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Molecular epidemiology of *Mycobacterium tuberculosis* and antibiotic resistance in Lao PDR

Silaphet Somphavong

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“Molecular Epidemiology of *Mycobacterium tuberculosis* and antibiotic resistance in Lao PDR”

Présentée par Silaphet Somphavong
le 18 décembre 2018

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Epidémiologie moléculaire de *Mycobacterium tuberculosis* et sa résistance aux antibiotiques en RDP Lao

RÉSUMÉ

La tuberculose (TB) reste parmi les 10 premières causes de décès dans le monde l'émergence/réémergence de la TB résistante aux antituberculeux aggrave la situation et représente un défi majeur pour l'éradication de la TB. Le Laos est entouré par des pays fortement touchés par la TB et la TB multi-résistante (MDR) et cette maladie représente une priorité en termes de santé publique dans ce pays. Il n'existe encore aucune donnée sur la structure génétique et la résistance aux antibiotiques de la population de *M. tuberculosis* au Laos.

Dans ce contexte, ce travail avait pour but d'analyser la diversité génétique et la structure des populations de *M. tuberculosis* ainsi que les déterminants génétiques associés à la résistance à partir d'échantillons collectés lors de l'enquête de prévalence nationale de la Tuberculose (TBPS) 2010-2011, l'enquête de résistance aux antituberculeux (DRS) 2016-2017 et chez les cas suspects de MDR-TB au Laos (2010-2014). Plusieurs techniques d'analyses ont été utilisées, comprenant les tests de sensibilité aux médicaments (phénotypique et génotypique), le séquençage et le génotypage par spoligotypage et MIRU-VNTR. Les données ont été analysées par des méthodes statistiques et phylogénétiques.

Premièrement, ce travail s'est focalisé sur la diversité des familles de *M. tuberculosis* circulant au Laos. Les familles EAI et Beijing (76.7% et 14.4% respectivement) ont été principalement observées dans les échantillons de TBPS, alors que la famille Beijing était plus fréquente dans les échantillons de DRS et chez les patients suspectés de MDR-TB (34.9% et 41.0% respectivement). La transmission récente était non-négligeable avec un taux de « clustering » global de 11.9%, et des taux pour Beijing de 20.7 % et EAI de 11.0 %. Deuxièmement, les résultats ont révélé des profils de résistance très diverses allant de la mono-résistance jusqu'à la pré-XDR (ultrarésistance). Les mutations associées aux profils de résistance ont montré une grande diversité, avec cependant certaines mutations majeures dans les gènes *rpoB*, *katG*, et *rpsL*. Le gène *pncA* a montré un pattern différent avec de la diversité

sans mutations prééminentes. En plus des mutations détectées, des délétions et insertions de bases ont été également observées. Le séquençage a montré son utilité pour la détection de la résistance aux antibiotiques dans les trois échantillons à l'étude. Enfin, la famille Beijing, famille la plus problématique au niveau mondial en termes de résistance et de transmissibilité, a été identifiée de manière significative dans le groupe de patients <35 ans, principalement dans les provinces du Nord, dans les cas de transmissions récentes et chez les isolats très résistants. Tous ces points suggèrent un risque d'émergence de la MDR-TB accrue au Laos dû à la famille Beijing.

En conclusion, cette étude permet d'avoir pour la première fois un aperçu de la structure des populations de *M. tuberculosis* au Laos. Les résultats soulignent le risque d'augmentation du nombre de cas infectés par la famille Beijing et donc des cas de résistance. Pour empêcher une dégradation de la situation, il est essentiel d'améliorer les stratégies pour le dépistage des résistances et de développer des tests moléculaires capables de couvrir un large nombre de mutations qui soit simple à implémenter dans les pays à ressources limités. Les résultats de ce travail serviront de base en termes de famille/sous-famille/génotype et de mutations associées à la résistance au Laos. Ces données pourront être comparées avec de futures études/analyses pour étudier l'évolution de la TB et de la TB résistante et ainsi d'évaluer l'efficacité des politiques de contrôle mises en place. La description des mutations associées aux résistances est utilisée pour créer une base de données régionale en collaboration avec le Vietnam et le Cambodge pour développer un outil de diagnostic basé sur la technologie des puces à ADN pour améliorer la détection de la résistance dans la région.

Les mots clés : Epidémiologie moléculaire, *Mycobacterium tuberculosis*, Résistance aux antibiotiques, RDP Lao, Asie du Sud-est, Déterminant génétique, Beijing, EAI, transmission récente, Séquençage de gènes ciblés.

Molecular epidemiology of *Mycobacterium tuberculosis* and antibiotic resistance in Lao PDR

SUMMARY

Tuberculosis (TB) is still one of the top 10 leading causes of death worldwide; the emergence/re-emergence of drug resistant TB aggravates the situation globally and challenges the prospect of ending TB by 2035. Lao PDR is surrounded by TB and MDR-TB high burden countries and TB continues to be one of the priority infection diseases in this country. The prevalence of TB in 2010 was almost twice as high than previous estimates and little is known about drug resistance. Up to now, *M. tuberculosis* population data regarding drug resistance and genetic structure are totally absent. In this context, we aimed to study the diversity and the structure of *M. tuberculosis* population and the genetic determinants associated to drug resistance using clinical samples collected from the TB prevalence survey (TBPS), 2010-2011; from the Drug resistance survey (DRS), 2016-2017 and from presumptive MDR-TB cases in Lao PDR (2010-2014). Various methods and analyses were used, including drug susceptibility testing (phenotypic and genotypic), DNA sequencing and genotyping of *M. tuberculosis* using spoligotyping and MIRU-VNTR. The data were analyzed by statistical and phylogenetic analyses.

Firstly, this work was focused on the diversity of *M. tuberculosis* families circulating in Lao PDR. According to the result from TBPS, EAI and Beijing family (76.7% and 14.4% respectively) were mainly observed, while Beijing family was more observed in DRS, and presumptive MDR-TB cases (34.9% and 41.0% respectively). The level of recent transmission in Lao PDR was non-negligible with a global clustering rate of 11.9% and in Beijing and EAI of 20.7% and 11.0%, respectively. Secondly, the results demonstrated the diversity of drug resistant patterns from mono-resistance to pre-extensively drug resistance (pre-XDR). A high diversity of mutations associated with drug resistance was also observed, however common mutations were mainly found (e.g. mutations in *rpoB* gene, *katG* and *rpsL*). The pattern was different for *pncA* gene, we observed a diversity of mutations without preeminent ones. Besides the number of known and unknown mutations associated with anti-TB drug resistance, deletion and insertion of bases

were also observed. The sequencing showed its usefulness for drug resistance detection. Lastly, Beijing family, which is the more problematic family in the world in terms of resistance and transmissibility, was observed on a significant manner in young age group, mainly in the northern provinces, in recent transmission cases and among highly drug resistant isolates, suggesting an increasing risk of highly drug resistance TB due to highly transmissible Beijing strains in Lao PDR.

In conclusion, this study provides the first genetic insights into the *M. tuberculosis* population in Lao PDR. The results underline the risk of increase of Beijing and drug resistant TB in the country. In order to prevent a more serious situation in the future regarding drug resistance as observed in neighboring countries, there is an urgent need of effective strategy improvement for drug resistance screening and the development of rapid molecular tests that cover a large number of drug resistance simultaneously with a feasible implementation in the limited resource countries. The results of genotyping from our study will be the baseline of families/subfamilies/genotype of *M. tuberculosis* population and of the mutations associated with drug resistance in Lao PDR. These data will be compared with further study/analysis to evaluate the trend of TB and drug resistant TB in the country and to determine if the drug resistance is under control after the set-up of new policies. The data of drug resistance associated mutations are used to build a regional database in collaboration with Vietnam and Cambodia in order to develop a diagnostic tool based on DNA chip technology to improve the drug resistance detection in the region.

Key words: Molecular epidemiology, *Mycobacterium tuberculosis*, Antibiotic resistance, Lao PDR, Southeast Asia, Genetic determinant, Beijing, EAI, Recent transmission, Target genes sequencing.

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List of Abbreviations

Abbreviation	Full word
ACF	Active Case Finding
AFB	Acid-Fast Bacillus
AIDS	Acquired immunodeficiency syndrome
AMK/Amk	Amikacin
ART	Antiretroviral therapy
BCG	Bacillus Calmette Guérin
BDQ/Bdq	Bedaquiline
BSL-3	Biosafety level 3
CAP/Cap	Capreomycin
CAS	Central Asian Strain
CI	confidence interval
CFZ/Cfz	Clofazimine
CILM	Center of Infectiology Lao-Christophe Mérieux
CS/Cs	Cycloserine
Cm	Capreomycin
CPC	Cetylpyridinium Chloride
CR	Clustering rate
CXR	Chest x-ray
DFB	Damien Foundation Belgium
DLM/Dlm	Delamanid
DNA	Deoxyribonucleic acid
DOT	Directly observed therapy
DOTS	Directly Observed Treatment Short course
DRS	Drug resistance survey
DST	Drug susceptibility testing
DR-TB	Drug resistant TB
DS-TB	Drug susceptible TB
EMB/E	Ethambutol
EQA	External Quality Assessment
ETO/Eto	Ethionamide
EAI family	East African-Indian family
ERDR	Ethambutol resistance-determining region
4FDC	4-drug fixed dose combinations
FLD	First line anti-TB drug
FQ	Fluoroquinolones
GAT/Gfx	Gatifloxacin
H fammily	Haarlem family
Hh	High-dose isoniazid
IDU	Injection Drug Use
INH/H	Isoniazid

HIV	Human immunodeficiency virus
IQR	Interquartile range
KAN/Km	Kanamycin
KIT	Korean Institute of Tuberculosis
LAM	Latin American Mediterranean
Laos / Lao PDR	Lao People's Democratic Republic
LED	light-emitting diode
LJ	Löwenstein–Jensen
LPA	Line probe assays
LSPs	Large Sequence Polymorphisms
LVX/Lfx	Levofloxacin
LZD/Lzd	Linezolid
MDR-TB	Multidrug resistant tuberculosis
Mfx	moxifloxacin
MIC	Minimal inhibitory concentration
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable
MoH	Ministry of Health
MXF/Mfx	Moxifloxacin
<i>M. tuberculosis/M.tb</i>	<i>Mycobacterium tuberculosis</i>
MTBc	Mycobacterium tuberculosis complex
NJ tree	Neighbor joining tree
NRL	National Tuberculosis Reference Laboratory
NPV	Negative predictive value
NTC	National TB Center
NTCP	National Tuberculosis Control Program
NTM	non-tuberculous mycobacteria
OFX/Ofx	Ofloxacin
PAS/Pas	p-aminosalicylic acid
PCR	Polymerase chain reaction
PLHIV	People living with HIV
PMTCT	Prevention of mother-to-child transmission
PNB	Para-nitrobenzoic acid
PPV	Predictive positive value
Pre-XDR	Pre-Extensive drug resistance
PTO/Pto	Prothionamide
PZA/Z	Pyrazinamide
QDR	Quadruple drug resistance
QRDR	Quinolone resistance- determining region
RRDR	Rifampicin resistance-determining region
RIF/R	Rifampicin
RR-TB	Rifampicin-resistant Tuberculosis
SIT/SITs	Spoligotype International Types
SLD	Second line anti-TB drug

SLID	Second line injectable drug
SNPs	Single Nucleotide Polymorphisms
SRL	supra national reference laboratory
STR/S	Streptomycin
TB	Tuberculosis
TRD/Trd	Terizidone
USA	The United States of America
VL	viral load
WGS	Whole genome sequencing
WHO	World health organization
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen

Box 1

Definitions of drug resistance terms used in this study

Mono drug resistance: resistance to only one anti-Tuberculosis drug (first line drug (FLD) or second line drug (SLD))

Poly drug resistance: resistance to more than one anti-Tuberculosis drug (FLD and/or SLD) other than both isoniazid (INH) and rifampicin (RIF)

Multidrug resistance (MDR): resistance to at least both INH and RIF

Quadruple drug resistance (QDR): MDR plus resistance to at least 2 more first line drugs (ethambutol (EMB) and pyrazinamide (PZA))

Pre-Extensive drug resistance (pre-XDR): MDR plus resistance to any fluoroquinolone (FQ) or to one second line injectable drug

Extensive drug resistance (XDR): MDR plus resistance to any fluoroquinolone (FQ) and at least one of the three second-line injectable drugs: Capreomycin (CAP), Kanamycin (KAN) and Amikacin (AMK)

Chapter 1 INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1.1. Introduction and objectives

Tuberculosis (TB) remains a public health problem worldwide and the ninth leading cause of death due to a single agent, ranking above human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) [1]. In 2016, the estimation of TB incidence was 10.4 million, 56 % of the incidence were in five countries located in Asia: India, Indonesia, China, the Philippines and Pakistan. The incidence of multidrug-resistant TB (MDR-TB, resistant to at least both isoniazid (INH) and rifampicin (RIF)), rifampicin resistant TB (RR-TB) and extensively drug-resistant TB (XDR-TB, MDR plus resistance to at least one fluoroquinolone (FQ) and one second line injectable drug (SLID)) is continuously increasing and is defined as a major threat to TB control. Moreover, only one-fourth of the 600,000 incident MDR-TB/RR-TB cases were detected in 2016 and the successful rate of treatment in MDR/RR-TB patients was 54 % and only 30 % in XDR-TB patients [1].

