Drug-resistant *Mycobacterium tuberculosis* and its genotypes isolated from an outbreak in western Thailand

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Abstract

Background

Multidrug-resistant tuberculosis outbreaks have occurred in the Thamaka district, Kanchanaburi province in Thailand. Here, we aimed to characterise this pathogen and its genotypes.

Methods

Seventy-two *Mycobacterium tuberculosis* isolates were collected and genotyped by spoligotyping 15-locus mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) and single nucleotide polymorphism genotyping, and their drug resistance was analysed.

Results

The spoligotyping results showed that Beijing SIT1 was the predominant sub-lineage (n=38, 52.8%) whilst the remaining were non-Beijing sub-lineages (n=34). The MIRU-VNTR analysis showed that the most Beijing isolates (n=37) belonged to the modern type, forming 5 clusters and 13 individual patterns. In *katG*, only mutation Ser315Thr was identified. In *rpoB*, Ser531Leu was the predominant except His526Arg and Leu533Pro found in two isolates. A cluster of 14 Beijing strains contained these common mutations and shared the MIRU-VNTR genotype with isolates spreading previously in the Thamaka district. Two U SIT523 isolates contained mutations A1400G in *rrs* and Asp94Gly in *gyr*A genes, indicating a spread of extensively drug-resistant tuberculosis (XDR-TB).

Conclusions

Most mutations were associated with drug resistance, and the specific MDR Beijing and XDR-TB in U SIT523 isolates are remaining. This genotyping was likely a key tool for tracking TB transmission in the Thamaka district, Thailand.

Keyword: MIRU-VNTR, Mutation, Outbreak, Spoligotyping, Thailand, Tuberculosis

Introduction

Tuberculosis (TB) caused by the *Mycobacterium tuberculosis* complex is a serious health-deteriorating disease and one of the leading causes of deaths in the world. Thailand has a high TB burden, with approximately 120 000 new cases in 2018, and ranked amongst the top 30 TB-burdened countries. TB infection in Thailand, with a population of about 69 million people, was reported to have an incidence rate of 153 new cases per 100 000 inhabitants. Multidrug-resistant tuberculosis (MDR-TB) caused by *Mycobacterium tuberculosis* (MTB) resistant to at least isoniazid (INH) and rifampicin (RIF), was detected at about 2.3% and 24% of both new cases and retreated patients, respectively.[1] The Kanchanaburi province, located in western Thailand, is one of the areas that has a high incidence of MDR-TB. In particular, the Thamaka district in Kanchanaburi had been documented as a hotspot, with multiple community outbreaks of MDR-TB from 2002 to 2010.[2] An epidemiological investigation, proper strategies, and rapid responses, including effective treatments, are deemed necessary to control TB transmission in the epidemic area.

Occurrence of drug-resistant TB can be categorised into primary and secondary drug resistance. The primary drug resistance is caused by an infection with drug-resistant MTB, whilst secondary drug resistance is an acquired resistance developed at a later stage by drug-susceptible strains.[3] Major mechanisms of drug resistance that primarily involve genetic mutations have been reported in a number of studies.[4-5] For example, mutations in the *katG* gene account for 30-75 % of INH resistance and are associated with a high-level resistance to INH. The most frequent mutation in the *katG* gene is Ser315Thr. In addition, a point mutation in the regulatory region of *inhA* promoter, mainly at the positions -15 and -8, accounts for 6-30% of INH resistance, resulting in a low-level resistance to INH.[6-7] Meanwhile, 90-96% of rifampicin resistance is caused by mutations in the *rpoB* gene in which the 81 bp of the rifampicin resistance determining region (RRDR) between codons 507–533 is considered a mutation hotspot region.[7] The increasing incidence of MDR and extensive drug-resistant tuberculosis (XDR-TB; MDR plus resistance to fluoroquinolone and aminoglycoside) have become major hurdles to treat TB effectively.[8] To date, molecular

epidemiological data from many countries have demonstrated that MDR and XDR-TB have been detected around the world, and hence become a serious threat to proper TB control. To overcome this epidemiological problem, efficient epidemiological strategies are needed.[9,10]

For the purpose of molecular epidemiology, a number of genotyping methods have been reported and used to differentiate MTB strains. For example, spoligotyping[11], analysis of MIRU-VNTR[12], single nucleotide polymorphism (SNPs)[13], region of differences (RDs), large sequence polymorphisms (LSP)[13] and restriction fragment length polymorphism (RFLP)[13] can be listed amongst the methods extensively used for typing MTB strains. Recently, whole genome sequencing (WGS) has been used to genetically characterise MTB.[14] This method can provide comprehensive genetic information and help deepen our understanding of the factors affecting TB transmission. The genotyping of MTB is a molecular tool that can be used to investigate possible epidemiological links between TB patients, detect suspected outbreaks and distinguish exogenous re-infection from endogenous reactivation in relapse cases.[12] For example, spoligotyping is a simple and rapid genotyping method that focuses on the detection of polymorphisms based on the presence or absence of 43 unique spacer sequences in the direct repeat (DR) regions.[11] Spoligotyping has been used to categorise MTB strains into several families, such as Beijing, East-African Indian (EAI), Central Asian (CAS), Latin American Mediterranean (LAM), Unclassified (U) and T families.[15] The analysis of variable-number tandem repeat of mycobacterial interspersed repetitive units (MIRU-VNTR) is another genotyping method that has been widely used for monitoring the global spread of TB.[16] The combination of spoligotyping and MIRU-VNTR typing methods has become a traditional and practical tool providing a resolution high enough for the clear identification of MTB strains.[17] Generally, MIRU-VNTR has been used after spoligotyping to further subtype major MTB groups including the Beijing family, which commonly causes several major outbreaks worldwide, is over-represented in drug-resistant isolates and seems to have unique virulence properties.[13]

Outbreaks of TB along the western border of Thailand with Myanmar are still a problem as genotyping is not routinely conducted during these events. The present study was conducted to gain

an insight into the spread of TB in the Thamaka district of Kanchanaburi province, Thailand and neighbouring regions. Genotyping was conducted by spoliogotyping, MIRU-VNTR, and SNP analysis. Mutation characteristics in targeted genes associated with INH, RIF, fluoroquinolone and aminoglycoside resistance in MTB isolates were also analysed.

