615A>G *gidB* was found in all East Asian and 32/34 Indo-Oceanic isolates in our study (Table 3). Whereas, the 276A>C (Glu92Asp) *gidB*, a known marker for the Beijing isolates[6,8], presented in 55/57 spoligotyped-Beijing (East Asian) and 2/34 EAI5 (Indo-Oceanic) isolates (Tables 1, 3). The discrepancies were further clarified by DTM-PCR, and it was found that the 55 Beijing and 2 EAI5 isolates with Glu92Asp carried RD105 deletion specific for Beijing lineage (Figure 2). In contrast, one of two spoligotyped-Beijing isolates lacking Glu92Asp carried the intact RD105 region while another carried mixed intact and deleted RD105 genotype (Figure 2).

In addition to 615A>G *gidB*, the 26/29 Indo-Oceanic (EAI) isolates in this study also carried 330G>T *gidB* silent mutation (Tables 1, 3). Interestingly, the two spoligotyped-Beijing isolates lacking Glu92Asp SNP but with intact RD105 region mentioned above, also carried the EAI marker, 330G>T *gidB*. Five of the Indo-Oceanic isolates, all exclusively belonging to EAI6-BGD1 sub-lineage, carried triple silent mutations (423G>A, 330G>T, and 615A>G) (Tables 1, 3), demonstrating the potentiality of 423G>A *gidB* SNP as an additional novel marker for EAI sub-lineage classification. Moreover, a *whiB7* frameshift mutation (188delG), unlinking to SMR, appeared to be present exclusively in the three Indo-Oceanic sub-lineage EAI2-Nonthaburi isolates[25].

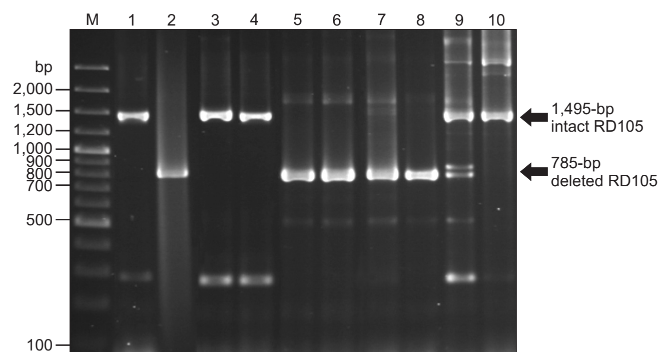

Figure 2. RD105 deletion-targeted multiplex polymerase chain reaction (DTM-PCR) amplicons of clinical *Mycobacterium tuberculosis* isolates. The arrows indicate RD105 DTM-PCR amplicons of intact RD105 (1,495 bp) and deleted RD105 (785 bp) regions, respectively. Lane M: GeneRuler 100 bp Plus DNA ladder; lane 1: H37Rv *M. tuberculosis* (non-Beijing lineage control); lane 2: *M. tuberculosis* isolate No. 19 (Beijing lineage control); lane 3–4: spoligotyped-non-Beijing lineage isolates; lane 5–6: spoligotyped-Beijing lineage isolates; lane 7–8: two spoligotyped-EAI5 isolates carrying deleted RD105 region; lane 9: spoligotyped-Beijing lineage isolates carrying both intact and deleted RD105 regions; lane 10: DTM-PCR amplicon of a spoligotyped-Beijing lineage isolate carrying intact RD105 region.

Discussion

The amino acid lysine at codons 43 and 88 are the key SM binding sites on the ribosomal protein S12 coded by *rpsL*[26]. Therefore, any substitution to lysine such as Lys88Arg (or Lys43Arg or Lys88Thr) would ultimately result in SMR of bacteria including *M. tuberculosis* as a consequence of the reduction in SM binding activities. The majority of SMR in *M. tuberculosis* isolates in this study was contributed by the *rpsL* mutations (73.9%). Although, this mutation frequency was higher than that of the average worldwide frequency (52%–59%)[4] and a single previous report from Thailand (63.6%)[8]. However, it was still lower than that from China, Vietnam, and Singapore[7,27,28]. Similar to those reported from South East Asian countries, the most predominant *rpsL* mutation associated to SMR in this study was Lys43Arg (69.6%)[8,27,28]. As the mutation in either codon 43 or 88 of *rpsL* had a strong connection to high-level SMR[3], the majority of SM resistant *M. tuberculosis* isolates in our collection would possess the same level of SMR. Furthermore, the mutation in these two codons of *rpsL* markedly predominated among Beijing isolates of our study (93.8%)[27,28].

It was found that 2/55 SM susceptible Beijing isolates carried Lys43Arg mutation. Previously, the genotype-phenotype discrepancy of SMR was verified by performing DST on *M. tuberculosis* isolates collected from the subsequent follow-up sputum samples of the same patients and it was then proved that the newly collected isolates had become SMR[29]. These data suggested the significance of re-performing DST on the isolates from the follow-up sample of a patient who was originally infected with SM susceptible *M. tuberculosis* carrying Lys43Arg *rpsL* that might exhibit the SM hetero-resistant phenotype. However, the follow-up samples were not available in this study.

