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Characterization of streptomycin and pyrazinamide resistance in clinical multidrug-resistant *Mycobacterium tuberculosis* isolates from Myanmar

(ミャンマー由来臨床分離多剤耐性結核菌株のストレプトマイシン並びにピラジナミド耐性に関する研究)

NAN AYE THIDA OO

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ABBREVIATIONS

TB	Tuberculosis
MTB	*Mycobacteria tuberculosis*
WHO	World Health Organization
DNA	Deoxyribonucleic acid
WT	Wild type
STR	Streptomycin
PZA	Pyrazinamide
PZA-R	PZA resistance
INH	Isoniazid
RIF	Rifampicin
EMB	Ethambutol
pncA	Gene encoding pyrazinamidase
rpsL	Gene encoding ribosomal protein S12
rrs	Gene encoding 16s ribosomal RNA
gidB	Gene encoding m7G methyl transferase
katG	Gene encoding catalase-peroxidase
inhA	Gene encoding Enoyl-acyl carrier protein reductase
rpoB	Gene encoding RNA polymerase β-subunit
PZA-S	PZA susceptible
DST	Drug Susceptibility Testing
FLD	First-line drugs
HR^r	Phenotyping resistance to isoniazid and rifampicin
HRS^r	Phenotyping resistance to isoniazid, rifampicin and streptomycin

HRE^r	Phenotyping resistance to isoniazid, rifampicin and ethambutol
$HRSE^r$	Phenotyping resistance to isoniazid, rifampicin, streptomycin and ethambutol
Indel	Insertion or deletion of nucleotide
RR-TB	Rifampicin resistant tuberculosis
LSP	Large Sequence Polymorphism
MDR-TB	Multi-drug resistant tuberculosis
XDR-TB	Extensively-drug resistant tuberculosis
DOTS	Direct Observed Therapy, Short-course
NTRL	National TB Reference Laboratory
Cat II	Previously treated cases
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeat

PREFACE

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains one of the leading causes of morbidity and mortality worldwide, especially in developing countries [1]. Although, effective therapy for TB is available, almost 10.4 million new cases, of which about 45% were people from WHO South-East Asia Region (Fig 1) and 1.7 million deaths in 2016.

Susceptible TB can be treated with first-line drugs consisting of a 2-month 'intensive' phase, where four drugs combination of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) are administered, followed by a 4-month 'continuation' phase (INH and RIF) [2].

Control of TB has become more challenging with the emergence of drug resistance, multidrug-resistant TB (MDR-TB), which is defined as TB resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs, and extensively drug resistant TB (XDR-TB) which is MDR-TB strains that have developed resistance to any fluoroquinolones and one of the injectable aminoglycosides used in anti-TB treatment, compromise the control of TB globally [1]. Every year, the emergence of estimated 480,000 new MDR-TB cases is threatening to end TB strategies in 2030. Precisely, an estimated 4.1% of new cases and 19% of previously treated cases had MDR/RR-TB (rifampicin resistant TB) worldwide [1].

Among the 30 high TB burden countries, Myanmar is the tenth-highest TB prevalence rate, 361 cases with 47 deaths per 100,000 populations (Fig 1) comprising the emergence of 180,000 new TB cases in each year, along with 9,000 MDR-TB cases. MDR-TB is a result of inadequate treatment of patients infected with drug-sensitive strains of TB and subsequent transmission of these strains.

As Myanmar is one of the developing countries, limited capacity to diagnose and manage drug resistance is more likely to promote the increasing rate of drug-resistant TB. In addition, XDR-TB has also been reported [1].

According to the second nationwide survey that carried out in 2008, the proportion of MDR-TB was 3.9% among the new cases and 10.0% among the previously treated cases [2]. In 2016, MDR-TB prevalence has been reported to be 5.1% in the new cases and 27.0% in previously treated cases which is seen to be similar with to other neighboring countries of Thailand (24%) and China (26%) (Fig 2-3) [1].

Not only that spread of drug-resistant TB but also increased rates of MDR-TB pose serious threats to TB-control programs around the world [1]. Although Myanmar adopted the Directly Observed Treatment-Short Course (DOTS) strategy since 1980 according to (WHO), TB is still included in one of the major public health problems in Myanmar. From 1999 to 2009, there are increasing rate of new TB cases and previously treated cases which can be considered as rate of MDR-TB in those cases is increasing from time to time (Fig 4).

Rapid detection is essential to treat patients with drug-resistant TB. However, a gold standard of drug susceptibility testing (DST) of culture-based methods takes several weeks, leading to delay in diagnosis and treatment of drug-resistance to TB. Consequently, molecular DST using the Hain GenoType MTBDRplus v.2.0 (Hain Life science GmbH, Nehren, Germany) as well as the Cepheid GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) has been established in Myanmar since 2014 [4]. However, these tools are only setup in the limited reference TB laboratories and can only detect the most frequent resistance-associated mutations for certain anti-TB drugs. Therefore, DNA sequencing for the detection of drug resistance-conferring mutation for specific genes has the potential to overcome the limitation and can be used for effective and early identification of patients with drug-resistant TB. In addition, the duration of treatment for drug-resistant TB is prolonged and high cost, with poor outcome and severe side effects [84]. Poor outcome predisposes to poor compliance, which results in the emergence of resistance [86]. Therefore, it is important to establish the rapid and easy accessible diagnostic tools for drug-resistant TB to improve TB control especially in countries with poor resources with high TB burden.

Therefore, DNA sequencing of the target gene conferring drug resistance has been performed to rapidly differentiate DR-TB for early and effective treatment and preventing the transmission of drug-resistant TB among patients.

There are limited studies on determination of MDR-TB isolates in Myanmar by both phenotypic and genotypic, and compromise the gap between the diagnosis and the treatment outcome. As death due to TB could be prevented with early diagnosis and appropriate treatment, this study was addressed to inform frequency and patterns in drug resistance-conferring mutation which can contribute in diagnosis, management, and policies for the prevention of MDR-TB in Myanmar.

This thesis is consisted of two chapters. In the chapter I, I have discussed about the characterization of the molecular mechanism of STR-resistance *M. tuberculosis* isolates in Myanmar. I also explored the variation and frequency of mutations in genes conferring STR resistance, compared them with those in isolates from other countries, and studied their association with different MTB genotypes. In chapter II, I have described the characterization of pyrazinamide resistance-associated mutation among MDR-TB isolates in Myanmar.

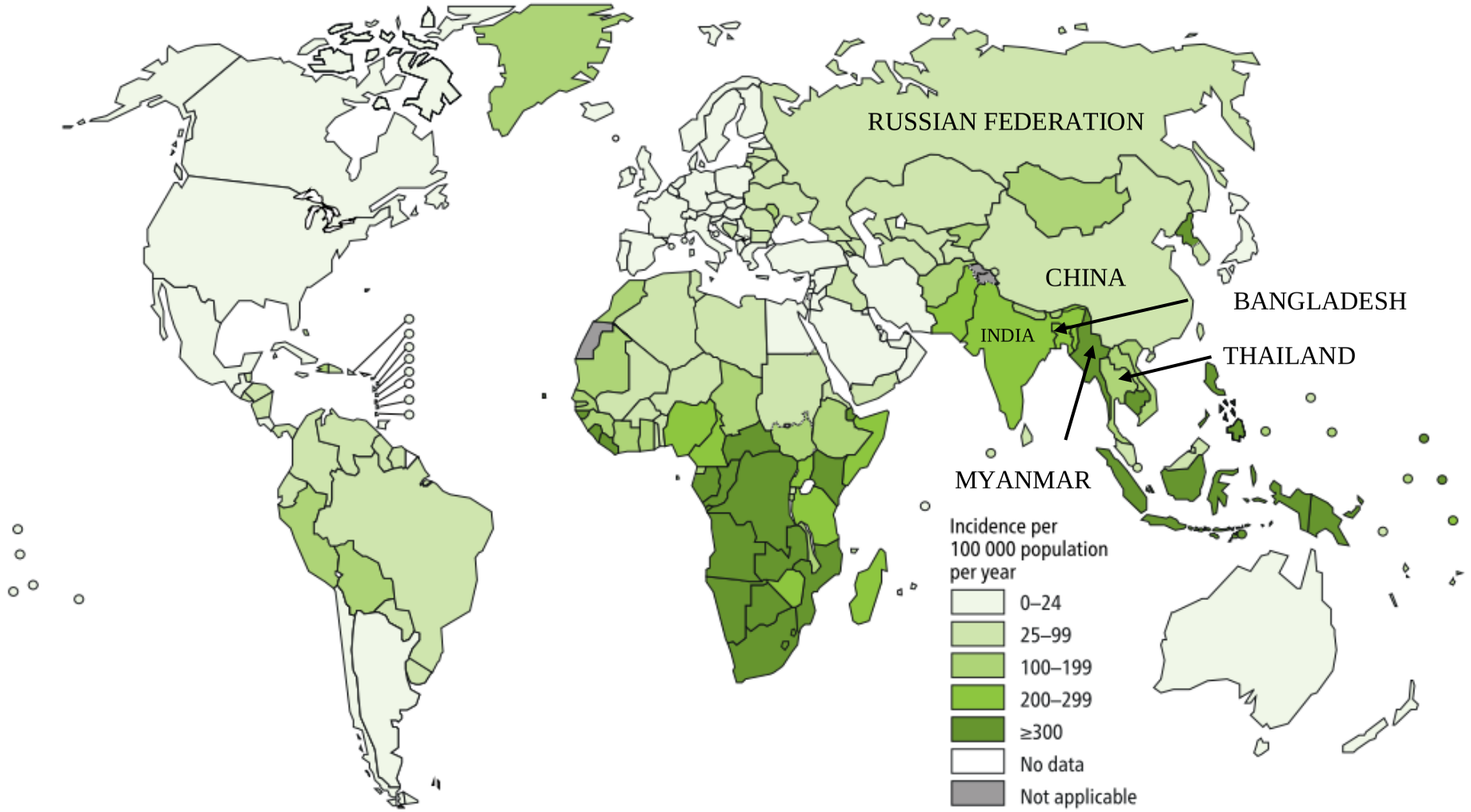

Fig 1. The estimated TB incidence rate at the country level, 2016 [1].

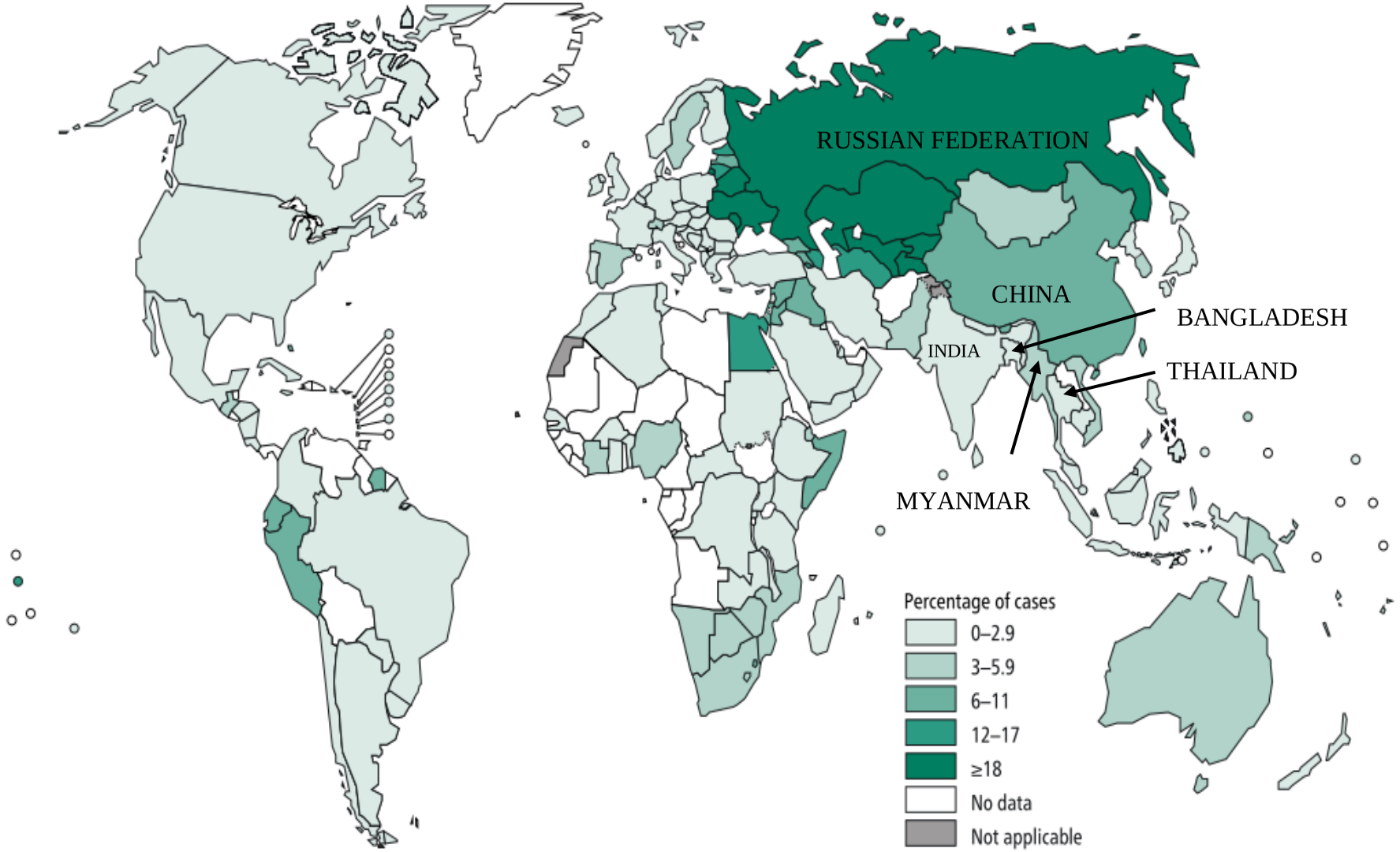

Fig 2. Percentage of new TB cases with MDR-TB/ RR cases, 2016.
[1]