Materials and Methods

Processing of MTB strains and samples

A total of 72 MTB isolates from Thai pulmonary-TB patients (registered for TB treatment during 2013-2014 MDR-TB outbreak in Thamaka District, Kanchanaburi Province) and strains isolated from surrounding areas in western Thailand, Samut Sakhon and Prachuap Khiri Khan Provinces, were analysed. The locations of the sample collection sites are showed in Figure 1. These isolates were obtained from decontaminated sputum samples that were inoculated into BACTEC MGIT™ liquid media (Becton Dickinson®,USA). Drug susceptibility testing (DST) for first-line drugs including INH, RIF, ethambutol (EMB), and streptomycin (STM) was conducted using BACTEC MGIT™ 960 SIRE Kit, as per the manufacturer's instructions. A second-line drug susceptibility test was carried out only on some isolates (data not shown).

DNA was extracted from 500 µl of MTB cell culture suspension recovered from the BACTEC 960 MGIT media using magnetic bead-based nucleic acid extraction (SEEPREP 12^{TM}, Seegene, Inc., Korea) as per the manufacturer's protocol. The extracted DNA with a final volume of 50 µl was kept at -20^{O} C until further molecular analyses.

Genotyping

MTB isolates were genotyped by spoligotyping as previously described.[11] Briefly, the direct repeat (DR) region in chromosomal DNA of MTB was amplified by PCR. The hybridisation of PCR products to specific DNA probes was then screened for spoligo-patterns. The obtained spoligo-patterns were converted to an octal code and compared with those in the international spoligotyping database. SpolDB4 was used to identify spoligo-international types (SITs).[15]

To subtype isolates, the selected 15 MIRU-VNTR (424, 580, 802, 960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, 4156, 4348) were analysed according to a method previously described.[17,18] After amplification, the copy numbers of tandem repeats were determined from the sizes of amplicons by agarose gel electrophoresis using 50 bp DNA ladders (New England, Biolabs®Inc.) as markers.[12] A numerical value profile was assigned to each strain according to the number of variable repeats in each VNTR allele.

The allelic diversity amongst the MIRU-VNTR isolates was determined. The Hunter-Gaston Discriminatory Indexes (HGDIs) were calculated by the following equation: HGDI = 1- (1/N(N-1)) ($\sum$ nj(nj-1), where N is the total number of isolates in the sample population, S is the total number of allelic, and n_j is the frequency of the allele in the locus. The discriminatory power was considered high if the HGDI value was >0.6, moderate if HGDI was between 0.3 to 0.6 and low if HGDI was <0.3, according to the definition by Sola *et al*., 2003.[19]

Analysis of clustering

Spoligotyping patterns and 15-locus MIRU-VNTR typing data were analysed by MIRU-VNTR*plus* (http://www.miru-vntrplus.org/MIRU/index.faces) to establish the lineages and sub-lineages.[20] Dendrograms by the unweighted pair group method with arithmetic means mode (UPGMA) were generated. In the present study, the genotypes of Beijing isolates were compared with those of a previously published study.[10] A mini spanning tree (MST) was constructed based on the data of 15- locus MIRU-VNTR analysis using BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium). Clusters were defined as two or more isolates sharing an identical 15-locus MIRU-VNTR pattern. The clustering rate was calculated using the following formula: number of clustered isolates/ total number of isolates.

SNP analysis and gene mutation detection by sequencing

Single nucleotide polymorphism (SNP) at the 1477596 locus was examined to subdivide modern and ancestral Beijing genotypes.[13] DNA samples of Beijing isolates were amplified by PCR. The PCR mixture contained 1x Go*Taq* buffer (Promega Co., Madison, WI), 0.26 mM of each dNTP,

0.3 µM of each primer, 0.5 M Betaine, 0.5 units of Go*Taq* DNA polymerase and DNA of MTB. DNA sequences were aligned through pairwise alignment of the *M. tuberculosis* H37Rv using Bio-Edit software version 7.09.[21]

DNA samples from drug-resistant strains were subjected to an analysis of mutations in genes associated with drug resistance, targeting the following: the *katG* coding sequence and *inhA* promoter region for INH; rifampicin resistance determining region (RRDR) in *rpoB* for RIF; quinolone resistance determining region (QRDR) in *gyrA* for fluoroquinolone; and 16S ribosomal RNA gene in *rrs* for kanamycin or aminoglycosides.[8,22] The PCR components used were the same as those described above. The amplified DNA fragments were subjected to sequence analysis using BigDye terminator V3.1 (Life Technologies Co., CA) reagents and a 3130 genetic analyser (Life Technologies Co., CA), as per the manufacturer's protocol. Sequences were compared with those of wild-type reference H37Rv and analysed using Bio-Edit software.

Results

MTB phenotyping by drug susceptibility testing

Based on the drug susceptibility testing, phenotypes resistant to four first-line anti-TB drugs were identified in all 72 MTB isolates. Of them, 62.5% (n=45) were susceptible to INH and RIF, whilst the rest (n=27) were drug-resistant MTB, including RIF mono-, INH mono-resistant, and MDR at 5.6% (n=4), 1.4 % (n=1) and 30.6% (n=22), respectively (Table 1).

Spoligotyping

The spoligotyping results showed clustering amongst 59 isolates and 13 different non-clustered spoligotypes (Table 2). Overall, there were 20 spoligotype patterns and the clustering rate was 82%. The largest cluster belonged to the Beijing SIT1 family (n=38, 53%). Non-Beijing (n=34, 47.2%) included EAI2_NTB (n=6, 8.3%), EAI5 (n=8, 11.2%), EAI6_BGD1 (n=4, 5.6%), H3 (n=1, 1.4%), T1 (n=2, 2.8%) U (n=6, 8.4%) and new spoligotypes (n=7, 9.8%) (Table 2). There were 6 small clusters of T1 SIT53 (n=2), EAI2_NTB SIT89 (n=6), EAI5 SIT236 (n=3), EAI5 SIT256 (n=4), U SIT523 (n=4), and EAI6_BGD1 SIT591 (n=2) amongst 21 non-Beijing, as shown in Table

2. The rest of the non-Beijing group (n=13, 18%) was non-clustered (Table 2). Of 38 Beijing (SIT1) genotypes, 19 (50%) were drug-resistant and 19 (50%) susceptible to both INH and RIF.