Despite the huge problem of public health that TB represents in the world, in many countries and especially in low-income countries such as in Lao people's democratic republic (Lao PDR) still little is known in terms of epidemiology of TB and drug resistance. This country is not notified as high TB burden countries but it is a landlocked country in Southeast Asia, surrounded by five of the 30 high TB burden countries in the world (China, Myanmar, Cambodia, Vietnam and Thailand) [1]. Despite the establishment in 1995 of the Directly Observed Treatment Short-Course (DOTS), many people have still no access to quality TB diagnosis and treatment services and many cases remain undiagnosed [2]. The first TB prevalence survey (TBPS) in 2010-2011 showed that the prevalence of TB in Lao PDR is two times higher than the WHO estimates [2]. And after the first national TBPS, WHO re-estimated the prevalence of all TB forms at 540/100,000 populations [2, 3]. Regarding drug resistant TB still little is known in this country. The conventional culture-based drug susceptibility testing

(DST) is available only at National Reference laboratory (NRL). Besides, the use of available molecular tests for detection of first line drug (FLD) and second line drug (SLD) resistance is limited that leads to a lack of knowledge concerning the genetic determinants linked to drug resistance in this country. Only data of resistance to INH and RIF was explored by a multicentric-study in three regional hospitals [4]. The resistance to the other FLDs (ethambutol (EMB), streptomycin (STR) and pyrazinamide (PZA)) has not been screened; the extent threat of the resistance and the associated genetic determinants to these drugs cannot be evaluated. Nevertheless, it is essential to detect the resistance and the associated mechanisms for both FLD and SLD in order to better know the processes of drug resistance emergence and spread. In addition, the analysis of specific genomic regions of *M. tuberculosis* known to be associated with anti-TB drug resistance is more and more considered as valuable tool for drug resistance detection, surveillance, providing new opportunities to monitor drug resistance in TB in resource-poor countries [5].

In addition, data on population genetic structure are totally absent in Lao PDR, although they are essential to evaluate the risk of highly drug resistance emergence, such as XDR-TB, and its spread in the population. As example, significant associations were frequently observed between *M. tuberculosis* genotypes and drug resistance [6]. More specifically, Beijing family is associated with epidemics and drug resistance in many countries all over the world [6–10]. Up to now, despite the numerous studies carried out in neighbouring countries like Vietnam, China, Thailand and Myanmar [7–12], no information is available yet in Lao PDR.

In this context and in order to acquire the first insight on genetic basis of *M. tuberculosis* and drug resistance in Lao PDR, this work aimed to study the diversity, the population structure and the genetic determinants of drug resistance in *M. tuberculosis* clinical samples collected from three different samplings: 1). a population based sampling (First National TB prevalence survey (TBPS), 2010-2011); 2). a routinely consecutive collection of patients with high risk of

MDR-TB (Presumptive MDR-TB), 2010-2014 and 3). a hospital based sampling (First national anti-TB drug resistance survey (DRS), 2016-2017) focused on drug resistant isolates. By analysing isolates from these three samplings, we expect to better understand the epidemiology and the extent threat of TB and drug resistant TB in the country; to define what are the different *M. tuberculosis* families involved in recent transmission and acquisition of drug resistance; what are the genetic determinants of drug resistance in our settings; and what kind of molecular methods can be used for detection of drug resistance TB in Lao PDR. Finally, the results from these studies will be crucial information for National Tuberculosis Control Program (NTCP) and Ministry of Health (MoH) in order to improve the TB control strategy and limit the increase of drug resistance in Lao PDR.

In this context, the specific objectives are as follows:

1. To determine the family/subfamily/genotype of *M. tuberculosis* population circulating in Lao PDR, using 43-spacer oligonucleotide typing (spoligotyping) and 24-locus MIRU-VNTR on *M. tuberculosis* isolates collected from three different samplings: 1). TBPS (2010-2011); 2). Presumptive MDR-TB (2010-2014) and 3).DRS (2016-2017).
2. To determine the transmission (recent versus ancient transmission) according to *M. tuberculosis* family and drug resistant patterns by estimation of the clustering rate in the different *M. tuberculosis* families present in Lao PDR.
3. To describe the structure of *M. tuberculosis* population by exploring the link between genetic diversity and epidemiological data and drug resistant patterns.
4. To determine the mutations in genes/regions of *M. tuberculosis* associated with first and second line drug resistance by DNA sequencing
5. To characterize drug resistance patterns and evaluate level of drug resistance by both methods phenotypic and genotypic drug susceptibility testing.

6. To evaluate the performance of different molecular methods of DNA sequencing compared to Xpert MTB/RIF, Genotypes MTBDR*plus*/Genotype MTBDR*s*/ for detection of first and second line anti-TB drug resistance

1.2. Literature review

1.2.1. Global tuberculosis disease burden

Tuberculosis (TB) is an ancient disease, caused by bacteria called “Mycobacterium tuberculosis”. It has affected humankind throughout known history and human prehistory [13]. TB has surged in great epidemics and continues to be a very significant global health problem. TB occurs in every part of the world, in 2016, the largest number of new TB cases occurred in South-East Asia and Western Pacific regions, with 62% of new cases, followed by the African region, with 25% of new cases [1]. The Figure 1.1 shows the TB incidence rates in 2016. Most of the estimated number of incident cases in 2016 occurred in the WHO South-East Asia Region (45%), the WHO African Region (25%) and the WHO Western Pacific Region (17%); smaller proportions of cases occurred in the WHO Eastern Mediterranean Region (7%), the WHO European Region (3%) and the WHO Region of the Americas (3%) [1].

Globally, the TB mortality rate is falling at about 3% per year and TB incidence is falling at about 2% per year; this needs to improve to 4–5% per year by 2020 to reach the first milestones of the End TB Strategy [1]. Regionally, the fastest declines in the TB mortality rate are in the WHO European Region and the WHO Western Pacific Region (6.0% and 4.6% per year, respectively, since 2010) [1]. High TB burden countries with rates of decline exceeding 6% per year since 2010 include Ethiopia, the Russian Federation, the United Republic of Tanzania, Viet Nam and Zimbabwe [1]. And the fastest decline in TB incidence is in the WHO European Region (4.6 % from 2015 to 2016). The decline since 2010 has exceeded 4% per year in several high TB burden countries, including Ethiopia, Kenya, Lesotho, Namibia, the Russian

Federation, the United Republic of Tanzania, Zambia and Zimbabwe [1]. Nevertheless, despite the decline in TB incidence and TB deaths, alongside HIV, it remains a top of cause of deaths from an infectious disease [1].

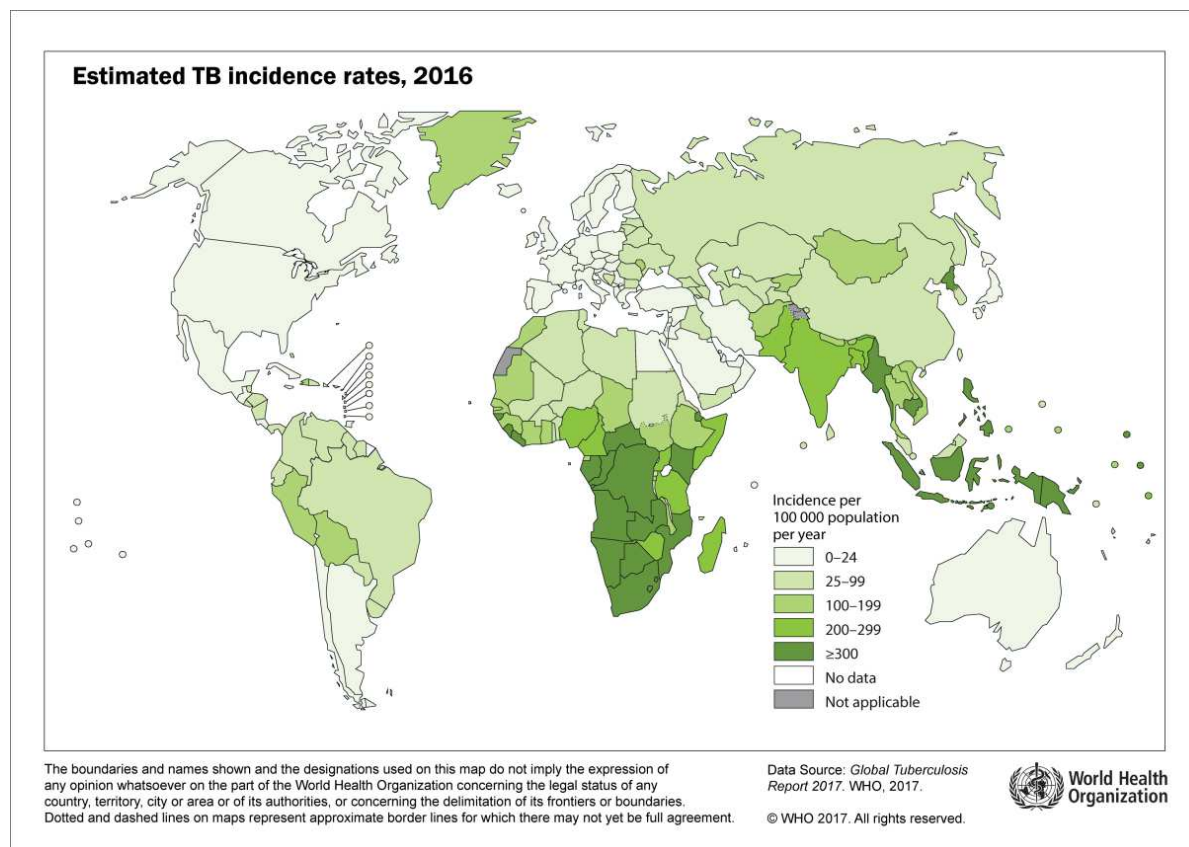


Figure 1. 1 Estimated TB incidence rates, 2016 (WHO, Global TB report 2017)

1.2.2. Global situation of drug-resistant tuberculosis

Drug-resistant TB threatens global TB care and prevention, and remains a major public health concern in many countries. Three major categories are used for global surveillance and treatment MDR-TB, RR-TB and XDR-TB [1]. Globally in 2016, an estimated 4.1% (95 % CI: 2.8–5.3%) of new cases and 19% (95% CI: 9.8–27%) of previously treated cases were MDR/RR-TB. The countries with the largest numbers of MDR/RR-TB cases (47% of the global total) were China, India and the Russian Federation (Figures 1.2 and 1.3).

There were an estimated 600,000 (range, 540,000–660,000) incident cases of MDR/RR-TB in 2016, with cases of MDR-TB accounting for 82% (490,000) of the total [1]. There were about 240,000 (range, 140,000–340,000) deaths from MDR/RR-TB in 2016 [1].

Despite the increase in testing, the number of MDR/RR-TB cases detected in 2016 only reached 153,000. In 2016, 8,000 patients with XDR-TB were reported worldwide, 123 countries have reported at least one XDR-TB case. On average, an estimated 6.2% of people with MDR-TB have XDR-TB [1]. In 2016, 130,000 patients were enrolled on MDR-TB treatment, equivalent to about 22% of the 600,000 incident MDR/RR-TB cases that year. Enrolments have increased over time and in several countries, the gap between detecting MDR/RR-TB cases and starting them on treatment has narrowed. In 2016, 8,500 patients with XDR-TB were enrolled in treatment, with 17% increase over 2015. However, the treatment success in MDR/RR-TB and XDR-TB patients were only 54 % and 30% respectively [1].

Beside the MDR/RR-TB and XDR-TB, drug resistance surveys have shown that mono- and poly-resistant TB (drug-resistant TB other than MDR-TB) are actually more common than MDR-TB. The global prevalence of MDR-TB in new cases is around 3% while the prevalence of mono- and poly-resistant strains is almost 17% [14]. Many of these cases contribute to the increase of resistance and, eventually, can lead to MDR if they are not properly managed [15]. The forms, mono-resistant and poly-resistant TB, often remain undiagnosed in resource-limited settings because they are not considered as priority. However, the risk of failure or relapse is well described [15]. INH is one of the most two powerful anti-TB drugs, used in all six months long for standard treatment regimen, used in some MDR-TB regimen and used as preventive therapy for people living with HIV [15–17]. However, INH mono resistance is the most common form of mono resistance, with estimated prevalence ranges between 0 to 9.5% (0-12.8% among new cases and 0-30.8% among retreated cases) [14].