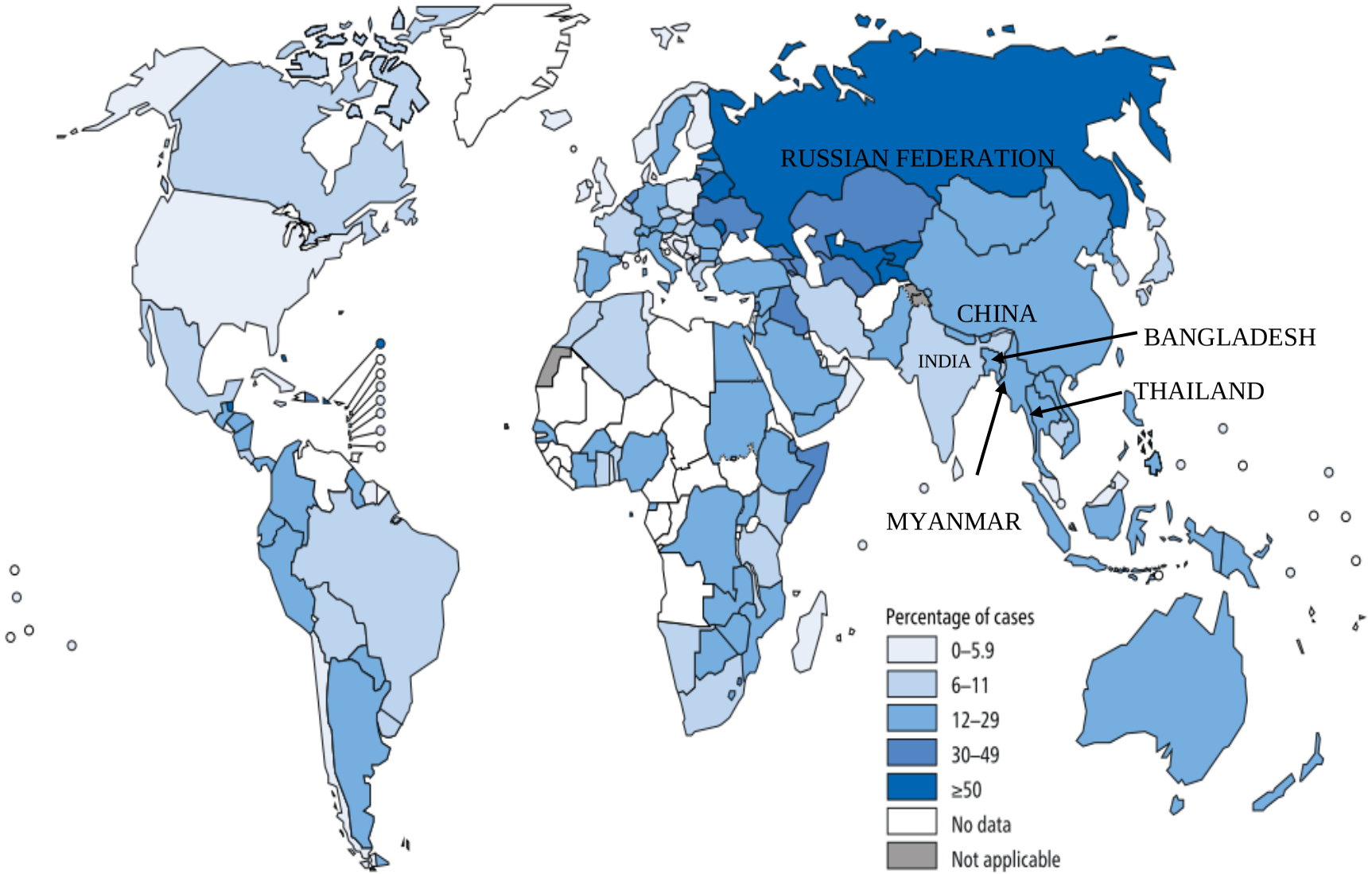

Fig 3. Percentage of previously treated TB cases with MDR-TB/ RR cases
[1]

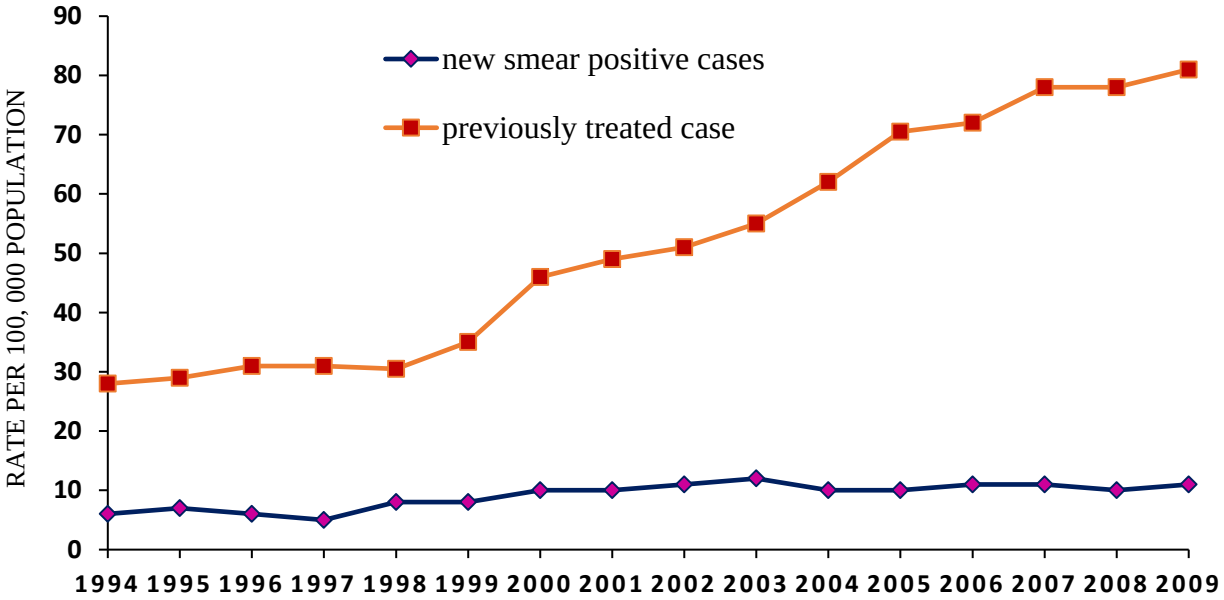

Fig 4. Tuberculosis case notification rate (new smear-positive cases and previously treated cases) per 100,000 populations in Myanmar, 1994-2009 [4].

Chapter 1

Characterization of mutations conferring streptomycin resistance to multidrug-resistant *Mycobacterium tuberculosis* isolates from Myanmar

Introduction

STR was the first antibiotic used to treat TB. Nonetheless, STR was removed as a first-line anti-TB drug due to 1) it caused a high rate of side effects, and 2) its addition as a single drug to an unsuccessful regimen risked developing drug resistance in TB [2]. An eventual development of STR resistance in TB would have led to the exclusion of STR from the group of first-line injectable agents for the Cat II retreatment regimen for all patients with a prior history of TB treatment in most developed countries [2]. However, STR was recommended by WHO for TB re-treatment [2] and MDR-TB cases [3]. In Myanmar STR has been used for years for re-treatment without conducting any DST [4], and this practice has continued despite the latest WHO guidelines [2] recommending to perform DST for regimens to be used for retreatment cases. Although DST focuses mainly on four anti-TB drugs, namely rifampicin (RIF), isoniazid (INH), STR and ethambutol (EMB), it is only conducted when the subjects are high-risk MDR-TB patients [4]. Furthermore, with an increment of MDR-TB cases in many countries including Myanmar, the use of STR to treat drug resistant TB has also increased [3]. A previous study on drug-resistant MTB in Myanmar detected resistance to STR in 25.1% of all TB cases [5]. Similarly, TB resistance to INH (23.0%) and RIF (18.3%) has been reported [5]. In MDR-TB cases, resistance to STR was reported to be 91.4% (32/35) in 2015 [5] and 92.8% (13/14) in 2016 [6]. The 4th global report on anti-tuberculosis drug resistance showed that 20% of new cases had resistance to at least one of the first line anti-TB drugs, of which 5.3% were MDR-TB and 11% had resistance to STR [7]. Thus, as in the case of resistance

to other important anti-TB drugs (INH, RIF, and PZA) [5, 6], understanding the molecular mechanism of resistance to STR in Myanmar is critical.

STR binds directly to the 16S ribosomal RNA (rRNA) in the small subunits of ribosome and inhibits protein synthesis. Mutations in genes *rrs* (encoding 16S rRNA) [8], *rpsL* (encoding the S12 ribosomal protein) [8-10], and *gidB* (encoding ribosome methyltransferase) [11] have been found to be strongly associated with resistance to STR. Amino acid substitutions in the S12 protein affect STR binding by disturbing the high conserved structure of 16S rRNA. Changes in the 16S rRNA cause lower affinity for STR. In particular, *rrs* mutation in 530 loop or 912 region and mutation in *rpsL* codon 43 or 88 have been associated with high-level or intermediate STR resistance in MTB [12]. Although almost 70% of all STR-resistant clinical isolates harbor the mutation either in *rpsL* or *rrs*, there are some clinical isolates with no mutation in these genes [13, 18]. GidB functions as an S-adenosylmethionine-dependent (SAM), 16S rRNA methyltransferase which methylate the nucleoside position 518 of the 16S rRNA to which SM directly interacts and which also located the 530 stem-loop pseudoknot region of the 16S rRNA site. Mutation in *gidB* may likely alter GidB's methyltransferase function which then changes the methylation status of the 16S rRNA at position 518G and consequently present with STR-R phenotype in clinical isolates. In contrast to *rpsL* or *rrs*, *gidB* polymorphisms have been reported as associated with low-level resistance to STR.

Moreover, the type and frequency of STR resistance-conferring mutations have been shown to vary according to the geographical region [11, 13-24]. For example, while mutations in *rpsL* were relatively higher in Asia [13, 17, 20-21], where Lys43Arg (K43R) mutation was found to be associated with the MTB Beijing genotype [17, 20, 21], mutations in *rrs* gene were relatively higher in Europe and the America [19, 22]. In contrast, *gidB* mutations have been found to be associated with either STR resistance or specific genotypes of MTB [11, 17].

Despite the high number of MDR-TB cases and evidence for the presence of STR-resistant MTB strains, no information on the molecular mechanism of STR resistance in Myanmar is currently available. Indeed, the only available gold standard DST for STR is based on the time-

consuming bacterial culture proportion method that exhibits low accuracy and poor reproducibility [25]. Although the WHO has endorsed BACTEC MGIT 960 (SIRE) for rapid diagnose of STR resistance, high contamination rate has been reported [3]. Nonetheless, in recent years, the molecular assay GenoType MTBDRplus (Hain Lifescience, Nehren, Germany) was developed for rapid detection of STR resistance-associated mutations [26]. Therefore, to take advantage of recent developments in drug resistance detection to further our understanding of the underlying molecular mechanism of STR-resistance would be an important first step to develop strategies to conduct rapid STR-DST and suggest effective TB treatments. Additionally, as the association between specific mutations (*rpsL*, *rrs* and *gidB*) in STR resistance have been recognized as highly-specific predictive markers of STR resistance, it is important to investigate the prevalence of each specific mutation among our isolates.

Among several genotyping methods to identity family or lineage, spoligotyping and Large Sequence Polymorphisms (LSPs) have been widely used. Spoligotyping is used to detect the presence or absence of spacer sequences by the PCR-based reverse-hybridization blotting technique and can differentiate MTB strains into distinct families such as Beijing, East African-Indian (EAI), Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), European family X, and family T. In contrast, LSP is PCR based method and used to detect genomic polymorphism based on large sequence deletions and known as regions of difference (RDs). Depend on region of deletion, Beijing, EAI, CAS and LAM family can be differentiated (30).

For the chapter I, I collected 141 STR-resistant MDR-TB isolates to characterize the molecular mechanism of STR-resistance in Myanmar. I explored the variation and frequency of mutations in genes *rpsL*, *rrs* and *gidB* and compared them with findings from other countries. Moreover, certain *M. tuberculosis* genotypes have been reported to be more frequently associated with STR resistance than other strain types [20, 21], MTB genotypes among the MDR-TB isolated were also analyzed to detect the association between the specific types of mutations with different MTB genotypes.

Materials and Methods

Sample collection and DST

From the MDR-TB isolates collected between 2009 and 2013 at a national TB reference laboratory in Yangon, a total of 141 MDR-TB STR-resistant isolates were randomly selected for the present study. The reference laboratory in Yangon was the only facility to perform DST in Myanmar during the present study was conducted.

Using anti-TB drugs INH, RIF, EMB and STR at the optimal concentrations of 0.2, 40, 2 and 4 ug/ml, respectively [27], DST was carried out by the standard Löwenstein–Jensen (LJ) medium proportion method. Briefly, from a freshly grown colony, a 1.0 McFarland standard mycobacterial suspension was prepared by serially diluting to four different concentrations (10^{-1} to 10^{-4}). Next, the suspension was inoculated onto LJ slants with and without drugs, and incubated at 37 °C until interpretation of results was completed at days 28 and 42. An isolate was determined to be resistant to a drug if it had ≥1% colony growth on drug-containing medium when compared with a control isolate.