MIRU-VNTR analysis

According to the 15-locus MIRU-VNTR analysis, 5 different subtypes were generated amongst 25 Beijing isolates consisting of Bj-I (n=16), Bj-II (n=2), Bj-III (n=2), Bj-IV (n=2) and Bj-V (n=3). The remaining of the 13 Beijing isolates were single or non-clustered Beijing subtypes (Figure 2A). The largest cluster was a group of Bj-I subtypes that contained 16 isolates (42%, 16 of 38 Beijing), including 14 MDR-TB isolates, 1 mono RIF- resistant and 1 susceptible isolate. All members of Bj-I cluster were from the Kanchanaburi Province. In addition, Beijing drug-susceptible strains were found in clusters of Bj-II, Bj-III, and Bj-IV. The other three Beijing isolates were categorised into the Bj-V cluster. These three isolates were from the Samut Sakorn Province. Two of these isolates were MDR and one was INH mono-resistant. The remaining Beijing isolate was from the Prachuap Khiri Khan Province and non-clustered (Figure 2A). In non-Beijing groups, the U SIT523 was found to have an identical MIRU-VNTR pattern in 2 of 4 isolates (Figure 2B). The MIRU-VNTR pattern in the matching U strains was 443824652641113. Overall, 59 clustered isolates that shared spoligotypes were subtyped by the MIRU-VNTR analysis, generating 38 different MIRU-VNTR patterns (Figure 2). Of these, 6 patterns formed major clusters containing 27 isolates, while 32 unique individual MIRU-VNTR patterns were identified. The dendrogram resulting from clustering analysis is shown in Figure 2.

Analysis of discriminatory power and diversity

A discriminatory power of the selected 15-locus MIRU-VNTR for differentiating the Beijing genotype was observed (Table 3). The comparison results showed that the HGDI value of 19 Beijing drug-resistant isolates (MDR, INH mono-resistant and RIF mono-resistant) was 0.38 lower than that of drug-susceptible Beijing isolates, with a value of 0.98. In the drug-susceptible Beijing group, allelic diversity was highly discriminated in locus 2163b and moderate in loci 0424, 0802, 1955, and 2996. Thus, 2 clusters consisting of 4 isolates and 15 single isolates were discriminated

from drug-susceptible Beijing strains. Nonetheless, although this selected 15-locus MIRU-VNTR had a high power to distinguish Beijing isolates susceptible to INH and RIF, it could not differentiate the MDR Beijing family that included 14 of 16 isolates in the Bj I cluster and 2 of 3 isolates in the Bj-V cluster.

SNP for identification of modern and ancestral Beijing

SNP data from locus 1477596 indicated that 37 of 38 Beijing isolates were either a SNP-T type or a modern Beijing sub-lineage (97%). Only one isolate was observed to have a SNP-C type (ancestral Beijing subtype) (Figure 2A).

Identical MTB genotypes

MIRU-VNTR data from Beijing isolates that were collected from past TB outbreaks occurring during the periods 2006-2012 and 2013-2014 were retrieved from a previous report[10] and compared with those from the present study (Figure 3). The results showed a major clonal expansion of 62 MDR-MTB isolates originated from previous outbreaks in 2006 (n=3), 2007 (n=1), 2008 (n=5, including 1 XDR-TB isolate), 2009 (n=8), 2010 (n=5), 2011 (n=1), and 2012 (n=1), and isolates in the Bj- I cluster (Figure 3) from the present study (n=38).

Mutations associated with drug resistance

The sequencing results showed a single type of mutation in the *katG* gene at a codon Ser315Thr in all INH-resistant isolates. Meanwhile, a substitution of Ser531Leu in *rpoB* was found in almost all RIF-resistant strains except for two isolates carrying His526Arg and Ser533Pro. Of those 22 MDR, 20 isolates, which included 16 Beijing, 1 EAI6_BGD1 (SIT591) and 3 U (SIT523), had a common mutation of Ser315Thr in the *katG* gene and Ser531Leu in the *rpoB* gene for typical INH and RIF resistances, respectively (Figure 2). Only one Beijing MDR strain was found to carry Ser315Thr in the *katG* gene but had a different mutation of His526Arg in the *rpoB* gene. This isolate was from the Samut Sakorn Province. Another one had no data of DNA sequences in any of the targeted genes. As for MDR, a common mutation of Ser531Leu in the *rpoB* gene was found in 3 of 4 mono-RIF-resistant isolates that were Beijing, EAI5 SIT526, and new spoligotypes, while

Leu533Pro was carried by EAI6_EGD1 SIT591. Most of the MDR isolates had no mutations in the *rrs* and the *gyrA* genes, which conferred resistance to kanamycin and fluoroquinolone, respectively. The exceptions were two isolates of the U SIT523 strain, which were observed to have the additional mutations A1400G and Asp94Gly in the *rrs* and the *gryA* genes, respectively (Figure 2B). Therefore, these two identical U SIT523 isolates were considered to be strains that cause a transmission of XDR-TB.

Discussion

A number of MDR cases has been detected in the Thamaka district, in the Kanchanaburi province, Thailand, and epidemic outbreaks of MDR-TB have since been continuously reported in these areas. Previously, the potential of a specific MDR-TB strain was reported to cause a continuous outbreak of drug-resistant TB, and showed the genetic diversity of MTB strains in these region.[2,3] Currently, molecular detection is being conducted for rapid detection of drug-resistant cases, but no genotyping is routinely carried out. Therefore, the genetic information of clinical isolates currently available is still limited. A previous study using retrospective isolates from the year 2006 showed that most of the MDR strains belonged to the Beijing genotype and that U SIT523 was the XDR-TB strain.[10] The findings of the present work confirmed that primary transmission of drug-resistant TB was the major factor driving the increase of TB drug resistance in local outbreaks in Thailand and that a drug resistance mechanism related to INH and RIF was caused by common mutations in genes *katG* and *rpoB*.