Through evidences that relied on simulations from modeling work, performing DST in all patients before treatment using a rapid test that detects resistance to INH and RIF would be the most cost-effective strategy for averting deaths and preventing acquired MDR-TB [18]. Performing rapid DST to INH and RIF at the start of treatment would help identify many more cases of mono- and poly-resistant TB. Clinicians should therefore expect to see more cases of mono- and poly-resistant TB in the future as rapid drug resistance diagnostic becomes more commonly used.

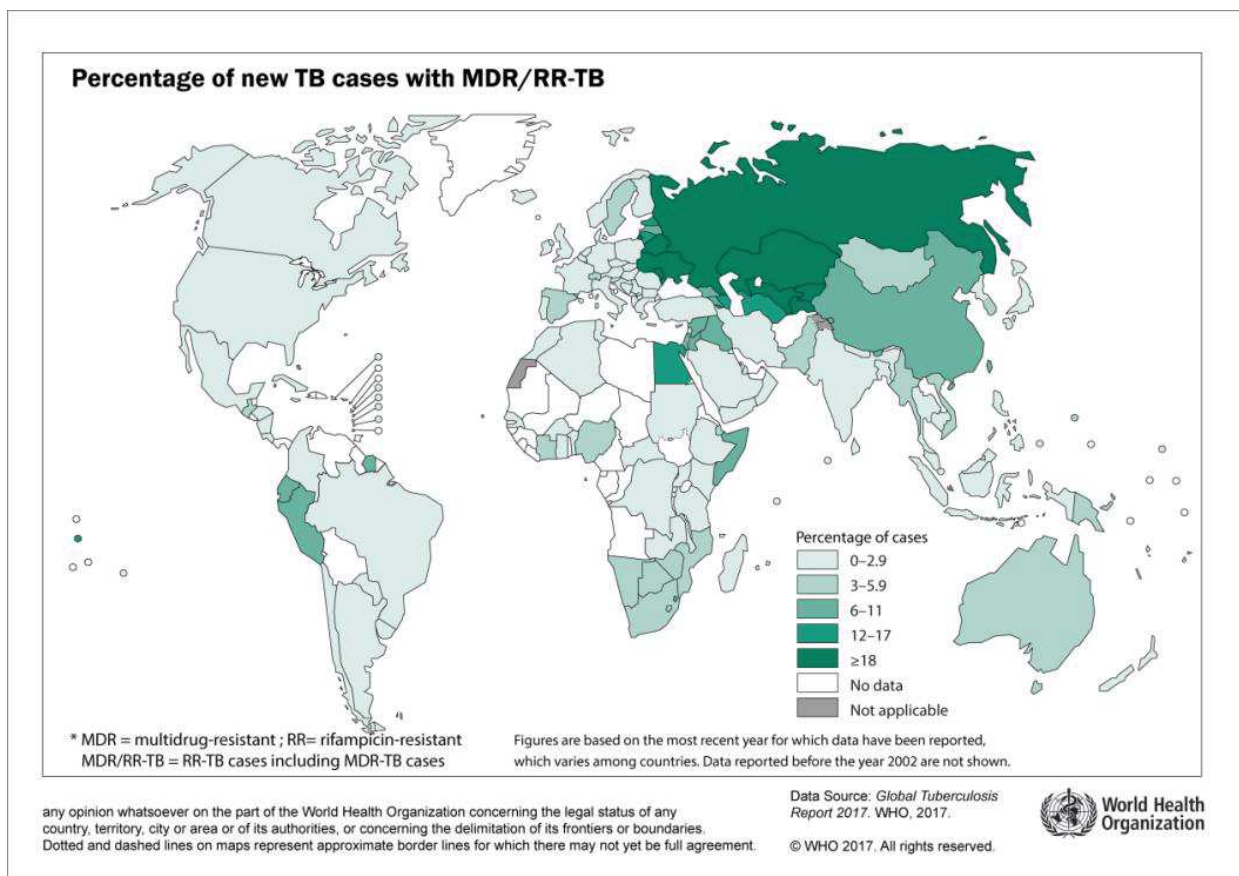


Figure 1. 2 Percentage of new TB cases with MDR/RR-TB (WHO, Global TB report 2017)

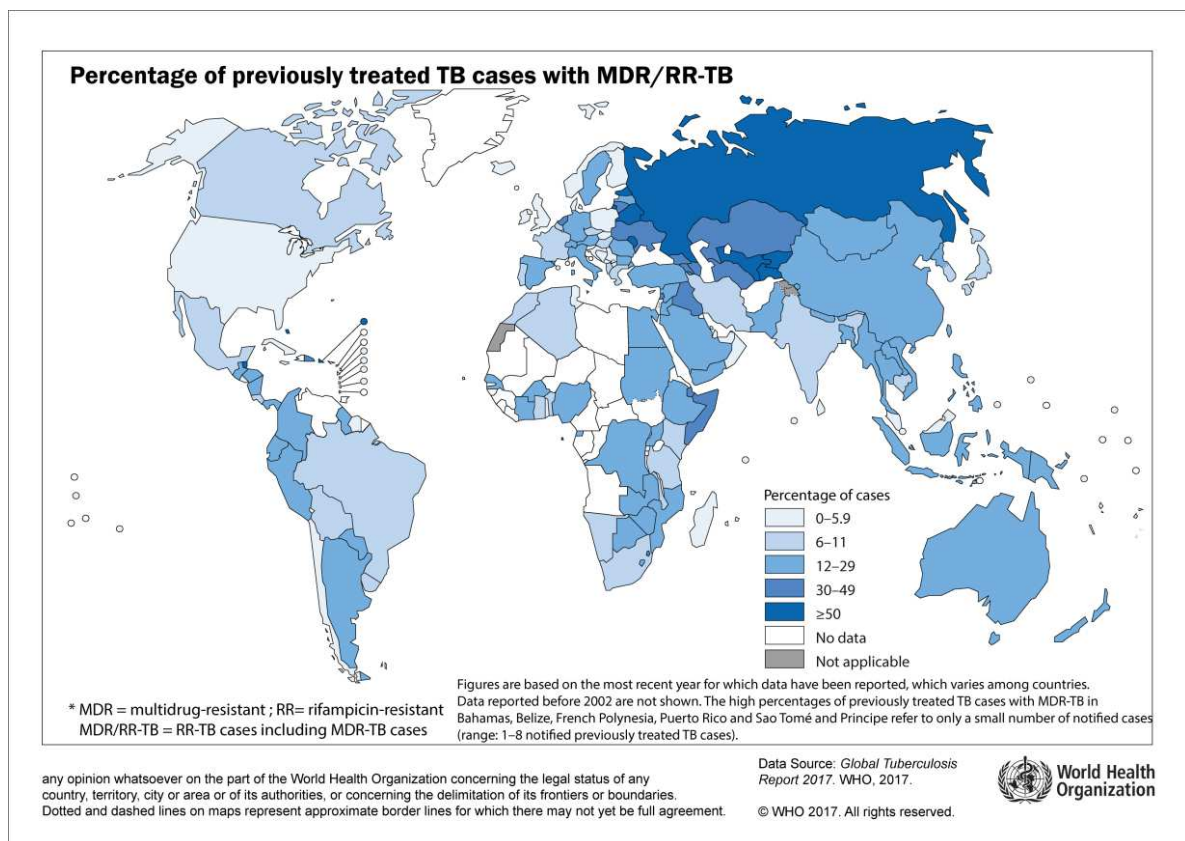


Figure 1. 3 Percentage of previously treated TB cases with MDR/RR-TB (WHO, Global TB report 2017)

1.2.3. Diagnosis of tuberculosis and drug resistance

The diagnosis of TB still relies primarily on the identification of Acid- Fast Bacilli (AFB) in sputum smears using a conventional light microscope in high burden countries [19]. The sputum specimens are smeared directly onto the slides (direct smears) and subjected to Ziehl-Neelsen (ZN) staining. This method, first developed in the 1880s and basically unchanged today, has the advantage of being simple, but is hampered by very low sensitivity. It may only detect half of all cases with active infection [20] and its usefulness is questionable for patients with reduced pulmonary cavity formation or reduced sputum bacillary load, such as children and HIV-coinfected patients. Moreover, this method cannot distinguish between drug-susceptible and drug-resistant *M. tuberculosis* or between different species of mycobacteria such as non-

tuberculous mycobacteria (NTM). Currently, WHO recommends the use of fluorescent light-emitting diode (LED) microscopy as alternative technique because it is more sensitive (10%) than ZN method [21]. Nevertheless, the use of LED microscopy is limited because of its high cost. Therefore only 7% of TB centers worldwide used this technology in 2014. Now a day, several methods can be used for determining the AFB, either culture based methods or Non culture based methods.

The culture-based method remains the gold standard for both diagnosis and drug sensitivity testing (DST). The detection rate often increases of 30-50% compared to microscopy [21]. However, this method is more complex and expensive than microscopy, time consuming (4-8 weeks by solid culture) and requires strict biosafety measures [21, 23, 24]. Besides, liquid culture media is a more sensitive and faster culture system but does not permit to identify contamination by other bacteria [21, 23, 24]. The reliability of DST varies with the anti-TB drugs. DST is more accurate in detecting susceptibility to INH, RIF, FQ and SLIDs, but results are less reliable and reproducible for EMB, STR, PZA and for drugs of groups 4 and 5 [21, 24, 25].

The development of molecular methods showed considerable advantages in *M. tuberculosis* and drug resistance detection. In order to rapid first-step identification of RR-TB, and MDR-TB, the two main molecular tools endorsed by WHO in 2008 and 2010 are Line Probe Assays (LPA) and Xpert MTB/RIF respectively [26, 27]. LPA allows rapid detection of *M. tuberculosis* and RIF resistance alone (INNOLiPA®Rif.TB assay, Innogenetics, Ghent, Belgium) or in combination with INH (GenoType® MTBDR assay, Hain Lifescience, Nehren, Germany) within 24 hours [21]. LPA is suitable for both AFB smear-positive sputum specimens and *M. tuberculosis* isolates grown by conventional culture method. This test showed high sensitivity for detection of RIF resistance (over 94%), but is less sensitive for the detection of INH resistance (approximately 85%) [21, 28]. Therefore, it may underestimate the number of MDR cases [21]. The Xpert MTB/RIF assay allows identifying *M. tuberculosis* complex and the RIF resistance directly from sputum specimens in less than two hours [21, 27]. The assays had similar

sensitivity, specificity and accuracy as culture on solid media and this tool has been recommended by WHO as initial diagnostic test for persons with a risk of MDR-TB and HIV. However, the tests for detection of RIF resistance alone cannot accurately predict RIF resistance and MDR-TB since about 10% of RIF resistant isolates were sensitive to INH [21]. Besides, despite the overall high sensitivity (99%), Xpert MTB/RIF sensitivity was 60-88% compared with liquid culture and false results have been reported [29]. Recently, a new version called Xpert Ultra showed similar performance as liquid culture and is able to detect *M. tuberculosis* in specimens with low numbers of bacilli, especially in smear-negative, culture-positive specimens (such as those from persons with HIV co-infection), in pediatric specimens and in extra-pulmonary specimens (notably cerebrospinal fluid). The WHO issued a recommendation in which Ultra can be used as an alternative to the existing Xpert MTB/RIF test in all settings [30]

For the rapid identification of resistance to SLDs or XDR-TB, MTBDRs/ assay version 1.0 was developed in 2009, followed by the MTBDRs/ version 2.0 in 2015. The assays detect the mutations associated with FQs and SLID resistance. Once a diagnosis of RR-TB or MDR-TB has been established, MTBDRs/ can be used to detect additional resistances to SLDs [31]. However, the moderate sensitivity (69%) for XDR-TB detection leads to an underestimation of XDR-TB cases [32]. The accuracy of MTBDRs/ by indirect testing for the detection of FQ resistance in patients with RIF-resistant or MDR-TB was 86% of sensitivity and 99% of specificity [31]. For detection of SLID resistance by indirect testing, this test showed 77% of sensitivity and 99% of specificity [31]. Therefore, the results obtained by MTBDRs/ may be used as initial test for detection of SLD resistance but cannot be used to properly guide the choice of SLIDs for the MDR-TB treatment [21, 24, 31].