DNA extraction

DNA was extracted from MTB colonies grown on LJ medium by the enzymatic lysis method [28]. Purified DNA was dissolved in TE buffer (10 mM Tris –HCl, 1 mM EDTA, pH 8.0), quantified by a Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) and stored at -20 ºC until further use.

Genotyping by spoligotyping and large sequence polymorphism (LSP)

All MTB isolates were genotyped by spoligotyping, as previously described [29]. Briefly, a primer set was used to amplify the direct repeat region, and the resulting PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes on the membrane. To determine

Spoligo-international types (SITs), hybridization patterns were converted into binary and octal formats. The resulting SITs were compared with the patterns previously reported in the SpolDB4 database provided by the Institute Pasteur de Guadeloupe (http:// www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) [30]. For those isolates considered to be undersigned or new spoligotypes, the genotype was determined by LSP using specific primers for the expected region of difference [31].

PCR amplification and DNA sequencing

461-bp fragment of gene *rpsL* and 595-bp fragment of gene *rrs* in MTB (including 530 and 912 regions) were amplified as previously described [32]. An 886-bp fragment of *gidB* was amplified as previously described [33]. The 20 µl reaction mixture consisted of 5X Green GoTaq® Reaction Buffer (Promega, CA, USA), 0.5 M betaine, 0.25 mM $MgCl_2$, dNTPs (0.25 mM each), primers (0.5 µM each), 1.25 U GoTaq® DNA Polymerase (Promega, CA, USA) and 1 µl of template DNA (approximately 10 ng/ul). The PCR was conducted in a thermal cycler (Takara Shuzo Co. Ltd, Shiga, Japan) with an initial denaturation at 95 ºC for 60 sec, followed by 35 cycles of amplification at 95 ºC for 10 sec, annealing at 50 ºC (*rpsL* and *rrs*) or 58 ºC (*gidB*) for 10 sec, an extension at 72 ºC for 30 sec, and a final extension at 72 ºC for 5 min. PCR products were sequenced using a BigDye ver. 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) in an ABI 3500xL Genetic Analyzer (Applied Biosystems, CA, USA). The sequencing results were analyzed with BioEdit version 7.05.3 [34] by aligning with wild-type sequences of the reference MTB H37Rv strain.

Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Appropriate Chi-square test or Fisher's exact test was used to determine associations between strain types and

specific mutations. Genotypes other than Beijing were determined as non-Beijing genotypes for statistical analysis. Data were considered statistically significant if the *P* value was <0.05.

Ethical approval

This study was approved by the Ethics Review Committee, Department of Medical Research, Yangon, Myanmar (15/Ethic, 2008).

Results

Drug resistance profile and *M. tuberculosis* genotypes

Ninety (63.8%) of 141 MDR-TB isolates with resistance to STR were also resistant to ethambutol (EMB). One hundred and five (74.5 %) of the isolates belonged to the Beijing genotype and the remaining 36 were considered as non-Beijing genotypes, including 23 (16.3 %) Indo-Oceanic/East African Indian (EAI) isolates and 13 (9.2 %) isolates of Euro-American genotype (including Latin-American Mediterranean family (LAM, 7 isolates) and (S, X2, and T1 sub-genotypes, 6 isolates) (Fig 5).

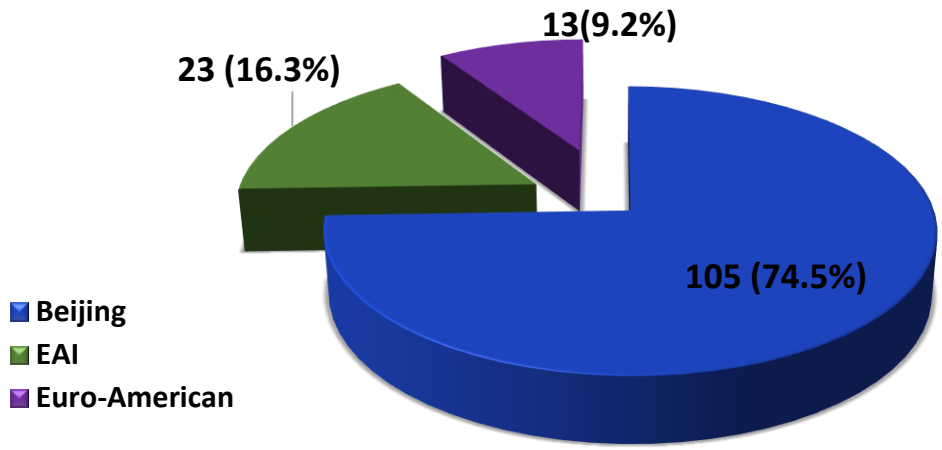

Fig 5. Distribution of genotypes in 141 MDR-TB isolates.

Mutations within *rpsL*

Mutations in *rpsL* were detected in 69.5% (98/141) of all STR-resistant MDR-TB isolates and 43 isolated harbored wild type sequences. Four different types of mutation, namely A128G causing amino acid substitution from Lysine to Arginine at codon 43 (Lys43Arg), A263G (Lys88Arg), A263T (Lys88Met) and A256G (Arg86Gly) were identified, of which A128G was the

most prevalent type (90/98, 92.0%) followed by A263G (6/98, 6.0%), A263T (1/98, 1.0%) and C256G (1/98, 1.0%) (Table 1).

Mutations within *rrs*

Mutations A514C, C517T and A905C were observed in five (3.5%) STR-resistant MDR-TB isolates and 136 showed no changes in the targeted *rrs* region (Table 1). While A514C mutation, frequent found mutant type, was found in three isolates, mutations C517T and C905A were each found in one isolate. All mutant strains carried a mutation in the 530 loop and 912 region of the secondary structure of MTB 16S rRNA. No *rrs* mutation was found in isolates with *rpsL* mutation, and vice versa.

Mutations within *gidB*

Twenty different types of mutation with polymorphisms were detected in gene *gidB* in STR-resistant MDR-TB isolates. Among them, 8 isolates were identified with four types of frameshift mutations (Gly33Fs, Gly71Fs, Val105Fs, Arg118Fs) causing by either a single nucleotide deletion (del) or insertion (ins). Fifteen isolates harbored 10 types of missense (Ser70Arg, Gly73Glu, Leu86His, Glu90Asp, Leu91Pro, Ala138Glu, Ala138Glu, Lys163Ala, His169Thr, Thr195His and Gly208Val) and one type of non-sense mutation (Glu170stop) (Table 1).

Hundred and five (74.5%) isolates showed A276C (Glu92Asp) missense mutation in *gidB* and of which 88 isolates showed co-mutation with Lys43Arg in *rpsL* (Table 2). A silent mutation of G330T (Val110Val) was found in 19 isolates, of which 13 isolates had an additional G423A (Ala141Ala) silent nucleotide substitution (Table 2). Hundred-four (73.7%) of the STR resistant isolates with *gidB* mutation had an additional mutation with either *rpsL* (99 isolates) or *rrs* (5 isolates) (Table 2). Furthermore, all 36 (25.5%) STR-resistant MDR-TB isolates with wild-type sequences on *rpsL* or *rrs* harbored a *gidB* mutation (data not shown).

Table 1. Distribution of mutations in each gene of *rpsL*, *rrs* and *gidB* in 141 STR-resistant *M. tuberculosis* isolates.

Genes	Mutation types		No. of isolates
	Nucleotide changes	Amino acid change	
rpsL	A128G	Lys43Arg	90
	A263G	Lys88Arg	6
	A263T	Lys88Met	1
	C256G[a]	Arg86Gly	1
	Wild-type		43
rrs	A514C		3
	C517T		1
	A905C		1
	Wild-type		136
*gidB**	G del 98[b]	Gly33Fs	5
	G del 211[a]	Gly71Fs	1
	A 208 C [c]	Ser70Arg	3
	G 218 A[a]	Gly73Glu	1
	T 257 A[a]	Leu86His	1
	T 269 G[a]	Glu90Asp	2
	T 272 C[d]	Leu91Pro	1
	T ins 314 [a]	Val105Fs	1
	G ins 352[e]	Arg118Fs	1
	C 413 A[f]	Ala138Glu	1
	A 487 T[a]	Lys163Ala	1
	C 502 G[a]	His169Thr	1
	G 508 T[a]	Glu170stop	1
	T 583 C[a]	Thr195His	2
	G 623 T[a]	Gly208Val	1
	Wild-type		118

*****= lineage-specific polymorphisms were not included. This table was made as according to mutation and wild type in each specific gene.

NM= No mutation, Fs= frameshift mutation, [a] =novel mutations

[b] = [19], [c] = [11], [d] = [15], [e]= [36], [f]= [15]

Table 2. Distribution of mutations in *rpsL, rrs* and *gidB* and different genotypes of 141 MDR-TB isolates

Mutations in			Genotypes			Total
rpsL [nucleotide, codon]	*rrs*	*gidB* [nucleotide, codon]	Beijing	EAI	Euro American	
A128G(Lys43Arg)		a	80			80
A263G (Lys88Arg)		a	5			5
A128G(Lys43Arg)		b			1	1
C256G(Lys86Gly)		d,e		1		1
A128G(Lys43Arg)		d,e		3		3
A128G(Lys43Arg)		C502G(His169Tyr),a	1			1
A128G(Lys43Arg)		G623T(Gly208Val),a	1			1
A128G(Lys43Arg)		G del nt 98(Gly33Fs),a	4			4
A128G(Lys43Arg)		T583C (Tyr195His),a	2			2
A263T(Lys88Met)		b,c			1	1
	C517T	a	1			1
	A514C	a	2			2
	A905C	d		1		1
	A514C	b			1	1
		T269G (Glu90Asp),a	2			2
		G218A(Gly73Glu),d,e		1		1
		G del 211 (Gly71Fs),a	1			1
		T272C (Leu91Arg),d,e		1		1
		a	5	1		6
		b			4	4
		b,c			2	2
		G ins at nt 352 (Arg118Fs)	1			1
		T ins at nt 314 (Val105Fs)		1		1
		e		2		2
		G508T (Glu170*)			1	1
		d		2		2
		A208C (Ser70Arg),b			3	3
		C413A (Ala138Glu),a	1			1
		d,e		7		7
		A487T (Lys163Ala),d		1		1
		G del nt 98 (Gly33Fs),d		1		
		T257A (Leu91Arg),d		1		1
Total			105	23	13	141

[a]= Lineage-specific polymorphisms and mutations not likely to cause STR resistance were excluded
*= Stop codon, EAI= East Africa India, Fs= Frameshift mutation; underline denote novel mutations
a=A276C (E92D), b=G615A (A205A), c=T47G (L16R), d=G330T (V110V), e=G423A (A141A)

Determining STR-resistance with results from DNA sequencing

The association between phenotypic STR resistance and the DNA sequencing data was analyzed to determine the sensitivity for detecting STR resistance from DNA sequencing (Table 3). Mutations in *rpsL* showed a sensitivity of 69.5% to predict STR resistance. A two-mutation analysis (*rpsL* and *rrs*) had a predictability to detect STR resistance with a sensitivity of 73.0%; however, a three-mutation analysis (*rpsL*, *rrs* and *gidB*) achieved a higher prediction with a sensitivity of 83.7%.

Table 3. Proportion of mutations in genes *rpsL, rrs* and *gidB* in STR-resistant *M. tuberculosis* isolates

Genes mutations	No. of MDR -isolates		(%)
	With mutation	Without mutation	
rpsL	98	43	69.5
rrs	5	136	3.5
gidB	23	118	16.3
rpsL or *rrs*	103	38	73.0
rpsL or *gidB*[a]	113	28	80.1
rrs or *gidB*[a]	20	121	15.0
rpsL or *rrs* and *gidB*[a]	118	23	83.7

[a]= Lineage-specific polymorphisms and mutations not likely to cause STR resistance were excluded

Association of genotypes with mutation in *rpsL, rrs,* and *gidB*

A comparison of mutations in genes *rpsL*, *rrs*, and *gidB* with genotypes in 141 STR-resistant isolates is shown in Table 2. Eighty-eight of 90 strains with an *rpsL* mutation conferring Lys43Arg amino acid substitution belonged to the Beijing genotype, and only two were non-Beijing genotypes (Table 2). Three of five *rrs*-mutated strains belonged to the Beijing genotype, and two belonged to the non-Beijing genotype.

Table 4 summarizes the association of frequently found mutations in *rpsL*, *rrs* and *gidB* with the specific genotypes. For example, nucleotide substitutions in *rpsL* 128 had a statistically

significant correlation with the Beijing genotype (P<0.001). Conversely, the association between the nucleotide substitutions in *rpsL* 263 and the Beijing family seemed to be low (P=0.573). There is no other specific mutations that showed the significant association with Beijing genotype. Compare with Beijing genotype, non-Beijing genotype were more likely to have wild types in the sequence fragment of *rpsL* or *rrs* (80.6% vs 8.6%; P<0.001, data not shown). The nucleotide substitution of A276C in *gidB* was significantly associated with the Beijing family (P<0.001) (Table 2). All isolates with synonymous G330T nucleotide substitutions belonged to EAI strains and isolates that harbored silent mutation of Ala 205 Ala were Euro-American genotype family (Table 2).