Molecular epidemiology has helped deepen the understanding of the TB epidemic in the studied Thai regions. Furthermore, our spoligotyping results indicated that more than 50% of MTB strains were clustered and belonged to the Beijing family. Based on the DST results and spoligotyping, we showed that the Beijing family was prevalent and the majority of the strains were drug-resistant. In addition, almost all Beijing members in the present study belonged to the modern type, which was determined by SNP at the position of 1477596.[13,24,25] The modern Beijing strain has become a great concern in epidemiology because it is spreading worldwide and causing

outbreaks in many countries.[26,27] In the present work, only one isolate was the ancestral Beijing type, which is usually associated with low virulence of tuberculosis.[13,24] This finding was in agreement with previous reports of TB incidence in many countries where the Beijing type was predominant.[26,27,28] A recently study reported that about 66% of Beijing strains were drug-resistant and that were prevalent in Thai tuberculosis patients.[29] Beijing strains have become a global health threat because of their frequent association with a high mutation rate, hypervirulence, immune evasion, treatment failure, and drug resistance.[30]

In the present study, the analysis of molecular data based on 15- locus MIRU VNTR showed diverse genetic backgrounds amongst drug-susceptible Beijing strains. In contrast, MDR Beijing strains had restricted genetic backgrounds. As a result, we proposed that the method provided a discrimination power high enough to differentiate MTB strains in the epidemic Thai areas. We found that the largest cluster of Beijing isolates circulating in these areas was the Bj-I subtype. Compared with a previous study (Figure 2), the specific Bj-I genotype of MDR-MTB was also found to be dominant, forming the largest cluster among Beijing strains. As shown in Figure 2A, the successful expansion of a specific clone of the Bj-I sub-lineage resulted in restricted genetic backgrounds. From this evidence, we proposed that the specific clone of Beijing sub-lineage with multidrug resistance was highly transmissible and may be the major cause of MDR-TB outbreaks in the Thamaka district. Our mini span diagram clearly showed that predominant cluster-sharing genotypes, identical to past strains, have been continuously spreading to date. Rapid identification of TB cases, patient isolation, and appropriate treatments are required for a future, more effective outbreak control.

Molecular drug resistance analysis by DNA sequencing showed that genetic alterations were highly associated with drug-resistant phenotypes and identified common mutations in the *katG* and the *rpoB* genes. Additional mutations conferring resistance to a fluoroquinolone and injectable aminoglycosides were further identified in two of three U SIT523 isolates, suggesting the emergence of XDR-TB. The absence of second-line drug resistance amongst a large number of

MDR-TB genotypes suggested that under a MDR treatment, acquisition of additional resistance to second-line drugs was limited. MDR was also found in non-Beijing strains, but in a low percentage, accounting for merely 12.5 % (4 of 22) (Figure 1). Occurrence of MDR with individual unique genotypes in Beijing and non-Beijing groups could be explained by the fact that MTB developed a spontaneous mutation or acquired drug resistance resulting in the development of new drug-resistant strains.

It should be mentioned that the present study had several limitations. For example, the sample size of the MTB population was limited by the short time of the sample collection. In addition, the demographic and clinical data obtained for each patient was limited. Nonetheless, even though the number of samples were small, we were able to find clusters of MTB amongst the isolates from the Kanchanaburi Province. It is also worth noting that in neighbouring provinces, no identical genotypes to those identified in Kanchanaburi were identified. These results may imply that there was no TB spread across the provinces, but the evidence remains inconclusive due to the sample sizes from other adjunct provinces were small.

Conclusions

Our data suggested that the specific Beijing genotype strain with MDR was the most prevalent MTB strain causing a continuous TB transmission in the Thai areas studied. Our results also showed that this specific MTB strain, which was dominant, virulent, and resistant to isoniazid and rifampicin, has not been eliminated from the region. In addition, it was demonstrated that XDR-TB concurrently emerged and caused transmission. Finally, in the present work, we showed that genotyping based on spoligotyping and 15-MIRU-VNTR analysis was a useful tool for successfully discriminating MTB strains.

Author Contributions

SB, KS, NT, YF conducted the analysis and interpretation of data. JR and BP designed the study, the analysis and the interpretation of data, and drafted the munuscript. CN and YS carried out the analysis and interpretation of data and edited and approved the final version.

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Legend

Figure 1. Map of sample collection sites and the number (*n*) of isolates from the Thamaka District, Kanchanaburi Province and surrounding areas in western Thailand, and Samut Sakhon and PrachuapKhiri Khan Provinces, Thailand.

Figure 2. Clustering analysis; A, Dendrogram of genetic relationships between 38 Beijing isolates from Kanchanaburi; B, Dendrogram of genetic relationships between 21 non-Beijing isolates based on the 15 loci-MIRU-VNTR analysis. Spoligotypes, phenotypic and genotypic characteristics of drug resistance were incorporated. **Abbreviations:** ID, identification; SIT, spoligo-international types; DST, drug susceptibility testing; MIRU-VNTR, mycobacterial interspersed repetitive unit-variable number tandem repeat; SNP, single nucleotide polymorphism; INH, Isoniazid; RIF, Rifampicin; Kan, Kanamycin; S, Susceptible; R, Resistance; WT, wildtype; Ser, Serine; Thr, Threonine; His, Histidine; Arg, Arginine; Leu, Leucine; Ser, Serine; Thr, Threonine; His, Histidine; Arg, Arginine; Leu, Leucine; Asp, Asparagine; Gly, Glycine; A, Adenine; G, Guanine; NA, Not applicable.

Figure 3. Comparison of genotypes among Beijing isolates in Thai outbreak areas in previous (during the year 2006-2012) and recent (during the year 2013-2014) outbreaks,based on MIRU-VNTR; A minimum spanning tree was constructed based on 15 loci of MIRU-VNTR genotyping of the Beijing strains (n=24) isolated during the period 2006-2013, as reported in a previous study and the present work (n=38). Circles denote different types discriminated by 15 locus-MIRU-VNTR genotypes. The origins of each isolate are representedby different colours.