Nowadays, many studies in different settings showed the potential use of whole genome sequencing (WGS) for getting rapid and full drug resistant-TB pattern [33–37]. Furthermore, WHO conducted a multi-country population-based surveillance study of drug resistance in TB in

highly endemic countries, using sequencing (either through WGS or targeted gene sequencing) and the study demonstrated that sequencing can be a valuable tool for surveillance of drug resistance, providing new opportunities to monitor drug resistance in TB in resource-poor countries. [5]

Overall, the TB case detection remains low. In 2016, only 6.6 million (63%) out of the 10.4 million estimated TB cases by WHO were reported [1]. Furthermore, only 59 % of estimated MDR/RR-TB were notified in 2016, and still only 16% of new TB cases and 60% of previously treated TB cases were examined for drug susceptibility [1]. As a result, many TB patients with undetected MDR were not potentially correctly treated, leading to treatment failure and an increased risk of MDR transmission in the community. Moreover, among the 1,694,000 MDR-TB patients enrolled in MDR-TB treatment according to WHO standards, only 35.5% received DST for both FQs and SLIDs [1] This suggests that many XDR-TB cases are never diagnosed. These data show the depth of the challenge for the management of MDR-TB and emergence of XDR-TB worldwide.

1.2.4. Characteristics and worldwide distribution of *M. tuberculosis*

A. Characteristics of *M. tuberculosis*

On 24 March 1882, the German doctor Robert Koch discovered the microorganism responsible for the deadly pulmonary TB [38]. It was in 1883 that the TB agent was named *Mycobacterium tuberculosis*. Further molecular analysis of these first isolates confirmed the identification of *M. tuberculosis* and indicated that Koch's isolates belong to the "modern" lineage of *M. tuberculosis* [38]. This bacteria belongs to the slow-growing bacterial group, characterized by one division every 18-24 hours [39]. Consequently, the growth on Löwenstein–Jensen (LJ) medium requires at least 3-4 weeks [39]. Faster results can be obtained using solid Middlebrook medium with growth supplement (OADC) or liquid medium (BACTEC) [40–43]. *M. tuberculosis* is non-pigmented, rough, dry colonies and forms a cord-like structure on LJ

medium (Figure 1.4 A). Using Ziehl-Neelsen staining or acid-fast staining method, the tubercle bacteria are rod-shaped and bright red (Figure 1.4 B). Under electron microscope, the bacteria are about 2 – 4 μm in length and 0.2- 0.5 μm in width (Figure 1.4 C). *M. tuberculosis* is classified as acid-fast Gram-positive bacteria due to their lack of outer cell membrane. Nevertheless, the membrane characteristics do not correspond to Gram-positive ones. Indeed, the bacteria does not retain the crystal violet dye as expected for Gram-positive bacteria, sometimes resulting in “ghost” appearance after washing with alcohol or acetone. *M. tuberculosis* has a specialized cell wall complex, which consists of four major components, mycoside, mycolic acids, arabinogalactan and peptidoglycan. Mycolic acids, the major lipids of the cell wall of mycobacteria in general, are major components of the outer permeability barrier and are responsible for the "acid-fastness" of this group of microorganisms [44]. Furthermore, the fatty acids are linked with carbohydrate components that form a unique envelope which inhibits phagolysosome fusion [39]. Like many other bacteria, *M. tuberculosis* does not form spores but has the capacity to become dormant, a non-replicating state characterized by low metabolic activity and prolonged persistence [39].

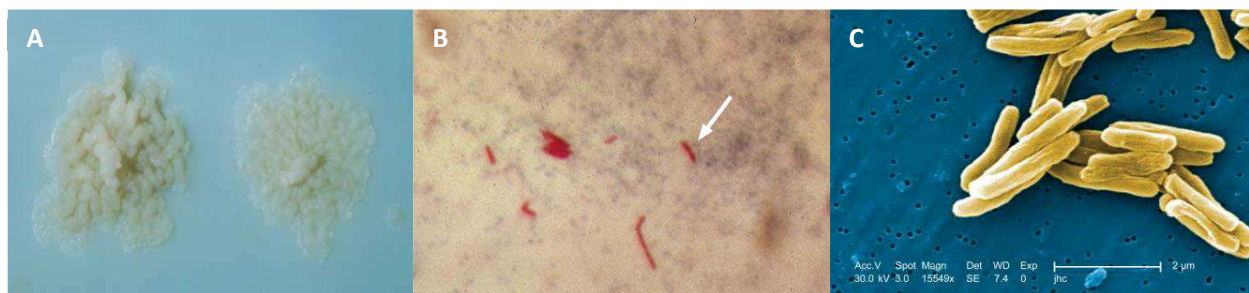


Figure 1. 4 *M. tuberculosis* colonies on Lowenstein-Jensen medium (A); *M. tuberculosis* stained by Ziehl-Neelsen method (B) and *M. tuberculosis* scanning electron microscopy (C)
(Source from <http://textbookofbacteriology.net/tuberculosis.html>)

The complete genome sequence of the reference strain of *M. tuberculosis* H37Rv, has been determined and analyzed in 1998. The genome comprises 4,411,529 base pairs, contains around 4,000 genes (Figure 1.5), and has a very high guanine + cytosine (GC) content (65 %)

[45]. Recently, thanks to the progresses in whole genome sequencing (WGS) technology, the sequences of many genomes have been determined covering all types of drug resistance from pan-drug sensitive to MDR and XDR strains [46–50]. *M. tuberculosis* is described as a clonal bacteria and the genome is highly conserved [45, 51, 52]. The pathogen is characterized by a low mutation rate about 10^{-9} mutation/bacterium/cell division. The genome evolutionary rate is very low, estimated between 0.4 - 0.5 SNP/genome/year [46, 53]

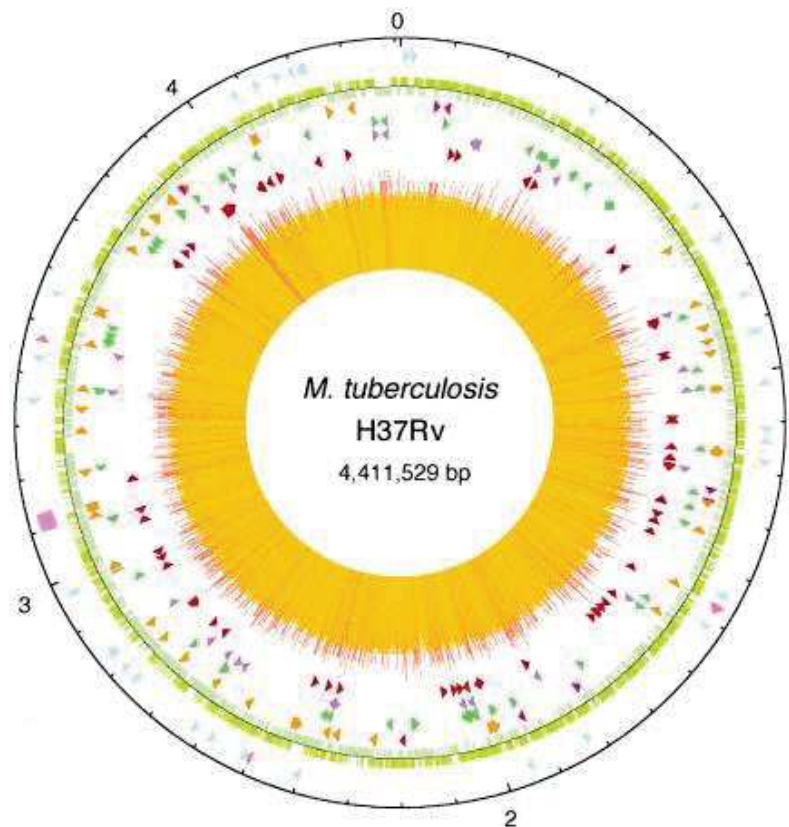


Figure 1. 5 Circular map of the chromosome of *M. tuberculosis* H37Rv
(Source from Cole et al 1998) [45]

The outer circle shows the scale in megabases, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red

B. *M. tuberculosis* lineages and families

M. tuberculosis is a member of the *M. tuberculosis* complex (MTBC), which comprises three human-adapted species (including 8 lineages), *M. tuberculosis* (5 lineages), *Mycobacterium africanum* (2 lineages) and *Mycobacterium canettii* and several animal-sourced lineages including *Mycobacterium bovis* (mainly pathogen of cattle), *Mycobacterium caprae* (pathogen of sheep and goats), *Mycobacterium microti* (pathogen of voles) and *Mycobacterium pinnipedii* (pathogen of seals and sea lions) (Figure 1.6) [54–59].

Among the eight human-adapted lineages, the 5 lineages belonging to *M. tuberculosis* are *M. tuberculosis* lineage 1 (The Philippines and Indian-Ocean), *M. tuberculosis* lineage 2 (East-Asian), *M. tuberculosis* lineage 3 (East-African-Indian), *M. tuberculosis* lineage 4 (Euro-American) and *M. tuberculosis* lineage 7 (Ethiopia) (Figure 1.6) [55, 57, 58]. From several detailed phylogenetic analyses, the lineages 1, 5 and 6 were defined as ancient lineages with *M. canetti* as the more ancestral branch; lineages 2, 3, 4 as modern lineages and lineage 7 appears to be intermediate between the ancient and modern lineages [51, 59–61].

Detailed phylogenetic analyses suggested that during these migration events, the *M. tuberculosis* lineages would have adapted to different human populations [51, 60]. Indeed, the lineages are strongly associated with geographical areas, their names reflecting the geographical origin of the *M. tuberculosis* population (Figure 1.6) [51].

The ancient lineage 1 (Indo-Oceanic, mainly EAI family) is reported in East Africa, but also spread all around the Indian Ocean and is frequently reported in Southeastern and Southern Asia, accounting for over 33–73% of total cases [62, 63]. This family is also prevalent in Northern Europe, Middle East and Central Asia, and in Oceania (22 – 25% of total cases) [62, 63].

Lineage 2 (East-Asian, mainly Beijing family) is one of the most virulent *M. tuberculosis* lineages and is spreading all over the world [48, 62, 63]. More specifically, the Beijing family is

predominant in East and South East Asia and in the countries of former Soviet Union, accounting for over 50 - 85% of total cases. This family is also highly prevalent in Oceania, Africa (except the West) and in North America (more than 17% of total cases) [62, 63]. However, this lineage is less detected in the other regions of the world, such as in Northern Europe, India, Central and South America, and in Middle East (less than 10% of total cases) [62, 63]. The Beijing family was found at very high frequency with more than 85% in the Beijing region of China [64]. This family is also found in high proportion in Mongolia, South Korea, Hong Kong, Taiwan, Vietnam, Thailand, and Malaysia [62, 63, 65]. Overall, this lineage accounted for 13% of all *M. tuberculosis* lineages [63].

The lineage 3 (East-African-Indian, mainly CAS family) is essentially localized in the Southern and Western Asia (9–30% of total cases, mainly in the South). This lineage is also found in the Eastern and Northern Africa (7 – 12% of total cases, mainly in the East). In several other regions including Central and North America, Europe, Far-East-Asia, and Oceania, this lineage is less frequent (0.1 - 5% of total cases). This lineage is predominant in India, Iran, and Pakistan, accounting for over 50% of total cases [62, 63].

The lineage 4 (Euro-American) consists of 10 different families, in which 5 main families LAM, T, X, H and S that are widespread throughout the world [62, 63]. Molecular epidemiology data showed that this lineage is the most frequent in Europe and Americas, but is also dominant in North Africa, Middle East and Oceania [60, 62, 63]. The distribution of these specific families varies according to the regions. The T family was found in all continents, accounting for 20 – 35% of total cases [62, 63]. The LAM family is the most represented in Americas (20 – 50% of total cases, mainly in the South), in Oceania (20% of total cases) and in all sub-regions of Africa (37% of total cases, except the West). The H family is the most represented family in Europe (24% of total cases) and in America (15 – 25% of total cases, mainly in the Caribbean region), while the X family is prevalent in Americas (8 – 21% of total cases, mainly in the North). Finally,

the S family is found in Africa (5 – 8% of total cases, mainly in the North) and in Southern Europe (5.8% of total cases) [62, 63].