Table 4. Comparison of mutation positions in *rpsL*, *rrs* and *gidB* with genotypes of *M. tuberculosis*

Gene	Position of base change	No. of STR- resistant isolates with different genotypes (n = 141)		*P* value
		Beijing (n = 105)	Non-Beijing [a](n = 36)	
rpsL	128	88	2	<0.001
	263	5	2	0.573
	256	0	1	0.255
rrs	514	2	1	0.430
	517	1	0	0.745
	905	0	1	0.255
gidB	98	4	1	0.621
	208	0	3	0.016
	211	1	0	0.745
	218	0	1	0.255
	257	0	1	0.255
	272	0	1	0.255
	269	2	0	0.553
	314	0	1	0.255
	352	0	1	0.284
	413	1	0	0.745
	487	0	1	0.255
	502	1	0	0.745
	508	0	1	0.255
	583	2	0	0.533
	623	1	0	0.745

[a]= Other genotypes including EAI and Euro-American

Discussion

In the first study, mutations in *rpsL* were present in 69.5% (98/141) of all STR-resistant isolates from Myanmar (Table 3), which suggested that these mutations could contribute to STR resistance. The detection percentage in these isolates was higher than that in isolates from Brazil, Mexico and Latvia (24% to 53.3%) [16, 22, 23], but similar to that in isolates from Thailand and China (Table 5) [13, 17]. Lys43Arg was the commonest type of the four different mutations in *rpsL*, being prevalent in 64.0% (90/141) of all STR-resistant isolates. This observation was in accordance with other studies that reported a prevalence of 47.0% to 80.4% [18, 20, 35], indicating that Lys43Arg mutation is profoundly involved in STR resistance. In the present work, I observed that 97.7% (88/90) of isolates with mutation *rpsL* Lys43Arg belonged to the Beijing genotype and that the association was highly significant ($P<0.001$). These observations may be due to the high prevalence of the Beijing family in our isolates, which was also reported in previous studies [13, 17, 20]. In contrast, mutation *rpsL* Lys88Met/Arg was not significantly associated with the Beijing family. Based on our results, it can be suggested that the Beijing genotype may be expanding due to an evolutionary advantage under STR pressure in Myanmar, similar to that reported in other countries [36, 37]. A nonrestrictive mutation of Lys43Arg can result in no additional fitness cost for MTB, leading to a broad transmission among the patients [36, 37, 38]. As *rpsL* Arg86Tyr mutation has been previously reported in isolates resistance to STR [13], it can be speculated that *rpsL* mutation Arg86Gly, first detected in the present study, may be the cause of resistance to STR.

I observed that 3.5% (5/141) of the isolates harbored *rrs* mutations, which showed a moderate frequency in comparison with isolates reported in previous studies as having a frequency range of 2.3 to 24.0% [19, 22] (Table 5). Changes in the 530 loop region were found in 2.1% (A514C) and 0.7% (C517T) of the STR-resistant isolates. These changes have been reported as frequently occurring *rrs* mutations in several studies [16-20, 31]. The 912 region showed one type of substitution, C905A, which has only been reported by Honore *et al* [10] in 1994. It can then be

Table 5. Comparison of genes *rpsL*, *rrs* and *gidB* in STR-resistant isolates reported in various countries

Gene mutations	Thailand (%) n=110	China (%) n=92	Japan (%) n= 57	Korea (%) n=53	Poland (%) n= 32	Brazil (%) n= 40	Myanmar (%) n=141
rpsL	63.6	78.3	59.6	56.6	50.0	27.5	69.5
rpsL codon 43	47.3	58.7	37.0	52.8	46.9	25.0	64.4
rpsL codon 88	16.4	19.6	19.3	3.8	3.1	2.1	4.0
rrs	17.3	9.8	14.0	15.1	21.9	25.0	3.5
rpsL or *rrs*	81.0	88.0	73.6	71.7	71.9	52.5	73.0
gidB *	7.3	6.5	17.5	20.8	15.6	27.5	9.9
rpsL or *rrs* and /or *gidB*	88.0	94.5	91.1	92.5	87.5	80.0	83.7
Wild type	12.0	5.5	8.9	7.5	12.5	28.8	16.3

*= Lineage-specific polymorphisms and mutations not likely associated with resistance were excluded

Reference sources: Thailand (17), China (13), Japan (11), North Korea (18), Brazil (43), Poland (19).

suggested that the majority of *rrs* mutations representing STR resistance occurs more frequently in the 530 region, as previously reported [16, 39]. Interestingly, no co-existing mutations were found in either *rpsL* or *rrs*, which indicated that mutations in *rrs* could exclusively confer STR resistance. In addition, as *M. tuberculosis* has only one copy of *rrs*, the single point mutation in this gene is enough for acquiring STR-resistance [40, 41].

In the present study, 20 different single, double and even triple nucleotide substitutions were found in *gidB*. The A276C single nucleotide substitution, found to be significantly ($P<0.001$) associated with the Beijing genotype (Table 2), occurred alone or in combination with *rpsL* or *rrs* mutation [15, 33]. In addition, it was found that all isolates with a synonymous mutation at codon

205 (G615A) in g*idB* belonged to the Euro-American genotype. This finding is in contrast with those from previous studies, which reported that the nucleotide substitution involving codon 205 belonged to the Beijing, the CAS or the EAI genotype [16, 17, 19, 23]. Only one study has reported a similar association of the Euro-American genotype with a nucleotide substitution at codon 205 to date [19].

All G330T nucleotide substitutions in our isolates belonged to the EAI genotype, which is in agreement with previous reports [16, 37]. Moreover, most of the isolates with G330T also carried a second nucleotide substitution of G423A in *gidB* and belonged to the EAI6-BGD1 sub-lineage (Table 2), which further supported the notion that these double mutations could be used as possible markers for the EAI sub-lineage [42].

Variations of *gidB* mutations detected in this study could help widen the range of known mutations in this gene. Five polymorphisms in *gidB* at positions 47, 276, 330, 423 and 615 have been reported to date as genetic markers of MTB lineages in the Beijing, the Euro-American and the EAI genotypes [17, 18, 43]. As the Beijing genotype is known to be the most frequently found with acquired DR-TB [17, 18, 42], the MDR-TB isolates in this study predominantly belonged to the Beijing family. Therefore, we have only identified the association of A276C changes in the *gidB* with Beijing genotype and in contrast, the association of other genotypes with specific mutations could not be determined clearly.

Except for lineage-specific mutations, of all 15 different *gidB* mutations detected (Table 2), only five (Gly33Fs, Ser70Arg, Leu91Pro, Arg118Fs and Ala138Glu) have been previously reported [11, 14, 17, 19] and hence, 10 mutations were novel. Similar high frequency of mutations was observed in previous work [13, 14, 17], indicating the highly polymorphic nature of *gidB*. This unique polymorphic feature can be explained by the presence of 16 amino acids at active sites of the entire GidB protein, which offers a higher probability of structure changes with an increasing tendency of novel mutations in *gidB* [43]. Except for Tyr195His, His169Tyr, Gly208Val, all eight

missense mutations had an exclusive *gidB* mutation with no mutation in *rpsL* or *rrs* might increase the likelihood of their association to STR resistance.

Missense mutations carrying Gly73Glu, Leu91Pro and Ala138Glu are yet to be reported. However, silicon-based protein modelling and docking analysis showed the presence of these amino acid residues at the interaction sites of GidB and S-adenosylmethionine (SAM), which are critical for methylation at the position G518 of 16S rRNA [44]. Therefore, these amino acids substitutions may affect the GidB function by interfering with STR binding and consequently leading to STR resistance. Despite being distantly situated from the active site, a nonsense mutation of *gidB* (G170stop) with no mutation in *rpsL* or *rrs* is presumed to inhibit the function of GidB protein, leading to STR resistance [44]. Co-occurrence of *rpsL* and *gidB* mutations (Table 2) can be hypothesized that mutation in *gidB* first presents with low-level STR-R and later it acquires *rpsL* mutation by reducing pressure for drug selection and then develops high-level STR resistance strain. As an opposite, it is possible that acquired *gidB* mutation can occurs as a compensatory mechanism for *rpsL* mutational. But there is no known evidence to support these hypothesis and further research focusing in the role of double mutation in two locus should be carried out.

Three frameshift mutations in *gidB* (V105Fs, R118Fs and G71Fs) were found in STR-resistant isolates with no mutation in *rpsL* or *rrs*, which probably play important roles in STR resistance. Other studies have also found the frameshift mutations in *gidB* G del at nucleotide 98 (5 isolates) in both STR-resistant and susceptible isolates [19, 24]. However, as the active center of 16S rRNA methylase in GidB is located at the 173 amino acid position, an early frameshift mutation at 33 codon loses its function and hence contributes to STR resistance.

Mutations in STR resistance-associated genes, namely *rpsL*, *rrs* and *gidB*, showed a highly predictive value for resistance with a sensitivity of 83.7%, as suggested by previous work [16, 19, 20]. Moreover, as only *rpsL* mutation was detected in most STR-resistant isolates, detection of mutation in *rpsL* can be proposed as a satisfactory predictor of STR resistance with a sensitivity of

about 70.0%. It is worth noting that in the present work, 16.3% (23/141) of the STR-resistant isolates showed a wild-type sequence in all three loci, which is in full agreement with the data from other Asian countries (Table 5). By contrast, our result was lower than that from a study in Brazil (ca. 28.8%) [16]. Wild-type sequence in STR-resistant isolates may be caused by either other STR resistance mechanisms [11] or poor reproducibility of the currently used culture-based proportion method [25]. It is important to mention that this study had a crucial limitation, we only focused on MDR-MTB isolates and most of them were STR-resistant as expected [5, 6]. And hence, we could not get sufficient number of STR-susceptible isolates for gene analysis.

Our study showed that the majority of STR-resistant MDR-TB isolates (83.7%, 118/141) had mutations in *rpsL*, *rrs* and *gidB*, which suggests that these mutations may play important roles in STR resistance found in Myanmar isolates. As a result, sequence analysis of these genes could be used to satisfactorily predict STR resistance in Myanmar. It was found that mutations in gene *rpsL*, in particular Lys43Arg, contributed the most to STR resistance, followed by mutations in *gidB* and *rrs*. In addition, results of the present work showed that the Beijing genotype was the dominant MTB strain with STR resistance in Myanmar and it was associated with Lys43Arg *rpsL* mutations. Thus, it can be suggested that A276C nucleotide substitution in *gidB* could be used as a phylogenetic marker for the Beijing family in Myanmar and then we can estimate the possible high frequency of Lys43Arg mutation in *rpsL* among those isolates other than mutation in other locus. Due to the diverse pattern of mutations in *gidB*, their role in STR resistance should be explored further by analyzing the changes of the intensity of methylation activity of mutants GidB.

Finally, our study successfully generated important molecular information on STR resistance in Myanmar, which is likely to be useful in developing molecular STR susceptibility assays, which in turn could contribute to develop TB treatments and control strategies in Myanmar.

Summary

Numerous studies report that mutations of *rpsL* (encoding the S12 protein), *rrs* (encoding 16S rRNA) and *gidB* (encoding rRNA methyltransferase) are responsible for conferring resistance to streptomycin (STR), which is usually used in both multidrug-resistant tuberculosis (MDR-TB) treatments and re-treatments in Myanmar. The aim of this study was to explore the variation and frequency of mutations in *rpsL*, *rrs* and *gidB* in 141 STR-resistant MDR-TB isolates from Myanmar. Most isolates belonged to the Beijing genotype (105, 74.5%). Moreover, mutations in *rpsL* were identified in 69.5% (98/141) of the STR-resistant isolates, where the most prevalent (91.8%, 90/98) and significantly associated mutation with the Beijing genotype ($P<0.001$) was Lys43Arg. Fifteen different mutations in *gidB* were found in 16.3% (23/141) of the isolates, and most of them were novel mutations. Moreover, based on our results, I suggest A276C nucleotide substitution in *gidB* as a phylogenetic marker for the Beijing family in Myanmar. Sequence analysis of *rpsL*, *rrs* and *gidB* with a sensitivity of 83.7% satisfactorily predicted STR resistance in Myanmar isolates. However, in 16.3% (23/141) of the isolates, none of the examined genes showed mutation. Hence, further studies are strongly recommended to elucidate other possible resistance mechanisms. The present findings may be useful in developing molecular STR susceptibility assays, which in turn could contribute to develop TB treatments and control strategies in Myanmar.

Chapter II

Characterization of *pncA* gene mutations associated with pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from Myanmar

Introduction

Pyrazinamide (PZA), a first-line anti-TB drug, is a pro-drug that is converted to its active form (pyrazinoic acid [POA]) by pyrazinamidase (PZase) produced by MTB. It especially affects on semi-dormant tubercle bacilli which survive in acidic environment of inflammatory sites that cannot be affected by other anti-TB drugs [45]. The detailed mechanism of action of PZA is not well known and it is believed that an acidic medium is needed to activate the action of PZA. POA is functioned in the neutral pH in the cytoplasm which has no activity against MTB. By the efflux pump, POA is pumped out of the cell which have in acidic environments and then protonated to HPOA which re-enters the cell and accumulation can eventually cause rupture of membrane which finally end with bacterial dead [50], (Fig 6).