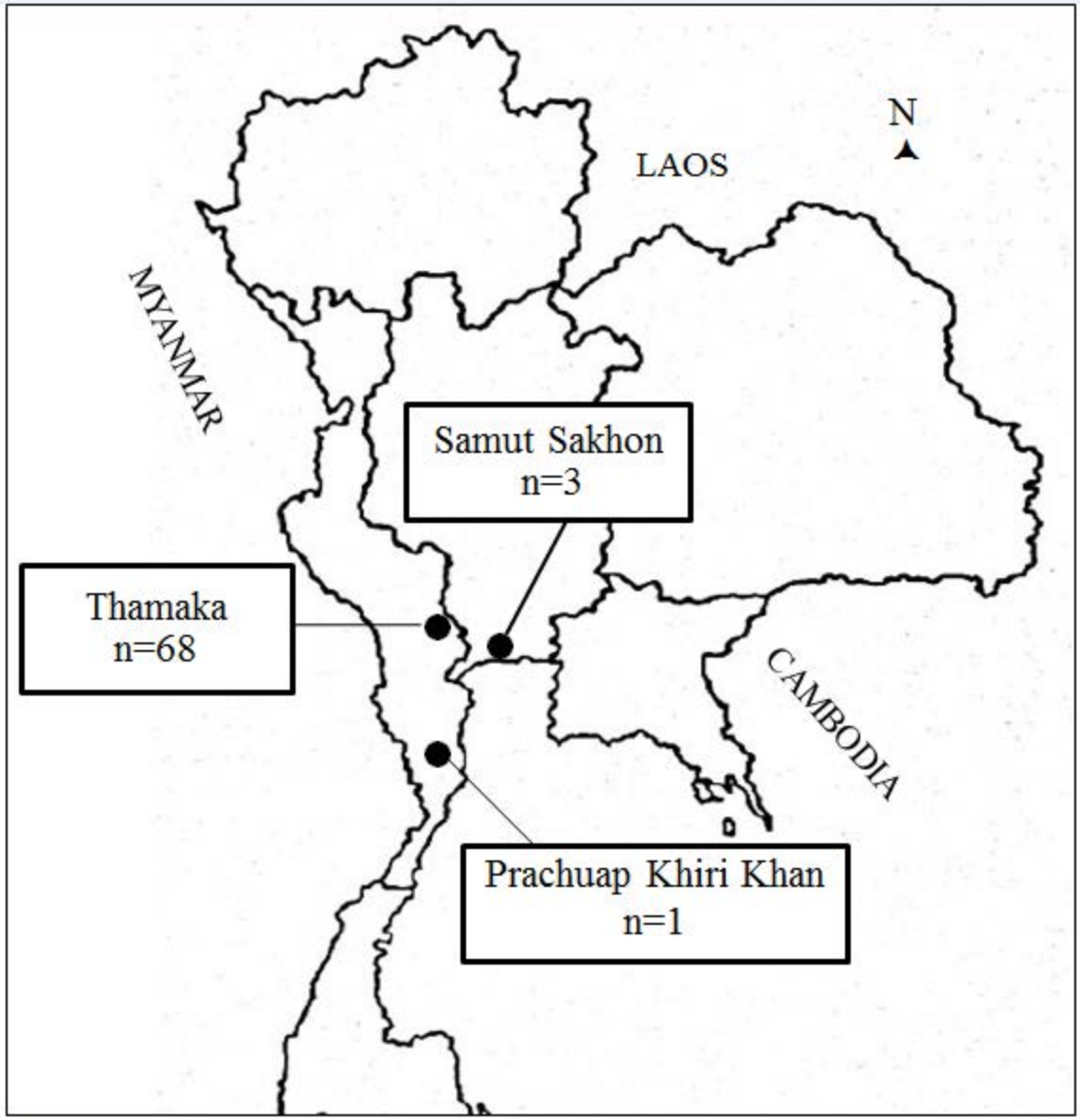
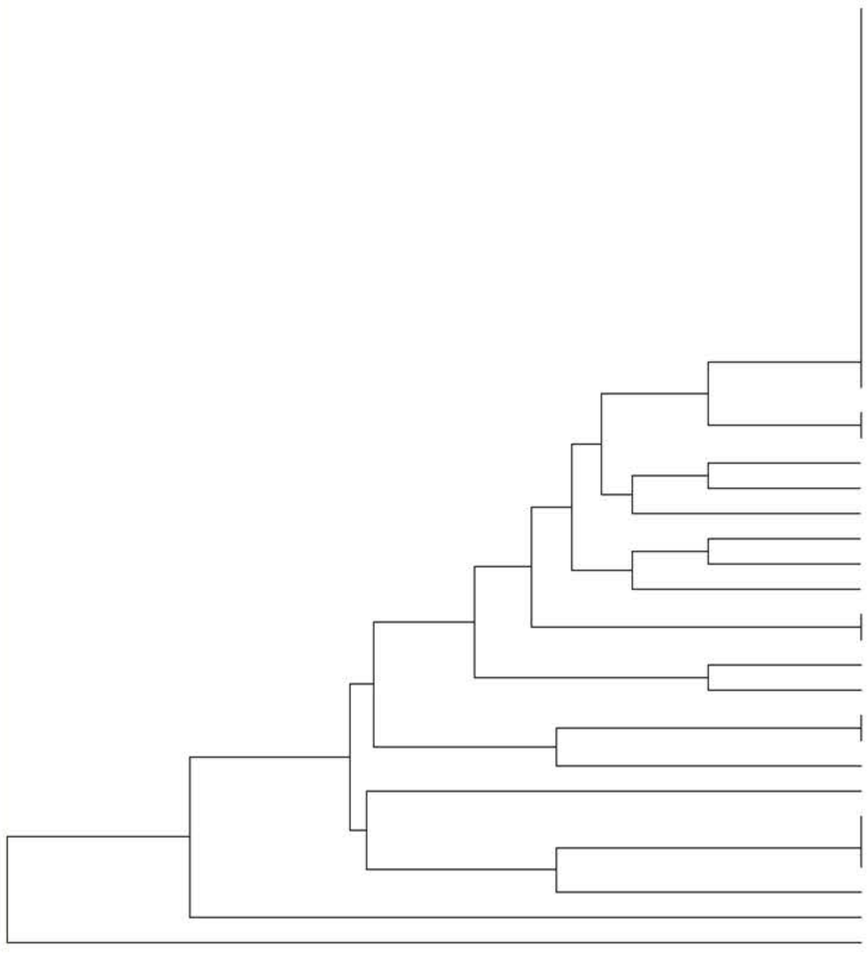
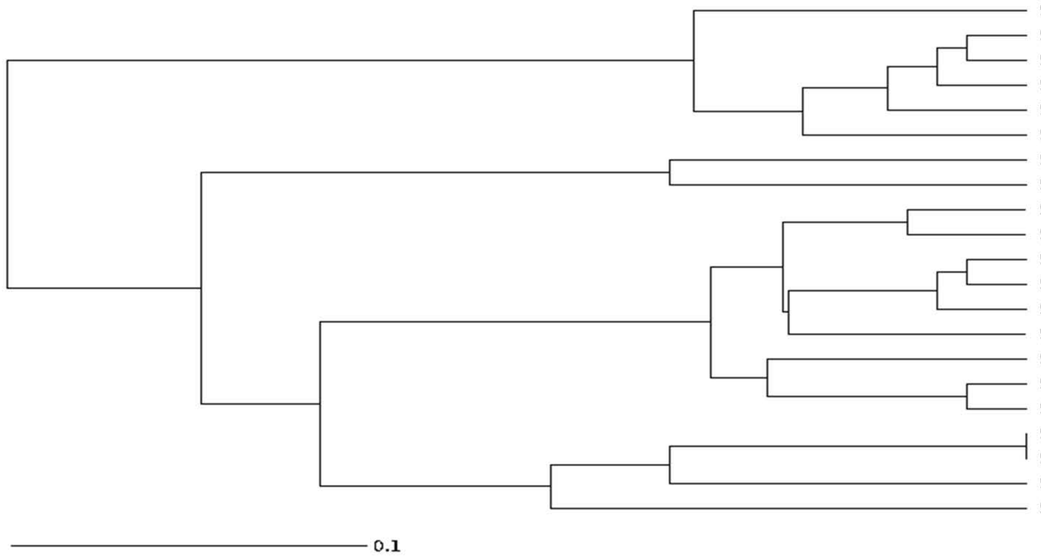