Surprisingly, none of SMR-related *rrs* mutations was present in *M. tuberculosis* isolates from our collection although the only previous study of *M. tuberculosis* Thai isolates collected from the different time and region revealed that 8.2% carried SMR-associated mutations in 530 and 900 regions of *rrs*[8]. The prevalence of *rrs* mutations responsible for SMR in this bacterium might be geographically dependent having with 0% for India and Sierra Leone[30,31], and less than 10% in East and South East Asian countries[7,8,27,28,32]. While, European and Latin American countries reported the higher prevalence (13%–28%)[6,33-35]. Although our sample size was smaller than that of the previous study from Thailand, the proportions of isolates with SMR (16% compared to 10%) and *rpsL* mutations-associated SMR (73.9% compared to 63.6%) was apparently higher[8]. Hence, it was possible that the absence of SMR-related *rrs* mutations was an outcome of the upsurge in SMR-related *rpsL* mutations. Moreover, our data implied the possibility that the genetic background of SM resistant *M. tuberculosis* isolates collected from the different period and

regions of Thailand might be substantially diverged.

Only two of six *gidB* mutations, Trp45Ter and Gly69Asp were likely to be responsible for SMR and exclusively found in Euro-American isolates. This finding coincided with the suggestion of the previous study that the isolates harbored only SMR-associated *gidB* mutations without those of *rpsL* and *rrs* were non-Beijing[8]. However, currently, the contribution of a novel *gidB* mutation found in our study, Gly69Asp, to SMR was unclear as this isolate also carried Lys88Thr *rpsL*.

For 12 SM resistant isolates missing of *rpsL* and *rrs* SMR-related mutations, three mutations, i.e., Arg81His, Arg83fs, and Gly64fs were found in the ORF of efflux pump activator, *whiB7*. Thus, they would contribute to the SM hyper-susceptibility rather than the SMR phenotype[9]. As a consequence, after excluding the *gidB* Trp45Ter mutant, the genetic constituents responsible for the SMR in 11 SM resistant isolates (24%) in the present study remained unknown. As a frameshift mutation, Gly64fs (188delG) was present in all three *whiB7*-sequenced Indo-Oceanic (EAI2-Nonthaburi) isolates and the EAI2-Manila sublineage from the Genbank database[25], the availability of this mutation as sub-lineage marker required further elucidation.

We found that SMR and its associated *rpsL* mutations were strongly linked to the Beijing lineage ($p=0.017$ and $p<0.001$, respectively), whereas there was no relationship between MDR isolates and the SMR or Beijing isolates. Therefore, the WHO recommendation for incorporation of SM as a standard regimen for retreatment and MDR-TB cases in this region would remain fully effective as long as the SMR-associated mutations and Beijing lineage differentiation were performed as a prerequisite.

Among four SMR-unrelated *gidB* SNPs identified, the Glu92Asp was the well characterized Beijing lineage-specific marker[5,6,8]. Moreover, the presence or absence of Glu92Asp and the carrying of RD105 deletion or intact types were better correlated than the spoligotying. It had been suggested that the phylogenetic accuracy of direct repeats (DRs) locus genotyping methods such as spoligotyping and MIRU-VNTR exhibited lesser discriminatory power for lineage classification compared to the gold standard DNA sequencing[36]. Accordingly, SNPs which bear almost no homoplasy have become more dependable genetic markers for phylogenetic differentiation in *M. tuberculosis*. Therefore, either the RD105 deletion in the same fashion as Beijing genotype in the EAI strains or retaining of intact RD105 in Beijing strains strongly suggested the presence of the convergent evolution between Beijing and EAI *M. tuberculosis* Thai isolates.

Furthermore, the 26/29 Indo-Oceanic isolates in our collection were EAI isolates and carried 330G>T *gidB* silent mutation, highlighting its specificity as a phylogenetic marker for EAI strains[8,31]. Interestingly, all five EAI6-BGD1 SpolDB4 family isolates belonging to Indo-Oceanic lineage carried triple silent mutations (330G>T, 423G>A, and 615A>G), demonstrating the potential of 423G>A *gidB* as a novel marker for EAI sub-lineage classification. Although the presence of this mutation was previously shown in 6/31 EAI *M. tuberculosis* Thai isolates, their association to the SpolDB4 family sub-lineage was not made[8]. However, its applicability as the EAI sub-lineage identification required further studies on the larger population of *M. tuberculosis* isolates.

In summary, we investigated the sequences of *rpsL*, *rrs,* and *gidB* in 101 *M. tuberculosis* Thai isolates to identify the mutations conferring SMR. The three *rpsL* SMR-associated mutations were found in 73.9% of SM resistant isolates suggesting that they possessed a high-level of SMR. Further analysis of the *whiB7* sequence in the SM resistant isolates lacking *rpsL* and *rrs* mutation revealed only the SNPs within *whiB7* ORF which could not confer intrinsic SMR. The present study confirmed the usefulness of four *gidB* mutations as a lineage phylogenetic marker for East Asian (Beijing), Indo-Oceanic and Euro-American. Moreover, 423G>A was identified in this study as a new putative surrogate makers for EAI subclade (EAI6-BGD1).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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