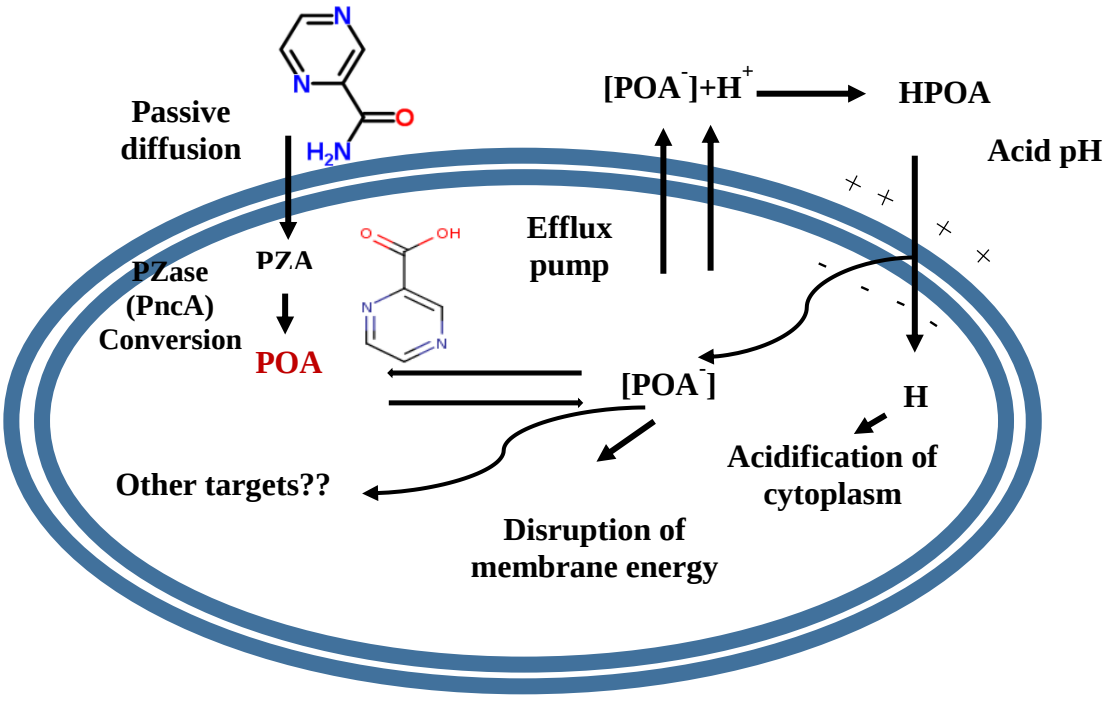

Fig 6. Mechanism of PZA/POA

Due to the unique ability of effectiveness and shortening of treatment duration from 9 to 6 months, PZA is widely used as short course anti-TB treatment regimen and recently has become an important role in MDR-TB treatment [45, 46]. Additionally, various studies have reported that the increasing rate of PZA resistance among MDR and non-MDR-TB patients indicating that drug susceptibility testing (DST) for PZA is important before it is included in the treatment regimen [45-47].

Culture base PZA susceptibility test is unreliable because of some limitations such as the need to adjust pH to acidic media and the standardized inoculum size which all might effect on PZA activity [48]. Currently, the phenotypic method by BD MGIT 960, the only test endorsed by WHO, have been widely used to detect PZA resistance in well setup laboratories in many countries. However, it has difficulty to be performed in limited laboratories setting and still present with high false-positive and negative resistance results [49].

PZase is encoded by *pncA* and several researchers had identified that mutations in *pncA* which lead to loss of PZase activity are responsible for PZA resistance [50-54]. Therefore, the alternative method to investigate PZase activity or Wayne test has been performed by many researchers. However the test gives inaccurate results of PZA susceptibility due to different *pncA* mutation produced variety of enzyme activity [50-54].

The frequency of *pncA* mutation among MDR-TB isolates varies from 27% to 60% depending on geographical areas while up to 98% of PZA phenotypic resistance isolates carried the mutation [55-60]. However, about 7-10% of PZA susceptible isolates that harbored *pncA* mutation have been reported so far [61, 62]. Although, PZA resistance has been well correlated with the *pncA* mutation, wide diverse and scattered nature of mutations [56-58] make difficulty in the development of easy and rapid detection method. Because of the detection of mutation in *pncA* not only provide the drug resistance mechanism to PZA but also give the chance to develop drug target for the killing of persister bacteria, the *pncA* mutation analysis have been proposed as the possible surrogate test for

rapid PZA susceptibility testing.

To date, in Myanmar, traditional PZA drug susceptibility testing by solid culture is not routinely carried out because of high false positive and negative rate [66, 67]. Up to our knowledge, limited data have been published for PZA resistance among MDR-TB and non MDR-TB in Myanmar [5, 6]. As PZA has the significant bactericidal effect, detection of possible prevalence of PZA resistance by genotyping method in clinical isolates is indispensable to give the rapid DST results. Additionally, this data can help in the proper treatment for MDR-TB cases and reduce the transmission of MDR-TB strains among the community. According to previous studies, *pncA* mutations were scattered throughout the coding region and promoter region, highlight the fact that the hot spot region comprising the majority of mutations in *pncA* could not be identified [51, 61, 62]. However, earlier reports demonstrated that the mutations have frequently occurred in a cluster that includes catalytic residues in the active site (Asp8Gly/Ala/Glu/ Asn, His51Gln/Tyr, His71Arg, Asp49Glu/Asn/Ala, His57Arg/Tyr/Gln/Pro, Trp68Arg/Gly/Cys/Stop/Leu,Gln10Pro/Arg, and His137Pro/Arg/Asp) and metal binding site (Ile6Thr, Val44Gly, Val139Gly/Leu, Met175Thr/Val, and Phe94Cys/Ser/Leu) [53, 62, 68, 69]. Additionally, 3D structure analysis reported that the specific activity of recombinant mutant PZase varies up to several folds based on the site of mutation [51]. Nowadays, molecular detection of drug- resistance associated gene mutations play the pivotal role in rapid diagnosis of drug susceptibility. More recently, genotypic PZA susceptibility test has been developed by observing mutations in the *pncA* which is being known as the primary mechanism of PZA resistance [58, 67–68]. Therefore, the characterization of *pncA* is necessary to estimates the prevalence of PZA resistance strains among MDR-TB isolates.

In this chapter, I investigated the frequency and patterns of *pncA* mutation among MDR-TB isolates. I described the possible prevalence of PZA resistance by comparing with previously reported PZA resistance-associated mutations. In addition, the association between the *pncA*

mutations frequency and other drug resistance-associated mutations (*rpoB* for RIF resistance and *katG* and *inhA* for INH resistance) as well as the patterns of *pncA* mutation according to *M. tuberculosis* genotype preference were also analyzed.

Materials & Methods

Collection of clinical samples and drug susceptibility test

Samples were collected from National Tuberculosis Program, Yangon between 2009 and 2013. A total of 285 MDR-TB clinical isolates, each corresponding to an individual TB patient, were randomly selected from the nation-wide collection (NTP), Myanmar. DST were carried out on LJ medium by the conventional proportional method at critical drug concentrations of 0.2, 2, 4, 40 mg/ml of INH, EMB, STR and RIF, respectively [27].

DNA extraction

DNA was extracted from MTB colonies grown on LJ medium by the enzymatic lysis method [28]. Purified DNA was dissolved in TE buffer (10 mM Tris –HCl, 1 mM EDTA, pH 8.0), quantified by a Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) and stored at -20 ºC until further use.

Genotyping by spoligotyping and large sequence polymorphism (LSP)

All MDR-TB isolates were genotyped by spoligotyping, as described in Chapter I. Briefly, a primer set was used to amplify the direct repeat region, and the resulting PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes on the membrane. To determine spoligo-international type (SIT), hybridization patterns were converted into binary and octal formats. The resulting SITs were compared with patterns previously reported in the SpolDB4 database [30]. For those isolates considered to be un-designed or new spoligotypes [30], the genotype was determined by LSP using specific primers for the expected region of difference and Run TB Lineage that is available in online [31, 70].

PCR amplification and DNA sequencing of drug resistance conferring genes

The targeted gene fragments (*rpoB*, *katG*, *inhA* regulatory region and *pncA* with upstream regulatory region) were amplified and sequenced by using the primers list in Table 6. PCR was performed in 20µl reaction mixture consisted of 5X Green GoTaq® Reaction Buffer (Promega), 0.5 M betaine, 0.25 mM $MgCl_2$, dNTPs (0.25 mM each), primers (0.5 µM each), 1.25 U GoTaq® DNA Polymerase (Promega) and 1 µl of template DNA (approximately 10 ng/ul). The following PCR condition were used. One cycle of initial denaturation 95 ºC for 60 sec followed by 35 cycles of amplification at 95 ºC for 10 sec, annealing at 50 or 55ºC for 10 sec, an extension at 72 ºC for 30 s, and a final extension at 72 ºC for 5 min. The amplified DNA fragments of target genes were confirmed by 1% agarose gel electrophoresis, purified to manual protocol of EtOH precipitation method. The purified DNA fragments were sequenced with the same primers as PCR using a BigDye ver. 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) in an ABI 3500xL Genetic Analyzer (Applied Biosystems). The sequencing results were analyzed with BioEdit version 7.05.3 [34] by aligning with wild-type *pncA* sequences of the reference MTB H37Rv strain.

Table 6. Primers used in the study.

Gene	Primers	Nucleotide sequences	Annealing T·(Time)	Expected size (bp)	Reference
rpoB	*rpoB*-F	5′-CAGGACGTGGAGGCGATCAC-3′	55ºC (10 sec)	278	71
	rpoB-R	5′-GAGCCGATCAGACCGATGTTGG-3′			
katG	*katG*-F	5′-ATGGCCATGAACGACGTCGAAAC-3′	55ºC (10 sec)	392	71
	katG-R	5′-CGCAGCGAGAGGTCAGTGGCCAG-3′			
inhA	*inhA*-F	5′-TCACACCGACAAACGTCACGAGC-3′	55ºC (10 sec)	231	71
	inhA-R	5′-AGCCAGCCGCTGTGCGATCGCCA-3′			
pncA	*pncA*-F	5′-GGCGTCATACCCTATATC -3′	50ºC (10 sec)	843	This study
	pncA-R	5′-CAACAGTTCATCCCGGTTC -3′	50ºC (10 sec)		

Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Two-tailed Fisher's exact test was used to compare the PZA resistance-associated mutations according to the resistance conferring mutation of other first-line drugs (INH, RIF, STR, and EMB). To realize the association between the frequency of *pncA* mutations and that of *rpoB, katG* and *inhA* mutations, odd ratio with 95% confident interval was calculated. Data were considered statistically significant if the *P* value was <0.05.

Results

Drug resistance patterns and *M. tuberculosis* genotypes

Among 285 MDR-TB clinical isolates with known first-line four anti-TB drugs susceptibility patterns (166 isolates), 8 isolates were resistant to only INH and RIF, 55 and 3 isolates showed additional STR or EMB resistance and the remaining 100 isolates were resistant to four first-line anti-TB drugs (Table 7). The other 119 isolates had noted as resistant to INH and RIF and other drug resistance patterns were unknown.

Table 7. Distribution of first-line drug susceptibility patterns in relation to MTB families

Drug susceptibility pattern	Genotypes				
	Beijing	EAI	CAS	Euro-American	Total
HR resistant	4	2	2	0	8
HR resistant*	108	8	3	0	119
HRS resistant	36	13	0	6	55
HRE resistant	2	1	0	0	3
HRSE resistant	80	13	0	7	100
Total	230	37	5	13	285

* Indicates the MDR-TB isolates with unknown Streptomycin and Ethambutol DST data
Isoniazid =H, Rifampicin=R, Streptomycin =S, Ethambutol=E

The spoligotyping analysis revealed 28 different SIT spoligotype patterns (Fig 7). The Beijing family was the most frequently detected (230, 80.7%), followed by the EAI (37, 13.0%), Euro-American (13, 4.6%) and CAS (5, 1.7%) families (Table 7). The unknown group (undersigned and new types) which determined by spoligotyping was later classified as EAI or Euro-American by Run TB lineage (free access online) or large sequence polymorphisms [30, 70]. The 'Euro-American' family included LAM (4, 1.4%), H37Rv (2, 0.7%), H3 (1, 0.4%), S (1, 0.4%), T1 (1, 0.4%) and X2 (4, 1.4%) spoligotype families. The LSP and Run TB lineage allowed assigning the 15 unknown spoligotypes into the EAI and Euro-American.

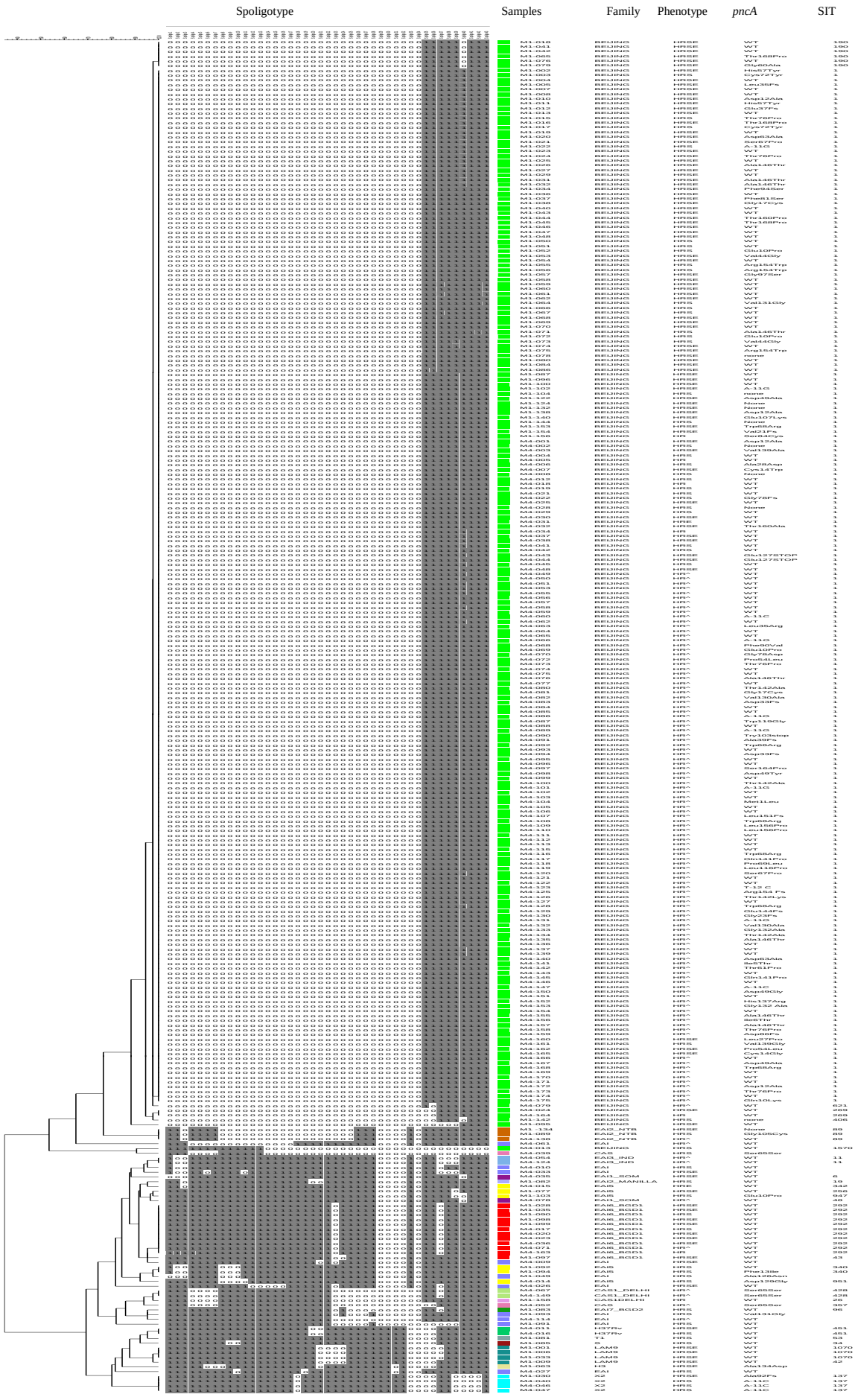

Fig 7. Phylogenetic tree based on spoligotypes patterns of the 285 MDR-TB isolates

Analysis of mutation in *rpoB, katG* and *inhA*

Mutations in the RIF resistant determining region of the *rpoB* gene were identified in 241 isolates (84.6%). No mutations were detected in 44 RIF-resistant isolates. Out of 285 MDR-TB isolates, 243 (85.2%) had an amino acid substitution in *katG* or mutations in the *inhA* regulatory region (Table 8).