Figure 1. Map of sample collection sites and the number (*n*) of isolates from the Thamaka District, Kanchanaburi Province and surrounding areas in western Thailand, and Samut Sakhon and Prachuap Khiri Khan Provinces, Thailand



ID	SIT	Clade	MIRU-VNTR Locus															Province	Drug resistance detection DST				Drug resistance detection Gene mutation					Cluster	SNP 1477596
			424	580	802	960	1644	1955	2163b	2156	2401	2996	3192	3690	4052	4156	4348		INH	RIF	EMB	STM	*katG*	*inhA* promoter	*rpoB*	*gyrA*	*rrs*		
56-0754	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0755	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0781	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	R	S	R	WT	WT	Ser531Leu	WT	WT		T
56-0830	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0840	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0926	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0949	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-1043	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT	Bj-I	T
56-1052	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-1103	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-0003	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-0287	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-0298	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-0302	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-0680	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0754	1	BEIJING	4	2	3	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-0836	1	BEIJING	4	2	3	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
56-1149	1	BEIJING	4	2	3	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-II	T
56-1023	1	BEIJING	4	2	3	3	3	5	4	4	4	5	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
56-999	1	BEIJING	4	2	3	3	3	5	6	4	4	5	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0423	1	BEIJING	4	2	3	3	3	5	6	4	4	4	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
56-0941	1	BEIJING	4	2	2	3	3	5	4	4	4	7	5	3	7	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0375	1	BEIJING	4	2	2	3	3	5	4	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0707	1	BEIJING	4	2	2	3	3	5	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0043	1	BEIJING	4	2	3	3	3	5	6	4	5	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-III	T
57-0114	1	BEIJING	4	2	3	3	3	5	6	4	5	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0198	1	BEIJING	4	2	3	3	3	5	6	4	4	8	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0312	1	BEIJING	2	2	3	3	3	5	6	4	4	8	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
56-0769	1	BEIJING	3	2	3	3	3	7	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA	Bj-IV	T
56-1010	1	BEIJING	3	2	3	3	3	7	6	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	R	NA	NA	NA	NA	NA		T
56-799	1	BEIJING	3	2	3	3	3	2	5	4	4	7	5	3	8	2	3	Prachuap Khiri Khan	S	S	S	S	NA	NA	NA	NA	NA		T
56-0952	1	BEIJING	5	2	1	3	3	5	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	R	NA	NA	NA	NA	NA		T
56-866	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	R	S	R	Ser315Thr	WT	His526Arg	WT	WT	Bj-V	T
56-998	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	R	R	R	Ser315Thr	WT	Ser531Leu	WT	WT		T
57-147	1	BEIJING	4	0	5	3	3	5	5	4	4	7	5	3	8	2	3	Samut Sakorn	R	S	S	R	Ser315Thr	WT	WT	NA	NA		T
56-868	1	BEIJING	4	0	3	3	3	3	5	4	4	7	5	3	8	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T
57-0530	1	BEIJING	4	2	3	3	3	4	5	4	4	7	5	3	10	5	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		C
56-0828	1	BEIJING	3	2	3	2	3	5	2	4	4	7	5	4	8	2	4	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA		T