In summary, the molecular epidemiology and clinical studies showed that the most geographically widespread lineages, which are the lineages 2 and 4, are the more virulent ones [51, 66–68]. In particular, Beijing family has been reported to be associated with young people, high virulence, drug resistance and MDR, relapses and treatment failures in many countries in the world [48, 51, 69–72]

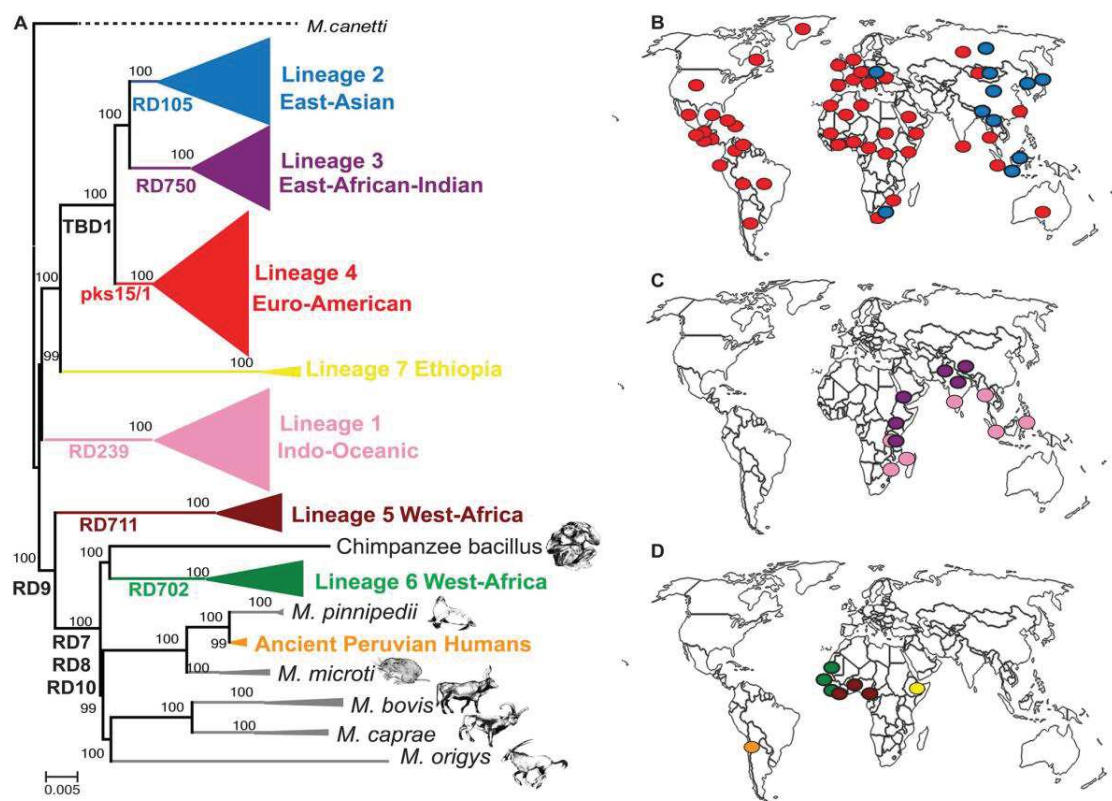


Figure 1. 6 Phylogeny of the MTBC and distribution of the 7 main *M. tuberculosis* lineages
(Source from Coscolla and Gagneux 2014) [51].

(A) Node support after 1000 bootstrap replications is shown on branches and the tree is rooted by the outgroup *M. canettii*. Large Sequence Polymorphisms (LSPs) are indicated along branches. Scale bar indicates the number of nucleotide substitutions per site. (B–D) Dominant MTBC lineages per country. Each dot corresponds to 1 of 80 countries represented in the 875 MTBC strains from the global strain collection analyzed by Gagneux et al 2006. [55]. The yellow dot represents the Lineage 7 in Ethiopia and the orange one the extinct MTBC strains from Peru, respectively. Panel (B) shows the most geographically widespread lineages, panel (C) the intermediately distributed lineages, and panel (D) the most geographically restricted lineages.

1.2.5. The tuberculosis situation in Lao PDR

A. TB and HIV epidemiological situation

Lao PDR (6.9 M population in 2017) TB burden remains considerable with incidence (include TB-HIV) at 168/100,000 (12,000 cases) and TB mortality 37/100,000, with the greater number in age above 14 years old (10,000 cases) [73].

The National TB Center (NTC) conducted the first National TB prevalence survey (TBPS) with WHO assistance in 2010-2011. The survey found that the prevalence of TB (≥ 15 years-old) is likely to be two times higher than the previous estimates (WHO re-estimated TB all form 540 per 100,000). The survey also remarked that case detection efforts remain the primary goal of NTCP with case notifications being very low in comparison with the estimated number of prevalent cases [2].

Prevalence of HIV is reported low (0.2% prevalence in population 15-49-year-old in 2011) with concentration in particular geographical areas and sub-populations, 6238 HIV cases in 2013 (53, 8 % M and 46.2% F), 3781 AIDS cases (58% men) and 1508 death (61% men) in 2013. Transmission is heterosexual 88%, mother to child 4.9%, homosexual and injection drug use (IDU) 4%, 11% among military and police. Among estimated 11,556 people living with HIV (PLHIV), 4730 are reported alive, 2787 (56%) are under antiretroviral therapy (ART), 2068 with access to viral load (VL) and 1954 VL <1000 copies. ART coverage is low among pregnant women HIV positive. Prevention of mother-to-child transmission (PMTCT) was 76% in 2013 (HIV Epi Review and impact analysis for Lao PDR, June 2014). 93% of the TB patients had an HIV test result available and 80% among 301 TB-HIV patients received ART during their TB treatment.

B. National Tuberculosis Center (NTC)

The NTC started directly observed treatment short-course (DOTS) in 1995 with the support of WHO and Damien Foundation Belgium (DFB). TB services are integrated in the primary health cares in five central, 18 provincial and 148 districts public hospitals since 2005 and currently in more than 1000 health centres. The DOTS has been reached full country coverage from central to village level since 2005 with high treatment success rates since then (treatment success reached 87% among new TB cases in 2016). Despite decades of TB control, and a 100% DOTS coverage, many people have no access to quality TB diagnosis and treatment services; Case notification has stagnated and many cases remain undiagnosed. Treatment coverage (% notified TB patients among estimated incident new TB cases) was 15% in 2000. Since then, thanks to Global Fund continued support, a progressive scaling-up of GeneXpert testing capacity among all presumptive TB since end of 2013, an accelerated implementation of systematic TB screening (using chest Xray) among TB contacts and other high-risk groups (22,295 people at higher risk in 8 prisons and 46 other high TB burden areas representing 1,040 additional new TB cases in 2017). TB treatment coverage has increased to 42% in 2016 and up to 50% in 2017 (Figure 1.7) of the estimated incidence in year 2017 (WHO country profile 2018). NTC outreach teams (5 teams in NTC Vientiane and one team in Khamouane province) screened for TB, 11,738 high risk people in 30 sites and notified 709 additional new TB cases in the first 6 months of 2018 (vs. 1,040 in all year 2017). The National TB Strategic Plan 2017-2020 has set the target of 70% treatment coverage by the end of 2020 in order to achieve a 20% decline in incidence between 2015 and 2020, in line with End TB targets. However, only half of estimated cases was notified (n= 5,934 cases), of which 93 % were pulmonary TB [73].

164 TB laboratories examined 45,356 presumptive TB patients, including 22,521 (50%) by Xpert MTB/RIF in 2017, in progress from respectively 41,314 and 19,450 (47%) in 2016. The

proportion of bacteriology positive among patients examined was 9.5% in 2013 and 9.2% in 2014.

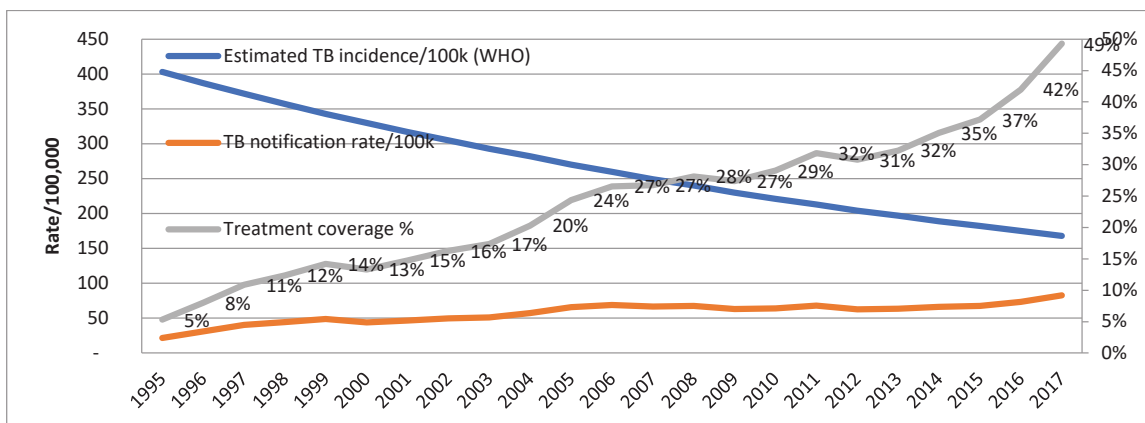


Figure 1. 7 TB case detection rate, Laos, 1995-2017

(Source: NTCP Laos)

C. National TB Reference Laboratory (NRL) and laboratory network

➤ Microscopy

164 TB laboratories perform quality assured AFB microscopy (ZN) at central, provincial and district levels. All laboratories participate in the External Quality Assessment (EQA) microscopy. Province laboratories re-read blindly a random sample of 25 district slides each quarter and NRL re-read samples of provincial and central levels laboratories. NRL confirms discrepant results (second level). Average 3% of all TB laboratories showed at least one major error in 2017 (Figure 1.8).

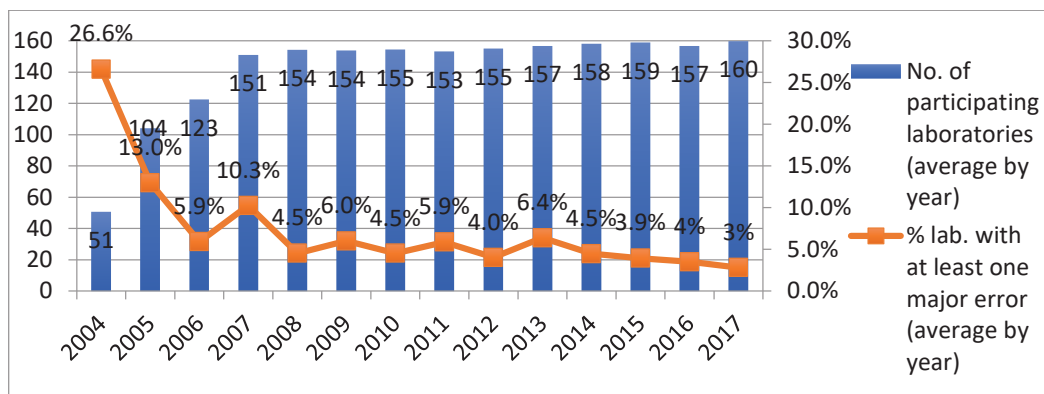


Figure 1. 8 External Quality Assessment of AFB microscopy (Ziehl Nielsen) in Lao PDR, 2004-2017
(Source: NTCP Laos)

➤ **Xpert MTB/RIF system**

By the end of 2013, the use of Xpert MTB/RIF testing was started in the central level (NRL and CILM) and Savannakhet provincial hospital in 2014. In 2018, there are total 20 Xpert MTB/RIF machine in the country. These machines are also distributed in provincial level for routine diagnosis and are used for active case finding (ACF) among high-risk groups.

The current situation towards Xpert MTB/RIF availabilities in 2018:

- NRL is equipped with 3 Xpert MTB/RIF machines for routine activities
- CILM (central level) is equipped with 1 Xpert MTB/RIF machine
- 10 provinces laboratories are equipped with one Xpert MTB/RIF machine
- 1 provincial laboratory (Champasack) is equipped with 2 machines for routine diagnosis
- Active case finding (ACF) : 2 machines are kept at central level and 1 is kept at Khammouane province
- 1 machine is kept as spare part at central level

➤ **Culture and DST**

The National TB Reference Laboratory (NRL) is equipped with BSL-3 room for culture and drug sensitivity testing (DST). The Korean Institute of Tuberculosis (KIT) supra national reference laboratory (SRL) performs DST external quality assessment (EQA) for FLDs and

SLDs on strains send by NRL and conducts regular on-site visit (two times per year) to the NRL. At the NRL, samples processed for culture are inoculated onto a solid culture medium (Löwenstein-Jensen). If no colony has appeared after 8 weeks, the culture is considered negative. If bacilli are present in the sample, colonies start to grow on the medium after 4 to 6 weeks. The colony growth will be graded according to the number of colonies observed: 1-19 colonies (Exact number counted), 20-100(1+), 101-200(2+), 201-500(3+), more than 500/confluent (4+). DST is performed following the growth of the culture and takes another 4 weeks. The following drugs are tested at NRL: INH (concentration tested 0,2 and 1 µg/ml), RIF (40 µg/ml), STR (4 and 10 µg/ml), EMB (2 µg/ml), these drugs were tested since 2015. And the following were started testing in late 2017 : KAN (30 µg/ml), CAP (40 µg/ml), Ethionamid (40 µg/ml), Cycloserine (30 µg/ml), Para-Amino Salicylic acid (1 µg/ml), Ofloxacin (4 µg/ml), Moxifloxacin (2 and 1 µg/ml), Amykacin (30 µg/ml), Levofloxacin (2 µg/ml), Rifabutin (20 µg/ml), Linezolid (2 µg/ml). However, no DST is performed for pyrazinamide.