Table 8. Comparison of *pncA* mutation frequencies with *rpoB, KatG* and *inhA* genes in 285 isolates of MDR-TB

Mutations patterns	No. of isolates		Relative frequency of *pncA* mutations (%)	Odd ratio, 95% CI	*P*-value
	with *pncA* mutations	Without *pncA* mutations			
rpoB mutation	121	120	50.2	4.0 (1.8-8.5)	<0.001
No *rpoB* mutation	9	35	20.5		
KatG or *inhA* mutation	121	122	49.8	3.6 (1.7-8.0)	<0.001
No *KatG* or *inhA* mutation	9	33	21.4		

Analysis of *pncA* mutations

Mutations in *pncA* were identified from 130 (45.6%) isolates while 156 (55.4%) isolates showed wild-type sequences. Seventy-four different types of mutations were distributed on the whole *pncA* including promoter region and among which 55 (55/74, 74.3%) have been previously reported and 19 (19/74, 25.7%) were found to be novel. The mutation types included single nucleotide substitutions, nucleotide deletions or insertions (Table 9). Among 74 distinct *pncA* mutations identified (Table 9), 7 were insertions (8 isolates), 5 were deletions (5 isolates), and 62 were single-nucleotide substitutions that lead to 61 amino acid substitutions and one silent mutations

(117 isolates); in both coding region and putative regulatory region. Among the amino acid substitutions identified (100 isolates), 49 types of mutations have been associated with phenotypic pyrazinamide resistance (90 isolates) (Table 10) and 9 amino acid substitutions (10 isolates) were not previously reported. No single mutation was particularly predominant and the most frequent mutation, observed in eight isolates, was Ala146Thr (nucleotide 359, T→C), followed by Trp68Arg presented in six isolates (nucleotide 202, T→C) (Table 10).

Table 9. Distribution of polymorphisms of *pncA* among 285 MDR-TB isolates of Myanmar.

Total types of mutations	Number of types of mutations (n=74, %)	No. of cases (n=130, %)
Nucleotide substitutions	62 (83.7)	117 (90.0)
Regulatory region (-11and -12)	3 (4.1)	13 (10.0)
Amino acid substitutions	58 (78.4)	100 (76.9)
Silent mutation	1 (1.4)	4 (3.1)
Nucleotide deletion*	5 (6.8)	5 (3.8)
Nucleotide insertion*	7 (9.5)	8 (6.2)

* mutation causing abnormal or truncated polypeptide

Table 10. Mutations found in *pnc*A gene and its promoter of the studied MDR-TB isolates of Myanmar. The diversity of *pnc*A mutations (74 different types) and the mutations found in PZA-resistant and/or PZA-susceptible isolates according to literature and its association with PZA resistance/susceptibility were described in the table.

Nucleotide changes	Amino acid changes	No. of isolates	Genotype (isolates)	Phenotyping susceptibility test/*	Reported	References
(-12 T-C)		1	Beijing	R	[Yes]	[61]
(-11 A-C)		5	Beijing (2), Euro-American (3)	R	[Yes]	[61]
(-11 A-G)		7	Beijing	R	[Yes]	[61]
A1T	Met1Leu	1	Beijing	N/D	Novel	
T14C	Ile5Thr	1	Beijing	R/S (*)	[Yes]	[62]
T17C	Ile6Thr	1	EAI	R	[Yes]	[66]
C28A	Gln10Lys	1	Beijing	R	[Yes]	[61]
A29C	Gln10Pro	4	Beijing(3), EAI(1)	R	[Yes]	[61]
A35C	Asp12Ala	4	Beijing	R	[Yes]	[61]
T37A	Phe13Ile	1	EAI	N/D	Novel	
T40G	Cys14Gly	1	Beijing	R	[Yes]	[6]
C42G	Cys14Trp	1	Beijing	R	[Yes]	[68]
G49T	Gly17Cys	2	Beijing	R/S (*)	[Yes]	[61]
AA ins 63	Val21Fs	1	Beijing	N/D	Novel	
TT ins 69	Gly23Fs	1	Beijing	N/D	Novel	
T80C	Leu27Pro	1	Beijing	R	[Yes]	[61]
C83A	Ala28Asp	1	Beijing	R	[Yes]	[61]
GC ins 95	Asp33Fs	2	Beijing	N/D	Novel	
T104G	Leu35Arg	1	Beijing	R/S (*)	[Yes]	[62]
G del 105	Leu35Fs	1	Beijing	N/D	Novel	
G del 109	Glu37Fs	1	Beijing	N/D	Novel	
CC ins 117	Ala39Fs	1	Beijing	N/D	Novel	
T131G	Val44Gly	2	Beijing	R	[Yes]	[68]
G145T	Asp49Tyr	1	Beijing	R	[Yes]	[60]
A146G	Asp49Gly	1	Beijing	R/S (*)	[Yes]	[61]
A146C	Asp49Ala	1	EAI	R	[Yes]	[69]
C161T	Pro54Leu	2	Beijing(1), EAI(1)	R	[Yes]	[62]
C169T	Thr57Tyr	2	Beijing	R	[Yes]	[75]
C176T	Ser59Phe	1	Beijing	R/S (*)	[Yes]	[78]
G179C	Gly60Ala	1	Beijing	N/D	Novel	
A181C	Thr61Pro	2	Beijing	R	[Yes]	[63]
A188C	Asp63Ala	2	Beijing	R	[Yes]	[63]
C195T	Ser65Ser	4	CAS	R/S [a]	[Yes]	[63]
T199C	Ser67Pro	1	Beijing	R	[Yes]	[61]
T202C	Trp68Arg	6	Beijing	R	[Yes]	[61]
C206T	Pro69Leu	1	Beijing	R	[Yes]	[61]
G215A	Cys72Tyr	3	Beijing	R	[Yes]	[63]
A226C	Thr76Pro	5	Beijing	R	[Yes]	[63]

G Ins 232	Gly78Fs	1	Beijing	R	[Yes]	[63]
G233A	Gly78Asp	1	Beijing	R	[Yes]	[62]
T242C	Phe81Ser	1	Beijing	R	[Yes]	[62]
A250T	Ser84Cys	1	Beijing	N/D	Novel	
TCCAG del 257	Asp86Fs	1	Beijing	N/D	Novel	
G ins 273	Gly92Fs	1	Beijing	N/D	Novel	
T280G	Phe94Val	1	Beijing	N/D	Novel	
T281C	Phe94Ser	1	Beijing	R	[Yes]	[61]
G289A	Gly97Ser	1	Beijing	R	[Yes]	[61]
C309G	Tyr103Stop	1	Beijing	R	[Yes]	[61]
G313T	Gly105Cys	1	Beijing	N/D	Novel	
G319A	Glu107Lys	1	Beijing	R	[Yes]	[63]
T347C	Leu116Pro	1	Beijing	R	[Yes]	[63]
G355A	Trp119Gly	1	Beijing	R	[Yes]	[66]
G376A	Asp126Asn	1	EAI	R	[Yes]	[61]
G379T	Glu127Stop	2	Beijing	R	[Yes]	[63]
A386G	Asp129Gly	1	EAI	N/D	Novel	
T389C	Val130Ala	2	Beijing	R	[Yes]	[61]
T392G	Val131Gly	1	Beijing	N/D	Novel	
G395C	Gly132Ala	2	Beijing	R	[Yes]	[63]
C401A	Ala134Asp	1	Euro-American	N/D	Novel	
A410G	His137Arg	1	Beijing	R	[Yes]	[63]
T416C	Val139Ala	1	Beijing	R	[Yes]	[61]
A422C	Gln141Pro	2	Beijing	R	[Yes]	[61]
A424G	Thr142Ala	4	Beijing	R	[Yes]	[61]
C425A	Thr142Lys	1	Beijing	R	[Yes]	[62]
GAG del 430	Glu144 del	1	Beijing	R	[Yes]	[62]
G436A	Ala146Thr	8	Beijing	R	[Yes]	[61]
AGGT ins 451	Leu151Fs	1	Beijing	N/D	Novel	
A460T	Arg154Trp	3	Beijing	R	[Yes]	[63]
13nt del 461	Arg154Fs	1	Beijing	N/D	Novel	
T467C	Leu156Pro	2	Beijing	R	[Yes]	[60]
A478C	Thr160Pro	1	Beijing	R	[Yes]	[64]
A478G	Thr160Ala	1	Beijing	R	[Yes]	[55]
T490C	Ser164Pro	1	Beijing	R	[Yes]	[58]
A502C	Thr168Pro	3	Beijing	R	[Yes]	[66]

Fs=Frame shift mutation, R= PZA resistance, S=PZA susceptible

*=Final interpretation as associated with PZA resistance in this study, N/D= not determined

[a] = The silent mutation that have been reported as CAS genotype specific polymorphisms

Of the 13 MDR-TB isolates with insertions and frameshift, 4 isolates showed single nucleotide insertions at codons 35, 37, 78 and three isolates with two-nucleotides insertion at codon 23, 33 and 35 whereas one isolate showed four-nucleotides insertion resulting frameshift at codon 151 (Table 10). One to thirteen bases deletions were observed in 5 isolates resulting in the loss of amino acids and changing the reading frame. Two single nucleotide substitutions (at codons 103 and 127) resulted in premature stop codons. With regards to the *pncA* promoter mutations, the most frequently found mutation was the nucleotide substitution at position −11 (12 isolates). Nucleotide substitutions at positions −12 was also detected (one isolate) (Table 10). Finally, 67.0% (87/130) of mutants carried *pncA* mutations in that specific three regions (codon 3–17, 61–85 and 127–154) (Fig 8).

Association of *rpoB, katG* and *inhA* mutations with *pncA* mutations

Among the 241 *rpoB* mutated isolates, 121 (50.2%) had at least one mutation in regulatory or encoding region of *pncA*, while 9 (20.5 %) of the isolates without *rpoB* mutations had one mutation in the *pncA* coding region (Table 8). This difference was statistically significant (P<0.001).

Similarly, the *pncA* mutation frequency in isolates that carried mutations in *katG* or *inhA* regulatory region (243, 49.8 %) was significantly higher than those in the isolates that does not carry these mutations (9, 21.4%) (Table 8).