ID	Spoligotype		MIRU-VNTR profile														Province	DST				Gene mutation				
			Locus																							
	SIT	Clade	424	580	802	960	1644	1955	2163b	2156	2401	2996	3192	3690	4052	4156	4348		INH	RIF	EMB	STM	*katG*	*InhA promoter*	*rpoB*	*rrs*	*gyrA*
56-0760	89	EAI2_NTB	1	9	2	4	2	10	1	4	2	2	3	2	5	1	2	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-0854	89	EAI2_NTB	1	5	2	4	3	5	1	4	2	2	4	2	8	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
57-0169	89	EAI2_NTB	1	5	2	4	3	5	1	4	2	2	4	2	7	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
57-0708	89	EAI2_NTB	1	5	2	4	3	10	1	4	2	2	4	2	8	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
57-0309	89	EAI2_NTB	1	5	3	4	3	10	1	4	2	2	4	2	7	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-1070	89	EAI2_NTB	1	5	2	4	2	6	1	4	2	2	5	2	8	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-0763	53	T1	2	0	2	3	3	7	5	3	2	5	3	1	7	1	2	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
57-0197	53	T1	2	0	5	3	2	10	2	3	2	5	3	3	8	1	2	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-0764	236	EAI5	2	0	3	4	3	5	2	7	2	2	5	7	6	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-0986	236	EAI5	2	9	3	4	3	5	2	7	2	2	5	6	6	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-0947	256	EAI5	2	5	3	4	2	5	2	5	2	2	5	5	6	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
57-0115	256	EAI5	2	5	3	4	2	5	2	5	2	2	5	4	6	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-1029	256	EAI5	2	5	3	4	3	5	2	5	2	2	5	4	6	1	3	Kanchanaburi	S	S	S	R	NA	NA	NA	NA	NA
56-940	256	EAI5	2	6	3	4	2	5	2	7	2	2	5	5	5	1	3	Kanchanaburi	S	R	S	S	WT	WT	Ser531Leu	NA	NA
56-0797	236	EAI5	2	3	3	4	2	4	1	7	2	2	5	4	6	1	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-1027	591	EAI6_BGD1	2	7	3	4	4	6	9	7	2	2	5	4	6	1	3	Kanchanaburi	R	R	S	S	Ser315Thr	WT	Ser531Leu	WT	WT
56-1047	591	EAI6_BGD1	2	7	3	4	4	5	9	7	2	2	5	4	6	1	3	Kanchanaburi	S	R	S	S	WT	WT	Leu533Pro	WT	WT
56-0851	523	U	4	4	3	8	2	4	6	5	2	6	4	1	1	1	3	Kanchanaburi	R	R	R	R	Ser315Thr	WT	Ser531Leu	A1400G	Asp94Gly
56-1146	523	U	4	4	3	8	2	4	6	5	2	6	4	1	1	1	3	Kanchanaburi	R	R	S	S	Ser315Thr	WT	Ser531Leu	A1400G	Asp94Gly
56-1086	523	U	4	2	3	9	2	4	6	5	2	2	7	4	1	2	3	Kanchanaburi	S	S	S	S	NA	NA	NA	NA	NA
56-1028	523	U	4	4	3	9	3	5	6	4	2	6	5	3	3	2	3	Kanchanaburi	R	R	S	R	Ser315Thr	WT	Ser531Leu	WT	WT