D. Programmatic Management of Drug-Resistant TB

Regarding drug resistant TB little is known. Only data of resistance to INH and rifampicin RIF was explored by a multicentric-study in three regional hospitals in Laos conducted in 2010. The study has shown that out of 87 MTBc isolates, seven (8 %) cases were INH mono-resistant (6.8 % (5/73) among new cases and 14.3 % (2/14) among previously treated cases), one (1.1 %) was MDR (7.1 % (1/14) among previously treated cases) [1]. National TB Control Program (NTCP) started the programmatic management of drug resistant TB in 2011 with 24 month treatment regimen and switched to shorter 9-month MDR treatment (4Km-Mfx-Pto-Cfz-H-E-Z/5Mfx-Cfz-E-Z) in 2013, which has become the standard MDR-TB treatment for the patients sensitive to FQs and SLIDs after LPA testing by GenoType MTBDRs/ test (HainLife Science) in CILM.

To date, the current national guidelines allow all presumptive TB and MDR-TB cases to

have access to rapid test for RR using Xpert MTB/RIF test. Once RR is detected, patients are referred to the MDR treatment unit, for sample collection for culture and DST; for fluoroquinolones (FQs) and second line injectable drugs (SLIDs) resistance testing (using GenoType MTBDRs/ test) and other examinations (liver and renal function, EKG) before initiating a SLD treatment. MDR-TB units are set up in Setthatthirath Hospital, Vientiane capital since 2011 and in Khammuane and Luangprabang provincial hospitals since 2016.

The estimation of total MDR-TB/RR-TB was 2.5 per 100,000 (170 cases) and 76 cases among notified pulmonary TB, while 38 MDR-TB/RR-TB cases were confirmed. There were about half (56 %) of new cases and only one third (36 %) of previously treated cases were tested for drug resistance (RR) [73].

Active case finding and search of MDR among new cases contribute to an increasing number of MDR-TB cases detected and started on SLD treatment. However early enrolment and MDR-TB patients management need to be improved. Indeed, the number of MDR-TB patients notified and enrolled in treatment increased from 14 in 2013, 25 in 2014 to 34 in 2017.

- Thirty two among 38 MDR-TB patients diagnosed were enrolled on treatment in 2015 and 27 MDR-TB patients (84%) were treated successfully in 2015 while 33/38 MDR-TB patients were enrolled and 27 (82%) were treated successfully in 2016 (Table 1.1). 34 MDR-TB patients started a shorter 9-month MDR treatment in 2017 and 24 during the first 6 months of 2018. MDR treatment success was 82% in the 2016 cohort.
- Most of the RR/MDR-TB patients are tested sensitive to FQs and SLIDs by LPA and can receive shorter 9-month MDR treatment. However, few RR/MDR-TB patients require an adjusted regimen due to either resistance or intolerance to some of the SLDs. Currently the programme has no direct rapid access to Bedaquilin or other SLDs. NTC has been reporting a relatively small number of treatment failure each year over the past ten years

Table 1. 1 RR/MDR-TB notification and treatment results (2011-2016) in Lao PDR

MDR-TB Lao PDR	2011	2012	2013*	2014	2015	2016
Diagnosed RR/MDR	8	18	13	27	38	38
Died before treatment	3	1	1	-	1	3
Other treatment	1	2	1	-	-	1
Refused treatment	-	-	3	2	3	1
No drug resistance	-	2	1	-	-	-
Transferred out	-	-	-	1	2	0
Started on MDR treatment	4	13	7	24	32	33
Cured	3	6	5	18	25	25
Complete	-	-	-	-	2	2
Died	1	5	1	5	2	4
Lost of follow up	-	2	1	1	3	2
<i>% successfully treated</i>	<i>75%</i>	<i>46%</i>	<i>71.4%</i>	<i>75%</i>	<i>84%</i>	<i>82%</i>

(Source: NTCP and MDR-TB treatment centre, *start of 9Month MDR-TB regimen)

E. TB treatment in Lao PDR

TB treatment in Laos is based on three main groups of patients: 1). Patients infected with drug susceptible TB, 2). Patients infected with RR/MDR-TB eligible for the short 9 month regimen, 3). Patients infected with RR/MDR-TB not eligible for the short 9 month regimen

➤ **Treatment regimens for drug-susceptible tuberculosis**

TB drugs treatment can be administrated in 2 forms: it can be a fixed-dose combination tablet or a separate drug formulation treatment. However, the WHO recommends the use of 4FDC (4-drug fixed dose combinations) tablets over the loose pills. FDCs may provide programme benefits by making the ordering of medication easier, simplifying supply chain management, reducing the occurrence of stock-outs, and facilitating drug delivery and prescription preparation. If at all possible, always use RHZE (4FDC) tablets in adult patients. 4FDC tablets are simple and easy to use. They make it easier for the health worker to prescribe

the correct treatment. One single type of pill is also easier to order, distribute and manage than 4 separate drugs. Most importantly, a patient taking 4FDC tablets always takes 4 drugs at the time. This will contribute to the prevention of drug resistance.

Loose pills still need to be available at the provincial and central hospitals to treat patients presenting with drug side effects. The dosage of the drugs depends on the weight of the patient. In order to make the drug administration as easy as possible, treatment prescription has been standardized according to weight categories. These categories are very broad for adults, but are more precise for children.

The standard 6-month treatment regimen of 2HRZE/4HR (Table 1.2) is recommended by the WHO for the treatment of patients with drug-susceptible pulmonary TB disease. Intensive phase of 2 months' (60 days) duration with 4 drugs (R, H, Z, E) daily followed by a continuation phase of 4 months' (120 days) duration with 2 drugs (R, H) daily. In patients with severe EP TB (TB meningitis, miliary TB, pericardial TB or osteo-articular TB) the continuation phase is extended to 10 months.

Table 1. 2 Standard 6-month treatment regimen of 2HRZE/4HR

Prescription using 4FDC tablets						
Treatment phase	Months	Drug	Administration	Weight in kg (adult)		
				25-37	38-54	>54
Intensive	1 st to 2 nd	RHZE (4FDC)	daily	2	3	4
Continuation	3 rd to 6 th	RH 150/75	daily	2	3	4
Prescription using loose pills						
Treatment phase	Months	Drug	Administration	Weight in kg (adult)		
				25-37	38-54	>54
Intensive	1 st to 2 nd	RH 150/75	daily	2	3	4
		Z 400		2	3	4
		E 400		1.5	2	3
Continuation	3 rd to 6 th	RH 150/75	daily	2	3	4

➤ **Treatment regimens for RR/MDR-TB patients eligible for the 9 months regimen**

The 2016 WHO conditional recommendation indicated that a shorter MDR-TB regimen of 9–12 months may be used instead of longer MDR-TB regimens. This regimen is specifically

designed for RR/MDR-TB patients who do not present resistance to the FQs or SLIDs. In Lao PDR, all patients who, after a RR Xpert result, present no resistance on the MTBDRs/ test, will receive the 9-month regimen, but women of child bearing age should receive contraception.

The 9-month regimen should not be prescribed in the following situations:

- The MTBDRs/ test shows resistance to FQs and/or SLIDs
- Pregnant woman in the first trimester of pregnancy
- Patient with MDR-TB meningitis
- Patient who is unable or unwilling to comply with the treatment
- Patient with a known allergy to any of the drugs used in the 9-month regimen
- Patient taking any medications contraindicated in combination with the drugs used in the 9-month regimen

The 9-month regimen consists of an intensive phase of 4 months with 7 drugs, followed by a continuation phase of 5 months with 4 drugs (Table 1.3). The intensive phase will be extended until smear conversion if smear conversion is not achieved within 4 months, with a maximum of 6 months:

Table 1. 3 Shorter MDR-TB regimen

Anti-TB drugs	Intensive phase	Continuation phase
	4 (+1 or 2) months	5 months
Kanamycin	X	
Prothionamide	X	
Isoniazid high-dose	X	
Moxifloxacin high-dose	X	X
Clofazimine	X	X
Pyrazinamide	X	X
Ethambutol	X	X

➤ **Treatment regimens for RR/MDR-TB patients non eligible for the 9 months regimen**

An individualized long-course regimen must be prescribed whenever it is not possible to administer the 9-month regimen. WHO has released a rapid communication with key changes to

treatment of MDR and RR-TB in August 2018. The individualized MTB-TB regimen is established on a stepwise approach and the drugs are selected according to a cascade of sequence.

In patients with RR-TB or MDR-TB, a regimen with at least five effective TB medicines during the intensive phase is recommended, including PZA and 4 core SLDs – one chosen from Group A, one from Group B, and at least two from Group C, based on the latest evidence about the balance of effectiveness to safety:

- Group A: Medicines to be prioritized: levofloxacin/moxifloxacin, bedaquiline and linezolid
- Group B: Medicines to be added next: clofazimine, cycloserine/terizidone

Group C: Medicines to be included to complete the regimens and when agents from Groups A and B cannot be used: ethambutol, delamanid, pyrazinamide, imipenem-cilastatin, meropenem, amikacin (streptomycin), ethionamide/prothionamide, p-aminosalicylic acid; Kanamycin and capreomycin are no longer recommended, given increased risk of treatment failure and relapse associated with their use in longer MDR-TB regimens.

In summary, TB continues to be one of the priority infection diseases to combat in Lao PDR. The screening of RR is routinely performed on presumptive TB and MDR-TB cases, however the resistance to other FLDs (INH, EMB and PZA) are not initially screened; the extent threat of the resistance to these drugs cannot be evaluated. The use of available molecular tests (XpertMTB/RIF and Hain tests) for detection of FLD and SLD resistance is limited and these tests target only some mutations associated with drug resistance in a limited number of genes or genomic regions. The conventional phenotypic DST method is available only at the National Reference laboratory (NRL), but the method is labor-intensive, time-consuming and requires competent staff and a biosafety Level 3 laboratory. Up to now, genetic data, regarding the complete antibiotic resistance profiles or the overall genotype of the strain, were totally absent in Lao PDR.

1.2.6. Treatment regimens for the mono-resistant and poly-resistant TB (drug-resistant TB other than MDR-TB) (WHO recommendations)

Mono-resistance cases in this section refer to resistance to a single first-line drug, and poly resistance cases refer to resistance to two or more first-line drugs but not to both isoniazid and rifampicin (i.e. not MDR-TB). WHO recommendations for building of treatment regimens according to individual drug resistant pattern (not currently used in Lao PDR) (Table 1.4)

Table 1. 4 Treatment regimens for the management of mono- and poly-resistant*

Pattern of drug resistance	Suggested regimen	Minimum duration of treatment	Comments
INH (± SM)	RIF, PZA, and EMB (± fluoroquinolone)	6–9 months	A fluoroquinolone may strengthen the regimen for patients with extensive disease. For additional options, see section: <i>Isolated resistance to INH in [15]</i> .
INH and EMB	RIF, PZA, and fluoroquinolone	6–9 months	A longer duration of treatment should be used for patients with extensive disease.
INH and PZA	RIF, EMB, and fluoroquinolone	9–12 months	A longer duration of treatment should be used for patients with extensive disease.
INH, EMB, PZA (± SM)	RIF, fluoroquinolone, plus an oral second-line agent, plus an injectable agent for the first 2–3 months	9–12 months	A longer course (6 months) of the injectable may strengthen the regimen for patients with extensive disease.
RIF	INH, EMB, fluoroquinolone, plus at least 2 months of PZA	12–18 months	An injectable drug may strengthen the regimen for patients with extensive disease. For additional options, see section: <i>Isolated resistance to RIF. [15]</i>
RIF and EMB (± SM)	INH, PZA, fluoroquinolone, plus an injectable agent for at least the first 2–3 months	12–18 months	A longer course (6 months) of the injectable may strengthen the regimen for patients with extensive disease.
RIF and PZA (± SM)	INH, EMB, fluoroquinolone, plus an injectable agent for at least the first 2–3 months	18 months	A longer course (6 months) of the injectable may strengthen the regimen for patients with extensive disease.
PZA	INH, RIF	9 months	Most commonly seen in <i>M. bovis</i> infections.