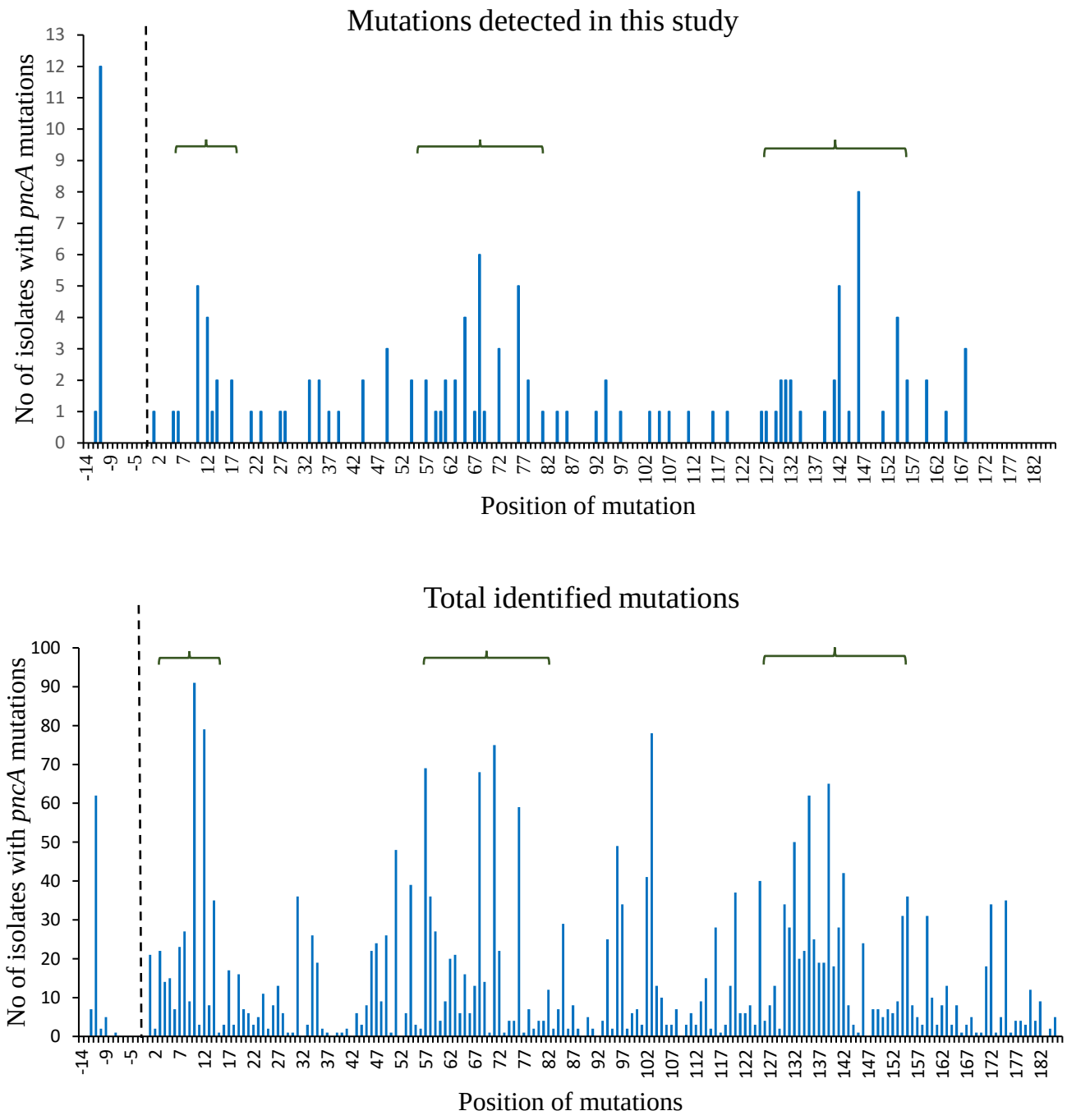

Fig 8. Frequency of *pncA* mutation on both promoter and coding region in MDR-TB isolates in Myanmar and reported mutations. Mutations at promoter region are indicated by nucleotide position and labeled by minus numbers. (The dotted line demarcate the promoter region and coding region). The brackets showed the three clusters of high frequent found mutations as described by previously reports (54, 68, 69).

(a) Location of *pncA* mutation (excluded frame-shift and stop codon mutations) found in *M. tuberculosis* isolates.

(b) Location of *pncA* mutation from previously reports.

Association of *pncA* mutations with *M. tuberculosis* families and drug resistance patterns.

Among 130 isolates with *pncA* mutation, 87.7% were isolates with Bejing genotype and each of 5.4% and 5.8% were EAI and Euro-American. The *pncA* mutation frequency was highest in Beijing isolates (115 out of 230, 50.0%), followed by Euro-American (5 out of 13, 38.5%) and EAI (6 out of 37, 16.2%). The association of each specific genotype with the frequency of *pncA* mutation were higher in Beijing and EAI genotypes than Euro-American genotype (Table 11). More specifically, the Fisher's exact test was significant, supporting a difference between the Beijing (115 isolates) and non-Beijing (15 isolates) families (P<0.001) (data not shown).

Moreover, when comparing each group of drug resistance pattern, the *pncA* mutation frequency was found to be distributed equally in the isolates with HR^r (3 out of 8, 37.5 %), HRS^r (22 out of 55, 40%) and $HRSE^r$ (39/100, 39%) (Table 12).

Table 11. Proportion of *M. tuberculosis* families and *pncA* mutation frequency among 285 MDR-TB isolates

Genotypes	Number of isolates		Relative frequency of *pncA* mutations	Odd Ratio	*P*-value
	With *pncA* mutation	Without *pncA* mutation			
Beijing	115	115	50.0	2.7 (1.4-5.1)	<0.001
EAI	6	31	16.2	0.2 (0.08-0.5)	<0.001
CAS	4	1	80.0*	N/D	N/D
Euro-American	5	8	38.5	0.6 (0.2-2.0)	0.777

*= the *pncA* mutations that have been reported as CAS specific polymorphism was not included in statically analysis

N/D=not determined

Table 12. Correlation of each group of first-line drug susceptibility patterns to *pncA* mutation frequency.

Drug resistant patterns	Number of isolates		Relative frequency of *pncA* mutations (%)
	With *pncA* mutation	Without *pncA* mutation	
HR resistant	3	5	37.5
HR resistant*	66	53	55.5
HRS resistant	22	33	40.0
HRE resistant	0	3	0.0
HRSE resistant	39	61	39.0

* Indicates the MDR-TB isolates with unknown Streptomycin and Ethambutol DST data

DISCUSSION

Although PZA is widely used for TB and MDR-TB treatment, because of technical challenging and high false resistant rate of standard phenotypic PZA susceptibility testing by BACTEC MGIT 960, the analysis of PZA resistance among MDR-TB isolates is seldom performed in Myanmar. As there is increasing rate of MDR-TB in Myanmar [1], it is needed to investigate PZA resistant level as an important public health issue. This study was the first to perform *pncA* mutation analysis in large number of MDR-TB isolates comparing to the two previous studies [5, 6]. Genotype data of *pncA* mutation found in this study was compared with previously reported PZA DST and the novel mutations were predict as the link with PZA-R according to the types of mutations (frame shift or silent) and positions of mutations (active sites or metal ion binding site) (Table 10).

In this study, 45.6 % of the selected MDR-TB isolates carry mutations in the *pncA* or its promoter, in line with findings from other countries such as Thailand (47%), China (43.1%), Belgium (42.7%), Japan (47%.0), Bangladesh (45.0%) and South Africa (52.1%). However, lower percentage comparing to Vietnam (72.0%) and Russia (68.0%) [51, 55, 60, 72-75, 87] (Table 13) was found. According to the whole genome sequencing study on selected MDR-TB isolates in 2015 in Myanmar, the detection rate of *pncA* mutation was 42.8 % (6/14) which showed lower than our finding [6]. Nevertheless, the study for the samples collected in 2015-2016 in Myanmar reported that 60.6 % (40/66) of MDR-TB isolates carried *pncA* mutation and were considered to be resistant to PZA [5].

Among totally detected 74 types of *pncA* mutations, the major mutation type was the amino acid substitutions (76.9%, 100/130) in coding region, while some deletions and insertions were detected with only small number (10.0%, 13/130) (Table 10).

Table 13. Comparison of *pncA* mutation from MDR-TB isolates of different countries

Countries	No. of isolates	Mutant (%)	Wild-type (%)	Reference
Myanmar	285	45.5	54.5	This study
Thailand	49	47.0	53.0	[58]
Belgium	32	42.7	57.3	[51]
Japan	35	53.0	47.0	[60]
China	274	43.1	56.3	[55]
South Africa	349	52.1	47.9	[72]
USA	143	58.0	42.0	[87]
Russia	75	68.0	32.0	[74]
Bangladesh	169	45.0	55.0	[73]
Vietnam	260	72.0	28.0	[75]

All mutations were scattered throughout in the *pncA* gene with only limited shared mutation patterns. Seventy different mutations were found in 61 of the 187 *pncA* codons (Fig 8 (a)) and this is in agreement with previous reports determined that 171 out of 186 codons carried more than 600 distinct *pncA* mutations (Fig 8 (b)) [61]. In addition, as some types of mutation have been found frequently at some specific codons (e.g., codons 10 and 68) and it can be proposed that the possible spread of the single mutant strain during any outbreak.

The *pncA* mutation residues are dispersed on the gene which demonstrated three clusters within the coding gene (codon 3-17, codon 61-85, codon 127-154) similar to previously observation [54,68,69] . This underscores the fact that there is the great diversity of *pncA* mutations at different positions depending on the geographical area [55-61] and highlighting that the development of molecular assay for detection of drug resistance-associated mutations might have more challenging compare with other genes (*rpoB* and *katG*).

To the best of our knowledge, 19 mutations (21 isolates) are reported here for the first time (Table 10). Eight types of novel amino-acid substitutions with an unknown link to PZA-R might also be considered as associated with PZA-R as the mutant that affect the same codon had been proved to be associated with PZA-R with high MIC [61]. For example, like a mutation at position

13 from Phe to Ile that found in this study, the changes from Phe to Ser/Leu have been reported to associate with PZA-R [61]. In contrast, although the changes from Met to Ile/Thr have been reported to be linked with PZA-R [61], a mutation in codon 1 from ATG to TTG found in this study may not probably associate to PZA-R because the changes may not alter start codon function. Nine out of 10 novel frame shift mutations or one codon deletion (Table 10) were determined as high confidence mutation not only as previously described that affect on the same codon [61] but also their effect on truncation of protein and loss of function.

In our sample, 60 (46.2%) isolates with amino acid substitutions provided the high confidence mutations according to literature in which recombinant PZase activity, MIC of PZA, and stability of 3D structure PncA have been confirmed (Table 13) [61]. In addition, 30 isolates (23.0%) with amino acid substitutions at *pncA* coding region have been reported as corresponded to the phenotyping PZA-R based on online mutation database as reported previously.

The most frequently found mutation was at -11 nucleotide positon in the putative regulatory region (12/130, 9.2%) and it has been reported to be the common site for PZA-R [61]. The frequent mutation found at codon 146 (8/130, 6.2%) have been reported to have an important suppression of metal iron binding, leading to decreasing or absence of PZase activity [76], while the highly variable mutation at codon 94 (Phe94Leu/Cys/Ser) has been noted to be involved in the hydrophobic core, important for PZase stabilization.

Based on our findings, 54 types of *pncA* mutations (excluding CAS specific type of single nucleotide polymorphism) were previously reported as PZA-R-associated mutations. As other types of mutation including novel ones were located at the site of the active residues or metal ions binding residues, suggesting that these mutations might cause loss of enzyme activity or chelation of ion-atom [52, 60, 65, 77], (Table 10) which then may play the role in emergence of PZA-R.

The five types of mutations (Ile5Thr, Gly17Cys, Leu35Arg, Asp49Gly and Ser59Phe) have previously been reported to be found in both PZA susceptible and resistant strains (Table 10). This

suggested that the limited accuracy of the currently used MGIT 960 might be the reason why these mutations can be found in susceptible strains. Some researchers excluded the mutations that have been found in both PZA-R and PZA-S isolates as being associated with PZA-R in their studies. However, as the large majority of mutations in *pncA* have been reported are associated with PZA-R in the literature [61-63, 64, 66], in this study, all these mutant isolates were interpreted to be associated with PZA-R.

Therefore, 125 isolates (43.8%) with *pncA* mutations were thought to be associated with PZA-R in this study and detection of *pncA* mutation could estimate the proportion of the PZA-R among MDR-TB isolates in Myanmar.

Among twenty-three types of mutations with multiple isolates, three types of frequently found mutation, six (Ala146Thr), six (Trp68Arg) and five (Thr76Pro) (Table 14 and Fig 7) with the same *pncA, rpoB, katG* and *inhA* mutation with Beijing genotypes indicating the possibility of small outbreak or primary transmission of these drug resistant isolates. It can be hypothesized that these strains are currently circulating in MDR-TB strain in Myanmar.

Table 14. Association of same spoligotypes, *rpoB*, *katG*, *inhA* and shared *pncA* sequences of MDR-TB isolates

Lineage	SIT	Mutation in				No. of isolates
		pncA	*rpoB*	*katG*	*inhA*	
Beijing	1	A-11C*	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	A-11G*	Ser 450 Leu	Ser 315 Thr		6
Beijing	1	Gln10Pro	Ser 450 Leu	Ser 315 Thr		3
Beijing	1	Asp12Ala	Ser 450 Leu	Ser 315 Thr		3
Beijing	1	Gly17Cys	His 445 Asn	Ser 315 Thr		2
Beijing	1	Asp33Fs	His 445 Asn	Ser 315 Thr		2
Beijing	1	Pro54leu	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	His57Tyr	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Asp63Ala	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Trp68Arg	Ser 450 Leu	Ser 315 Thr		6
Beijing	1	Cys72Tyr	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Thr76Pro	Ser 450 Leu	Ser 315 Thr		5
Beijing	1	Glu127STOP	His 445 Arg	Ser 315 Thr	C -15 T*	2
Beijing	1	Val130Ala	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Gly132Ala	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Gln141Pro	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Thr142Ala	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Ala146Thr	Ser 450 Leu	Ser 315 Thr		6
Beijing	1	Arg154Trp	Ser 450 Leu	N/D	C -15 T*	2
Beijing	1	leu156pro	Ser 450 Leu	Ser 315 Thr		2
Beijing	1	Thr168Pro	N/D	Ser 315 Thr		2
X2	137	A-11C	Ser 450 Leu	Ser 315 Thr		3
CAS1-DELHI	428	Ser65Ser	Ser 450 Leu	Ser 315 Thr		2

*= nucleotide substitutions on regulatory region of each gene

N/D = Not detected

Table 15. Association of spoligotypes, shared *pncA* sequences with different *rpoB*, *katG*, *inhA* sequences among MDR-TB isolates

Lineage	SIT	Mutation in				No. of isolates
		pncA	*rpoB*	*katG*	*inhA*	
Beijing	1	Ala146Thr	Ser 450 Leu	Ser 315 Thr		1
Beijing	1	Ala146Thr	His 445Tyr	Ser 315 Thr		1
Beijing	1	Gly132Ala	Asp435Val	Ser 315 Thr		1
Beijing	1	Gly132Ala	His 445 Asn	Ser 315 Thr		1
Beijing	1	Asp49Ala	Ser 450 Leu	Ser 315 Thr		1
Beijing	1	Asp49Ala	His 445 Asn	Ser 315 Thr		1
Beijing	1	Gly17Cys	His 445 Asn / Ser 450 Leu	Ser 315 Thr		1
Beijing	1	Gly17Cys	Ser 450 Gly	Ser 315 Thr		1

Totally 83 isolates with shared *pncA* mutations with different genotypes (Table 10 and Fig 7) and can be suggested that these shared mutations may not correlated to specific genotype. For example, three isolates with A-11C mutation at the promoter region with same genotype of X2 showed the same drug resistance-associated genes mutation patterns (*rpoB*, *katG* and *inhA*) and can also be strongly suggested as there is the small outbreak or recent transmission of these strains (Table 14 and Fig 9). In contrast, same *pncA* mutations (Gly17Cys, Gly132Ala and Ala146Thr) with the same genotypes, but different drug resistance-associated mutation patterns may propose that *pncA* mutations were acquired independently in that MDR-TB strains (Table 15 and Fig 9).