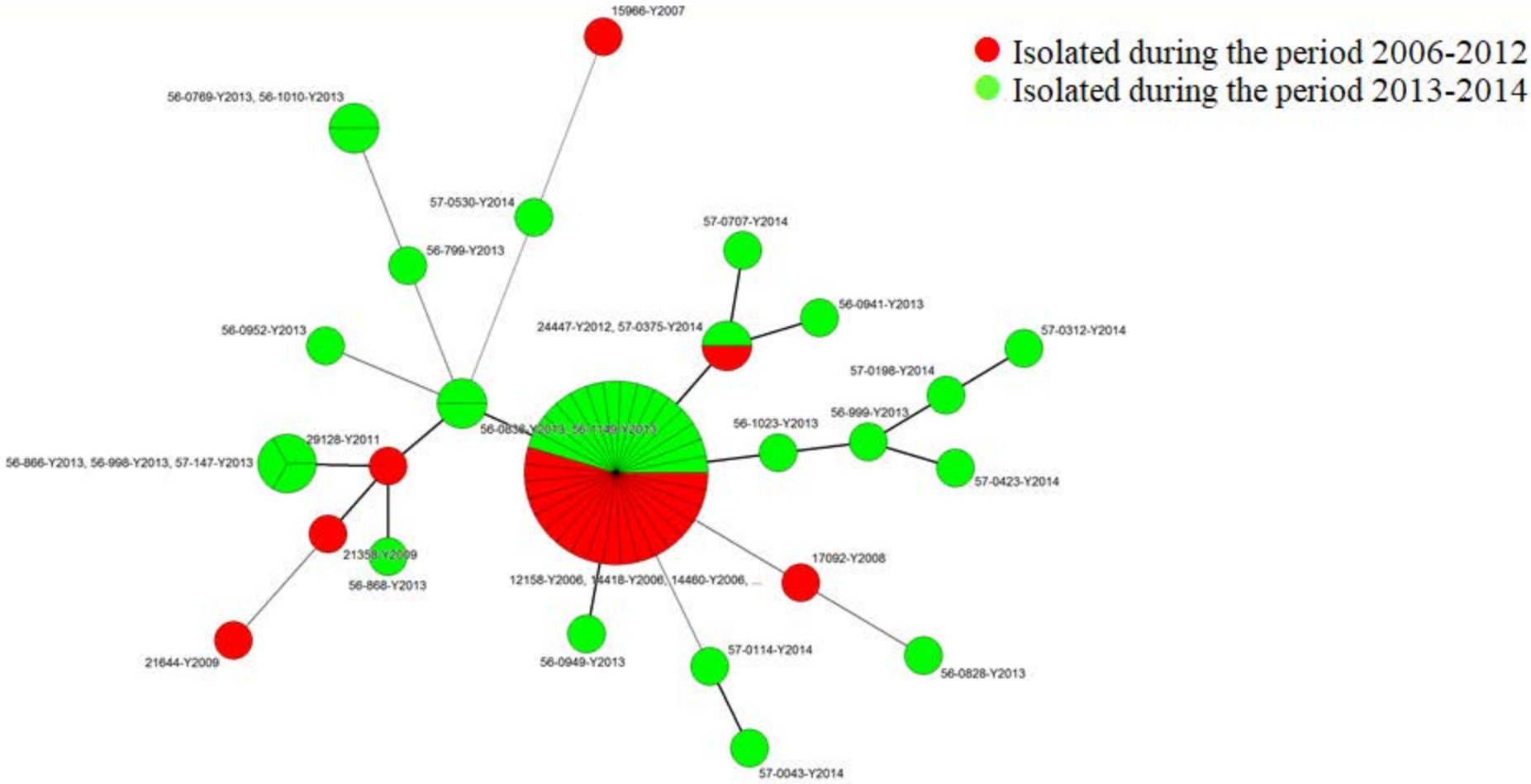

Isolated during the period 2006-2012
Isolated during the period 2013-2014
15966-Y2007
56-0769-Y2013, 56-1010-Y2013
57-0530-Y2014
56-799-Y2013
56-0952-Y2013
57-0707-Y2014
56-0941-Y2013
24447-Y2012, 57-0375-Y2014
57-0312-Y2014
57-0198-Y2014
56-999-Y2013
56-1023-Y2013
29128-Y2011
56-866-Y2013, 56-998-Y2013, 57-147-Y2013
57-0423-Y2014
21358-Y2009
56-868-Y2013
17092-Y2008
12158-Y2006, 14418-Y2006, 14460-Y2006, ...
21644-Y2009
56-0949-Y2013
57-0114-Y2014
56-0828-Y2013
57-0043-Y2014

Table 1. Phenotypic characteristics of MTB isolates according to drug susceptibility tests with first-line anti-TB drugs

Strain	Drug resistant patterns				Frequency		Proportion (%)	
	INH	RIF	EMB	STM				
Susceptible to INH and RIF					**45**		**62.5**	
	S	S	S	S	42		58.3	
	S	S	S	R	3		4.2	
Resistant to INH and RIF					**27**		**37.5**	
RIF mono-resistance	S	R	S	S	3	4	4.2	5.6
	S	R	S	R	1		1.4	
INH mono-resistance	R	S	S	R	1		1.4	
MDR and XDR	R	R	S	R	13		18.1	
	R	R	S	S	2	22	2.8	30.6
	R	R	R	R	7		9.7	
					72		**100**	

Abbreviations: MTB, *Mycobacterium tuberculosis;* INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STM, streptomycin; S, susceptible; R, resistance; MDR, multi-drug resistance; XDR, extensively-drug resistance

Table 2. Classification of MTB strains based on spoligotyping

No.	Clade	SIT	Octal Code	Frequency	%		Clustering rate (%)
				59			82
1	BEIJING	1	000000000003771	38		52.8	53
2	EAI2_NTB	89	674000003413771	6	8.3		8
3	EAI5	236	777777777413771	3	4.2		4
4	EAI5	256	777777777413671	4	5.6	11.2	6
5	EAI5	1395	474377767413771	1	1.4		
6	EAI6_BGD1	292	777777757413371	1	1.4		
7		591	777777757413771	2	2.8	5.6	3
8	EAI6_BGD1	1414	777757757413371	1	1.4		
9	H3	50	777777777720771	1	1.4		
10	T1	53	777777777760771	2	2.8		3
11	U	523	777777777777771	4	5.6		6
12	U	1189	677777477403771	1	1.4	8.4	
13	U	1391	777777700003371	1	1.4		
14	NEW1	ND	737777747413771	1	1.4		
15	NEW2	ND	12004377763771	1	1.4		
16	NEW3	ND	674000003412771	1	1.4		
17	NEW4	ND	367777670020731	1	1.4	9.8	
18	NEW5	ND	774177774000071	1	1.4		
19	NEW6	ND	000000007777711	1	1.4		
20	NEW7	ND	674000003413731	1	1.4		
	Total			**72**	**100**		

Abbreviations: SIT, spoligo-international type number; ND, no data

Table 3. Allelic distribution and discrimination power of MIRU-VNTR in Beijing genotypes resistant and susceptible toisoniazid and rifampicin

locus	DST result	N	MIRU-VNTR												
			Copy number											Discreminatory powe	
			1	2	3	4	5	6	7	8	9	10	ND	HGDI	Level
424	DR & MDR	19				19								0.00	Low
	Susceptible to INH&RIF	19		1	4	13	1							0.51	Moderate
580	DR & MDR	19		16									3	0.28	Low
	Susceptible to INH&RIF	19		18									1	0.11	Low
802	DR & MDR	19			16		3							0.28	Low
	susceptible to INH&RIF	19	1	3	15									0.37	Moderate
960	DR & MDR	19			19									0.00	Low
	Susceptible to INH&RIF	19		1	18									0.11	Low
1644	DR & MDR	19			19									0.00	Low
	Susceptible to INH&RIF	19			19									0.00	Low
1955	DR & MDR	19					19							0.00	Low
	Susceptible to INH&RIF	19		1	1	1	14	2						0.46	Moderate
2163b	DR & MDR	19				16	3							0.28	Low
	Susceptible to INH&RIF	19		1		3	6	9						0.68	High
2165	DR & MDR	19				19								0.00	Low
	Susceptible to INH&RIF	19				19								0.00	Low
2401	DR & MDR	19				19								0.00	Low
	Susceptible to INH&RIF	19				17	2							0.20	Low
2996	DR & MDR	19					1		18					0.11	Low
	Susceptible to INH&RIF	19				1	1		15	2				0.38	Moderate
3192	DR & MDR	19					19							0.00	Low
	Susceptible to INH&RIF	19					19							0.00	Low
3690	DR & MDR	19			19									0.00	Low
	Susceptible to INH&RIF	19			18	1								0.11	Low
4052	DR & MDR	19								19				0.00	Low
	Susceptible to INH&RIF	19							1	17		1		0.20	Low
4156	DR & MDR	19		19										0.00	Low
	Susceptible to INH&RIF	19		18			1							0.11	Low
4348	DR & MDR	19			19									0.00	Low
	Susceptible to INH&RIF	19			18	1								0.11	Low

Abbreviations: MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem repeat; DR, drug resistance; INH, isoniazid; RIF, rifampicin