* Table from the companion handbook to the WHO Guidelines for the programmatic management of drug resistant tuberculosis, 2014 [15]

1.2.7. Anti-tuberculosis drugs

A. Classes of anti-TB drugs

The classes of anti-TB drugs have traditionally been divided into first- and second-line anti-TB drugs with isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STR) being the primary first-line anti-TB drugs. While this classification is used in this document, it also uses a system that classifies the drugs into five different groups. The five-group system is based on efficacy, experience of use, safety and drug class [15]. WHO recommended the drugs for the treatment of RR-TB and MDR-TB in the 2016 update [74]. The different groups are shown in Table 1.5. The drugs in the same group do not come from the same “drug class” or have the same efficacy or safety. The individual description of each group is described in the Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-Resistant Tuberculosis 2014 [15]

Table 1. 5 Anti-TB drugs and medicines recommended for the treatment of RR/MDR-TB*

	Anti-TB Drugs (Abbreviation used in regimen)	Recommended for the treatment of RR-TB and MDR-TB^a
1-First-line oral anti-TB drugs	Isoniazid (H or INH) Rifampicin (R or RIF) Ethambutol (E or EMB) Pyrazinamide (Z or PZA) Rifabutin (Rfb) Rifapentine (Rpt)	Isoniazid high dose (Ad on agent/Group D1) Ethambutol (Ad on agent/Group D1) Pyrazinamide (Ad on agent/Group D1)
2-Injectable anti-TB drugs (injectable agents or parenteral agents)	Streptomycin (S or STR) Kanamycin (Km or KAN) Amikacin (Am) Capreomycin (Cm or CAP)	Streptomycin c (Group B) Kanamycin (Group B) Amikacin (Group B) Capreomycin (Group B)
3-Fluoroquinolones (FQs)	Levofloxacin (Lfx) Moxifloxacin (Mfx) Gatifloxacin (Gfx) Ofloxacin (Ofx or OFX)	Levofloxacin (Group A) ^b Moxifloxacin (Group A) ^b Gatifloxacin (Group A) ^b
4-Oral bacteriostatic second-line anti-TB drugs	Ethionamide (Eto) Prothionamide (Pto) Cycloserine (Cs)	Ethionamide (Group C) ^b Prothionamide (Group C) ^b Cycloserine (Group C) ^b

	Terizidone (Trd)	Terizidone (Group C) ^b p-aminosalicylic acid (Ad on agent/Group D3)
	p-aminosalicylic acid (PAS) p-aminosalicylate sodium (PAS-Na)	
5-Anti-TB drugs with limited data on efficacy and/or longterm safety in the treatment of drug-resistant TB (This group includes new anti-TB agents)	Bedaquiline (Bdq)	Bedaquiline (Ad on agent/Group D2)
	Delamanid (Dlm)	Delamanid (Ad on agent/Group D2)
	Linezolid (Lzd)	Linezolid (Group C)
	Clofazimine (Cfz)	Clofazimine (Group C)
	Amoxicillin/Clavulanate (Amx/Clv)	Amoxicillin/Clavulanate (Ad on agent/Group D3) ^d
	Imipenem/Cilastatin (Ipm/Cln)	Imipenem/Cilastatin (Ad on agent/Group D3) ^d
	Meropenem (Mpm)	Meropenem (Ad on agent/Group D3) ^d
	High-dose isoniazid (High dose H)	
	Thioacetazone (T)	Thioacetazone (Ad on agent/Group D3) ^e
	Clarithromycin (Clr)	

* Adapted from the companion handbook to the WHO Guidelines for the programmatic management of drug resistant tuberculosis, 2014 and the WHO treatment guidelines for drug resistant tuberculosis, 2016 update

^a This regrouping is intended to guide the design of longer regimens; the composition of the recommended shorter MDR-TB regimen is standardized

^b Medicines in Groups A and C are shown by decreasing order of usual preference for use

^c Refer to the text for the conditions under which streptomycin may substitute other injectable agents, Resistance to streptomycin alone does not qualify for the definition of XDR-TB

^d Carbapenems and clavulanate are meant to be used together; clavulanate is only available in formulations combined with amoxicillin.

^e HIV-status must be confirmed to be negative before thioacetazone is started

B. Mechanisms of action and resistance of anti-TB drugs

Tuberculosis drugs target various aspects of *M. tuberculosis* biology, including inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis. Table 1.6 summarizes the mechanisms of action and main mechanisms of resistance to FLDs and main SLDs. Figures 1.9 and 1.10 illustrate briefly the mechanisms of action of current TB drugs and drugs under development respectively.

Regarding drug resistance, there are two main mechanisms: 1). primary or transmitted drug resistance, occurs when resistant strains are transmitted to a new host, and 2). secondary or

acquired drug resistance, which occurs through the acquisition of drug resistance mutations to one or more drugs [74–76]. The acquisition of mutations in genes that code for drug targets or drug-activating enzymes is the primary vehicle driving drug resistance in *M. tuberculosis*. These are mainly in the form of SNPs, insertions or deletions and to a lesser extent, large deletions. Unlike other bacteria, resistance is not acquired via horizontal gene transfer by mobile genetic elements [77]. Other mechanisms of drug resistance in *M. tuberculosis* include compensatory mechanism, efflux-mediated resistance and deficient DNA repair mechanisms. Compensatory mechanism: The presence of co-occurrence of secondary mutations that act as compensatory mechanisms for the impaired fitness of the pathogen. These compensatory mutations are believed to occur in genes encoding the same protein or genes involved in similar metabolic pathways [78]; Efflux-mediated resistance: Efflux pump systems are involved in expelling drugs from the bacterial cell, enabling acquisition of resistance mutations in the bacterial genome. The overexpression of efflux pumps is believed to mediate the build-up of resistance mutations, which confers high-level drug resistance allowing *M. tuberculosis* to survive and persist at clinically relevant drug concentrations. The ability of the efflux pumps to extrude a diversity of compounds allows them to expel multiple drugs leading to the MDR phenotype [79, 80]; Deficient DNA repair mechanisms: Mutations occurring in DNA repair systems alter the ability of such systems to repair efficiently the damaged DNA, thereby increasing mutation rates. This provides a selective advantage to bacteria that bear resistance-conferring mutations [81, 82].

Table 1. 6 Mechanisms of action and main mechanisms of resistance to FLDs and SLDs

Anti -TB drugs	Mechanisms of action	Mains mechanisms of resistance
Isoniazid (INH)	<ul style="list-style-type: none"> - INH (prodrug) activated by the catalase/oxidase enzyme encoded by the <i>katG</i> gene. - Once activated, INH inhibits mycolic acid synthesis via the NADH-dependent enoyl-acyl carrier protein reductase, encoded by the <i>inhA</i> gene [75, 76] 	<ul style="list-style-type: none"> - INH resistance mediated by mutations in the <i>katG</i>, <i>inhA</i> (promoter and coding gene), leading to inefficient INH NAD product inhibiting the antimicrobial action of INH (<i>katG</i>), overexpression of <i>inhA</i> (<i>inhA</i> promoter); decreased affinity of the INH–NAD product (<i>inhA</i> coding) [83–87]

Rifampicin (RIF)	<ul style="list-style-type: none"> - RIF effective against actively metabolizing and slow-metabolizing bacilli. - Binding to the β subunit of the RNA polymerase, resulting in the inhibition of elongation of mRNA [75, 76]. 	<ul style="list-style-type: none"> - Resistance to RIF is mediated by mutations clustered in RRDR (codons 507–533) of the gene coding for the RNA polymerase β subunit (<i>rpoB</i>) [75, 76]
Pyrazinamide (PZA)	<ul style="list-style-type: none"> - Inhibit semi-dormant bacilli located in acidic environments [88] - Activated by the pyrazinamidase/nicotinamidase (PZase) enzyme, encoded by the <i>pncA</i> gene [89] - Disrupts the bacterial membrane energetics, inhibiting membrane transport and damage cell [90] 	<ul style="list-style-type: none"> - Mutations in the <i>pncA</i> (promoter and gene coding), the most common mechanism mediating pyrazinamide resistance [91] - Diversity of mutations (600 unique mutations in 400 positions) [92]
Ethambutol (EMB)	<ul style="list-style-type: none"> - Active against actively multiplying bacilli, disrupting the biosynthesis of the arabinogalactan in the cell wall. - The <i>embCAB</i> operon encodes the mycobacterial arabinosyl transferase enzyme 	<ul style="list-style-type: none"> - Resistance to EMB is mediated via mutations in the <i>embB</i> gene [93, 94] - Alteration in codon 306 of the <i>embB</i> gene, the most common resistance mechanism [95, 96]
Streptomycin (STR)	<ul style="list-style-type: none"> - Active against slow-growing bacilli and acts by irreversibly binding to the ribosomal protein S12 and 16S rRNA, which are the components of the 30S subunit of the bacterial ribosome. - Blocks translation thereby inhibiting protein synthesis [97, 98] 	<ul style="list-style-type: none"> - The main mechanism of resistance to STR is believed to be mediated via mutations in the <i>rpsL</i> and <i>rrs</i> genes, encoding the ribosomal protein S12 and the 16S rRNA, respectively [77]
Second-line injectable agents: <ul style="list-style-type: none"> - Aminoglycosides (KAN, Am) - Cyclic polypeptide (CAP) 	<ul style="list-style-type: none"> - All three drugs are protein synthesis inhibitors that act by binding to the bacterial ribosome resulting in a modification of the 16S rRNA structure 	<ul style="list-style-type: none"> - High-level resistance has been associated with mutations in the 1400 bp region of the <i>rrs</i> gene and additional resistance to capreomycin has been associated with polymorphisms of the <i>tlyA</i> gene. - The A–G polymorphism at position 1401 of the <i>rrs</i> gene, the most common mechanism of resistance to all three drugs [99] - Cross-resistance between KAN, AM, CAP occurred.
Fluoroquinolones (FQs)	<ul style="list-style-type: none"> - Targets the DNA gyrase enzyme, thereby preventing transcription during cell replication. - DNA gyrases encoded by the <i>gyrA</i> and <i>gyrB</i> genes 	<ul style="list-style-type: none"> - Resistance to the FQs linked to mutations occurring in a conserved region known as the quinolone resistance-determining region (QRDR) in the <i>gyrA</i> and <i>gyrB</i> genes [76, 100–102]

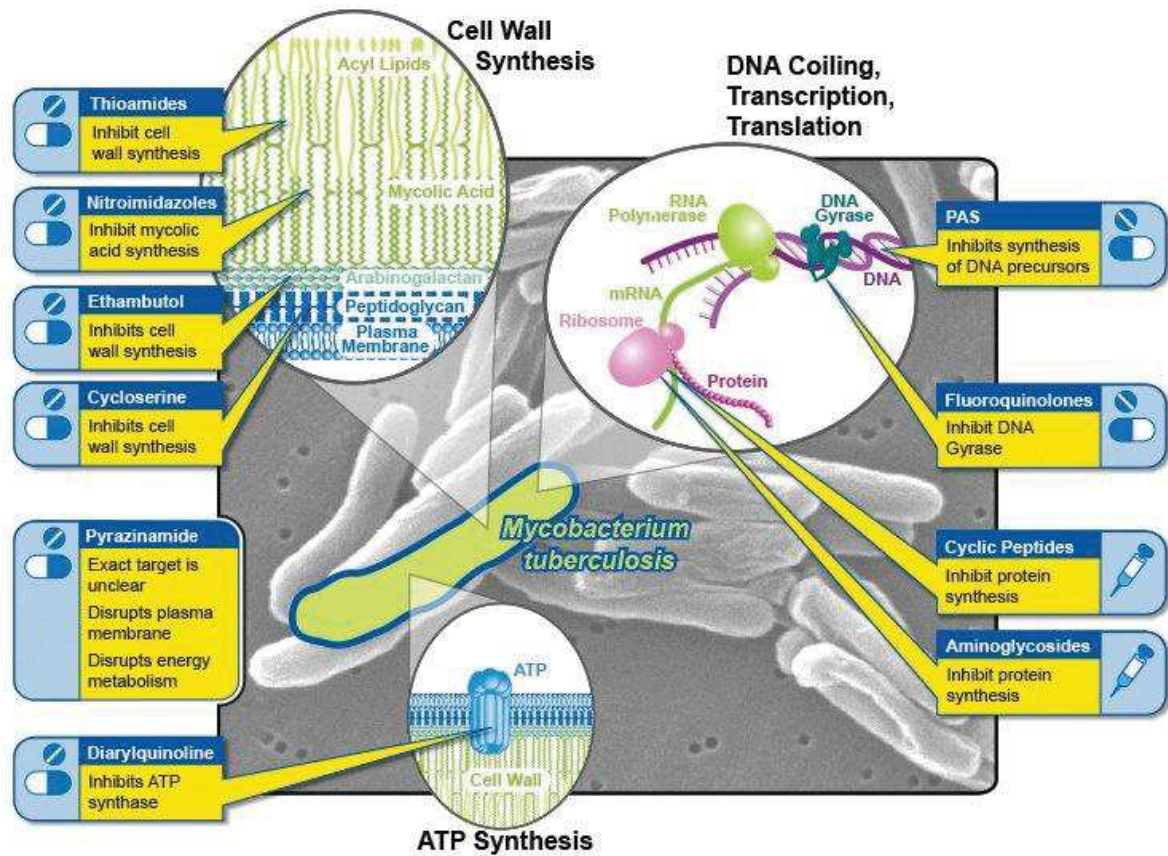


Figure 1. 9 Mechanisms of action of current anti-TB Drugs

Thioamides, Nitroimidazoles, Ethambutol, and Cycloserine act on cell wall synthesis. Diarylquinoline inhibits ATP synthase. PAS, Fluoroquinolones, Cyclic Peptides and Aminoglycosides act on the DNA