Our spolijgotyping also confirmed that 4 out of 5 CAS family carried silent mutation (AGT65AGC, Ser65Ser) and similar to the results from other studies which described that not all isolates of CAS family carried this mutation [70, 73].

Interestingly, the frequently found mutation at codon 146 was exclusively reported from the Asian countries where the high prevalence of Beijing genotypes [61] and further studies should be carried out any possibility of lineage specific PZA-R associated mutation like other anti-TB drug

(*rpsL* in STR resistance) [78]. In contrast, the second frequently found mutation of Trp68Arg that located in active site of PncA have been previously reported from all over the world, suggesting that the mutations affect active site or metal ion changes might present as frequently found mutation and consequently the hot-spot regions can be determined. The fact to that each of 48 types of mutation was found in one isolate (Table 10), suggested that most of *pncA* mutation developed independently among MDR-TB isolates in Myanmar.

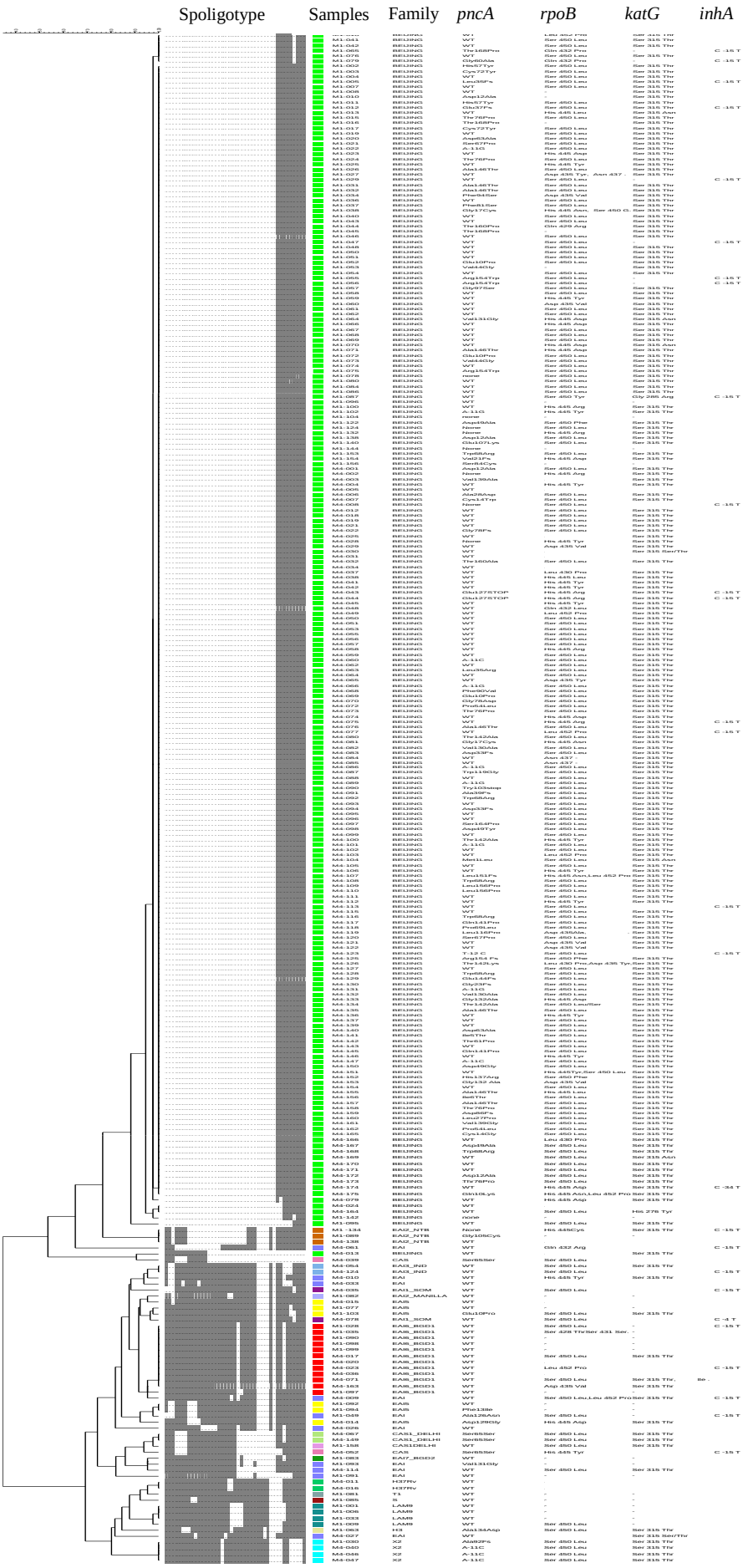

Fig 9. Phylogenetic tree based on spoligotypes patterns and drug resistances associated mutations of the 285 MDR-TB isolates

As nearly 50% of isolates with *rpoB* and *katG* mutations carried *pncA* mutations. This highlighted that half of the clinical MDR-TB cases may have the risk of PZA-R. And it can be proposed to include *pncA* sequencing in every isolate with an *rpoB* mutation, allowing for decision of choosing MDR-TB treatment.

The genotyping analysis did not highlight any association between specific *pncA* mutations and the different MTB families or clusters, in agreement with other studies. [48, 79, 80]. Nevertheless, the mutation frequency was higher in Beijing isolates compared with other families or genotypes, suggesting the strong association of this family with PZA-R ($p<0.001$). I thus hypothesize that the Beijing family may be more prone to acquiring *pncA* mutations. Although the situation differs by country [79, 81] the Beijing family seems to be strongly linked to PZA-R in Myanmar MDR-TB isolates like other neighbored countries [58, 60, 75].

Not only the findings from this study but also the evidence from other parts of the world, the rate of PZA-R is high in MDR-TB patients and in most cases the resistance results from the mutation in the *pncA* gene. The major limitation of the study is the fact that phenotyping PZA susceptibility test was not be able to perform because it is not routinely done for the diagnosis of drug resistant TB and strains were not stored at the NTRL, Myanmar. For genotyping analysis, it is better to investigate the MIRU-VNTR typing to identify more precise cluster or shared mutation too. Nonetheless, most of the *pncA* mutations (125 isolates, 43.8%) detected in our study are likely to be associated with phenotypic PZA-R according to previous reports and may suggest that the estimated PZA-R among MDR-TB is highly similar to other previously reports. As *rpsA* and *panD* mutation might have no or little role in PZA-R as previously described [53, 54, 59], I did not analyse these genes among the isolates. Furthermore, as all of the isolates from this study were the only representative of MDR-TB, non MDR-TB isolates should be analyzed to better identification of the possible prevalence of PZA-R among DR-TB isolates.

The significantly high prevalence of mutations in *pncA* or its putative regulatory region that likely to be associated with PZA-R in MDR-TB isolates (43.8 %) highlights the need for PZA susceptibility testing in patients infected with MDR-TB. Furthermore, this data can suggest that there is a need to reconsider the empirical usage of PZA in the MDR-TB treatment regimens. It can also be concluded that the study of PZA–R mechanisms among MDR-TB isolates and identification of PZA-R TB were fundamental for controlling of TB and MDR-TB.

The prevalence of a high level PZA-R among MDR-TB patients in Myanmar and many other countries highlighted the fact that PZA susceptibility test should be performed to have significant effect on the treatment outcome. As 92.2% (125/130) of the isolates with *pncA* mutation are suggested to be likely associated with *pncA* mutations, mutation analysis by DNA sequencing might be the suitable alternative tool for rapid detection of PZA-R in that one of the resources limited countries like Myanmar.

Summary

Pyrazinamide (PZA) is a first line anti-tuberculosis drug and included in both short course and multi-drug resistant tuberculosis (MDR-TB) treatment regimens. It is a pro-drug and needed to be converted to an active form by the mycobacterial enzyme pyrazinamidase encoded by *pncA*. Numerous studies reported that *pncA* mutations was responsible for PZA-R by reducing or abolishing PZase activity in *M. tuberculosis*. This study aimed to detect the frequencies and patterns of *pncA* mutations and their association to *M. tuberculosis* families in MDR-TB isolates in Myanmar. First-line drug susceptibility testing, *pncA* gene sequencing and spoligotyping were performed for 285 MDR-TB isolates. The frequency of *pncA* mutation was 45.6% (130/285) while 54.4% (155/285) showed wild-type sequences. Among 130 isolates with *pncA* muttaions, 125 isolates (96.2%), were suggested to be likely associated with PZA-R. Seventy-four different types of mutations were distributed on the whole *pncA* including putative regulatory region, of which 26 varity of mutation presented in two or more isolares. Eighteen-types of 19 novel mutaions were suggested to be associated with PZA-R according to the mutation patterns. Although the highest frequency of *pncA* mutation belonged to Beijing Family isolates (50%, 115/230), genotyping data highlighted that *pncA* mutation can occur in any *M. tuberculosis* family.

Finally, it can be suggested that the rates of *pncA* mutations likely associated with PZA-R in MDR-TB strains, 43.8 %, were found to be similar with the rates (nearly 50%) reported in other countries. The findings pointing out that the high frequency and diverse mutations of *pncA* that associated with PZA-R in MDR-TB isolates from Myanmar and rapid and simple genotypic PZA susceptibility assays is needed to be developed. As PZA has synergestic action with other new drugs or compounds agaist TB¸ rotuine assement of the possible prevalence of PZA-R by DNA sequencing should call an action to decide the effcetive treatment regime for TB as well as to consider the developrment of new target anti-TB drugs. According to the findings of previous studies, as not all

pncA mutation are associated with PZA-R, further studies on the correlation between mutations in *pncA* and pyrazinamidase activity are needed. As 50% of *pncA* mutation harbored *rpoB* mutaions, it can be proposed that *pncA* sequencing in every isolates with *rpoB* mutations can allow for the seletion of MDR-TB treatment. In addition, as according to the national guideline of drug resistance TB, Myanmar, patients with RIF resistance detected by GeneXpert were started the MDR-TB regimes which include PZA, and therefore the information of this study can somehow helpful in the decision for usage of PZA in MDR-TB treatment regime.

CONCLUSION

Although MDR-TB is one of the emerging public health problems in Myanmar, limited studies had been reported to determine the prevalence of drug resistance-conferring mutations among MDR clinical isolates. In addition, information regarding to the frequency and pattern of drug-resistance associated genes mutations among MDR-TB isolates is necessary for the developing of rapid DST to achieve successful TB treatment and control in Myanmar.

Numerous studies report that mutations of *rpsL* (encoding the S12 protein), *rrs* (encoding 16S rRNA) and *gidB* (encoding rRNA methyltransferase) are responsible for STR resistance. In chapter I of this thesis, I explored the variation and frequency of mutations in *rpsL*, *rrs* and *gidB* in 141 STR-resistant MDR-TB isolates from Myanmar. Most isolates belonged to the Beijing genotype (105, 74.5%). Mutations in *rpsL* were identified in 69.5% (98/141) of the STR-resistant isolates, where the most prevalent (92.0%, 90/98) and significantly associated mutation with the Beijing genotype ($P < 0.001$) was Lys43Arg. Fifteen different types of mutations in *gidB* were found in 16.3% (23/141) of the isolates, and most of them were novel. Sequence analysis of *rpsL*, *rrs* and *gidB* with a sensitivity of 83.7% satisfactorily predicted STR resistance in Myanmar isolates.

In chapter II, I had characterized the prevalence and pattern of mutations occurred in the drug target for pyrazinamide (PZA) in MDR-TB isolates in Myanmar. Forty-four percent of MDR-TB isolates had mutations likely to associate with phenotypic PZA resistance. In addition, the genetic backgrounds of these isolates were analyzed by spoligotyping and investigated whether any association of different genotypes with specific mutations in *pncA*. The most frequent mutations among MDR-TB isolates were found in three hot spot regions (codon 3-17, codon 61-85, codon 127-154) similar to those found worldwide. Majority of the isolates showed diverse *pncA* mutation with the same spoligotype, indicating that there is no large outbreak. However, 23 kinds of *pncA* mutation were found in multiple isolates. This fact may suggest that there is the possible

transmission of those mutant strains in Myanmar because mutations in *pncA* occurred randomly and it might be rare same mutation to be carried by isolates without any correlation.

The findings from this study can provide the expanded knowledge on molecular drug resistance mechanism of anti-TB drugs of STR and PZA. Moreover, the frequency and patterns of drug resistance-associated mutations can support the development of rapid and easy applicable DST tools which could contribute to the development of control strategies for the increasing rate of DR-TB in Myanmar.

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