Source: National Institute of Allergy and Infectious Diseases (NIAID).
<https://www.niaid.nih.gov/diseases-conditions/tbdrugs>

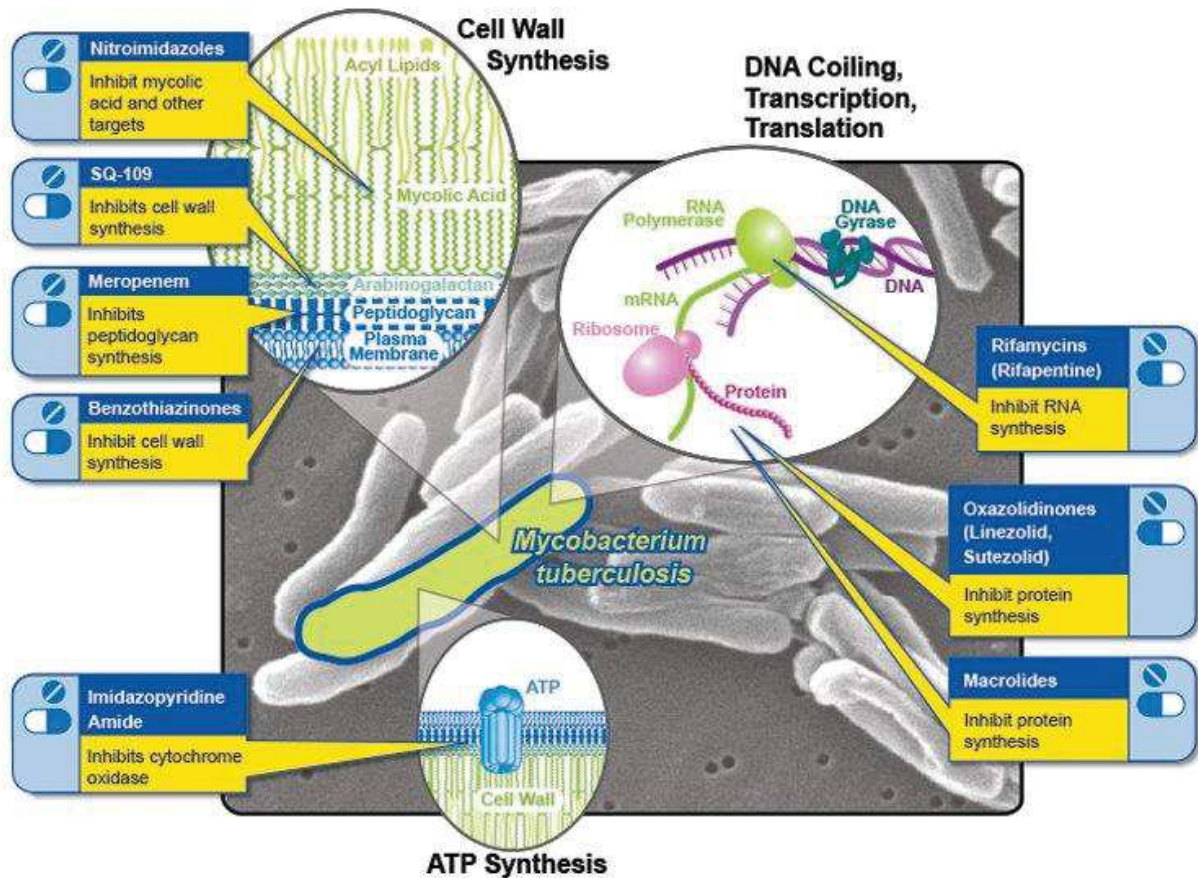


Figure 1. 10 Mechanisms of action of anti-TB drugs under Development

Nitroimidazoles, SQ-109, Meropenem, and Benzothiazinones act on cell wall synthesis. Imidazopyridine Amide inhibits ATP synthesis. Rifamycins, Oxazolidinones and Macrolides act on DNA.

Source: National Institute of Allergy and Infectious Diseases (NIAID).
<https://www.niaid.nih.gov/diseases-conditions/tbdrugs>

C. Common genes involved in resistance of *Mycobacterium tuberculosis*

There are two types of drug resistance in *M. tuberculosis*: genetic resistance and phenotypic resistance. Genetic drug resistance is due to mutations in chromosomal genes in growing bacteria, while phenotypic resistance or drug tolerance is due to epigenetic changes in gene expression and protein modification that cause tolerance to drugs in non-growing persister

bacteria [103]. At present, there are several mutations (SNPs, insertions or deletions of bases) in genes or genomic regions of *M. tuberculosis* described associated with anti-TB drug resistance (Table 1.7); however the most studies are the ones related to the FLD and the core SLD resistance, such as *katG* gene, *inhA* gene coding and the *inhA* promoter (INH resistance); *rpoB* gene (RIF resistance); *rpsL* gene and *rrs*-F1 fragment (STR resistance); *embB* gene (EMB resistance); *pncA* gene and its promoter (PZA resistance), *gyrA* and *gyrB* genes (FQ resistance), *rrs*-F2 fragment (SLID resistance).

Table 1. 7 Common genes involved in resistance of *M. tuberculosis* to classical, new and repurposed anti-TB drugs*

Drug/gene	Associated MIC (mg/L)	Mutation frequency among resistant isolates (%)	Compensatory mechanisms
Isoniazid			
<i>katG</i>	0.02–0.2	70	<i>oxyR0</i> and <i>ahpC</i>
<i>inhA</i>		10	
<i>kasA</i>		10	
Rifampicin			
<i>rpoB</i>	0.05–1	95	<i>rpoA</i> and <i>rpoC</i>
Ethambutol			
<i>embB</i>	1–5	70	unknown
<i>ubiA</i>		45, occurs with <i>embB</i> mutations	
Pyrazinamide			
<i>pncA</i>	16–100	99	unknown
<i>rpsA</i>		no clinical evidence	
<i>panD</i>		no clinical evidence	
Streptomycin			
<i>rpsL</i>	2–8	6	unknown
<i>rrs</i>		<10	
<i>gidB</i>		clinical relevance to be determined	
Fluoroquinolones			
<i>gyrA</i>	0.5–2.5	90	<i>gyrA</i> (T80A and A90G)
<i>gyrB</i>		<5	putative <i>gyrB</i>

Capreomycin, amikacin and kanamycin			
<i>rrs</i>	2–4	60–70	<i>rrs</i> (C1409A and G1491T)
<i>eis</i>		80 (low-level kanamycin)	
<i>tlyA</i>		3 (capreomycin)	
Ethionamide			
<i>ethA</i>	2.5–25		unknown
<i>mshA</i>		mutations occurring in various combinations in these genes account for 96% of ethionamide resistance	
<i>ndh</i>			
<i>inhA</i>			
<i>inhA</i> promoter			
Para-aminosalicylic acid			
<i>thyA</i>	1–8	40	unknown
<i>folC</i>		to be determined	
<i>ribD</i>		90	
Bedaquiline			
<i>rv0678</i>	0.06–1	clinical relevance of mutations to new drugs is to be determined.	
<i>atpE</i>		<i>atpE</i> described in two clinical isolates to date. Rv0678 occurs intrinsically, without prior exposure to drug. PepQ not detected in clinical isolates	
<i>atpE</i>			
Clofazimine			
<i>rv0678</i>	0.1–1.2	clinical relevance of mutations to new drugs is to be determined.	unknown
<i>rv1979c</i>		80% in <i>rv0678</i> with cross-resistance to bedaquiline. 20% <i>rv1979c</i> with resistance to clofazimine only	
<i>rv2535c</i>			
<i>ndh</i>			
<i>pepQ</i>			
Delamanid/pretonamid			
<i>fgd</i>	1 0.006–0.24	clinical relevance of mutations to new drugs is to be determined. Fdg1 emerging in clinical resistance	unknown
<i>fbiC</i>	(delamanid)		
<i>fbiA</i>	0.015–0.25		
<i>fbiB</i>	(pretonamid)		
<i>ddn</i>			
Linezolid			
<i>rpIC</i>	0.25–0.5	90	unknown
<i>rrl</i>		1.9–11	

*Source: Dookie et al 2018. [104]

Chapter 2 MATERIALS AND METHODS

2.1. Samplings and methods used in the study

The figure 2.1 presents the flowchart of the study. Each sampling (the first national tuberculosis prevalence survey in Lao PDR (TBPS) 2010-2011, the presumptive MDR-TB 2010-2014 and the first national anti-tuberculosis drug resistance survey (DRS) 2016-2017) is detailed in “Results” section.

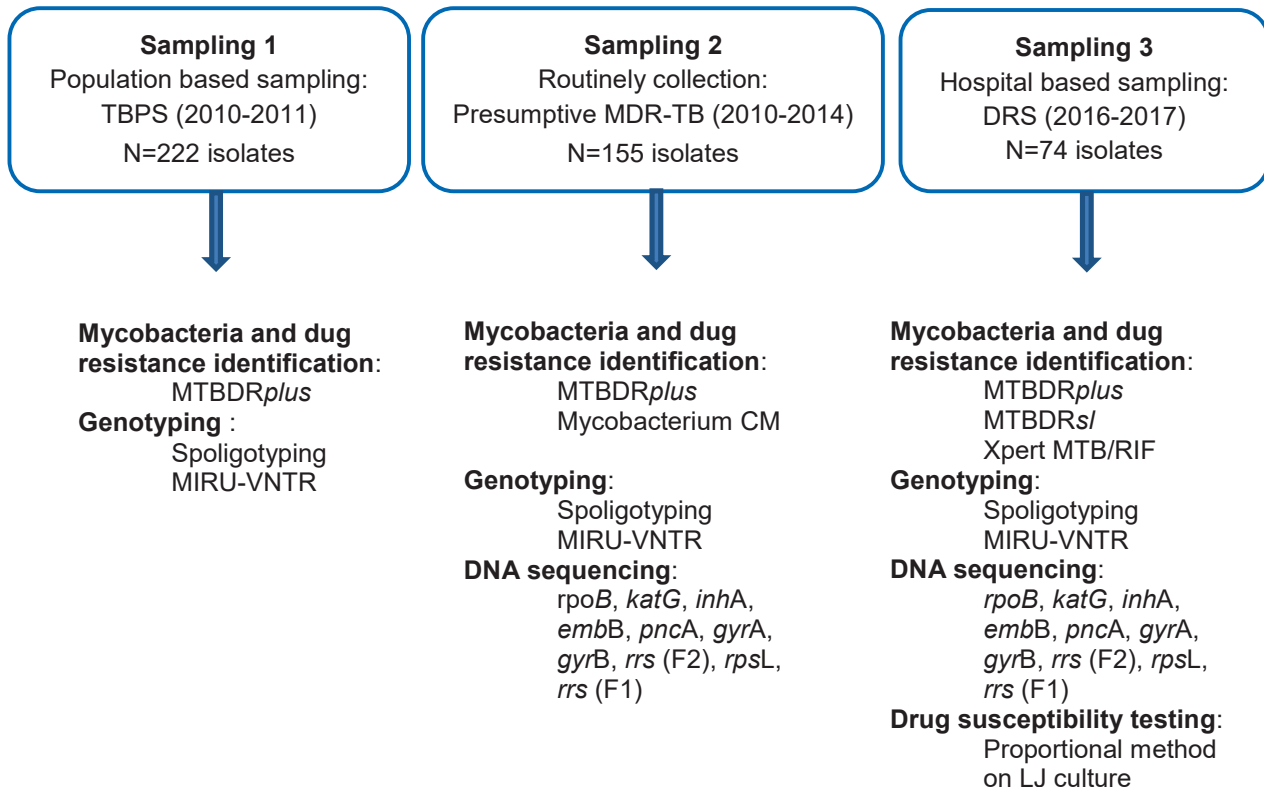


Figure 2. 1 Flowchart of the study presenting the three different samplings used in the study and the methods applied on each sampling

2.2. Ethic approval of research

This study proposal was approved by the National Ethics Committee of Health Research of Lao PDR. Written informed consents were obtained from all study participants.

2.3. Methods

2.3.1. Drug susceptibility testing (DST)

Drug susceptibility testing (DST) was recently available at NRL (2015). Among the three sampling of this thesis, only DRS (2016-2017) had available DST results. The proportion method on solid Löwenstein–Jensen culture was used. A loop of colonies was scrapped from the subculture, transferred into a tube containing glass beads and vortexed to separate the colonies. The concentration of bacilli was adjusted to a Mac Farland No 1 standard. Tenfold dilution was realized until the 10⁻⁵ dilution. One hundred µl of the 10⁻³ dilution was inoculated onto 2 LJ drug free media and all drug containing LJ media. One hundred µl of the 10⁻⁵ dilution was inoculated onto 2 LJ drug free media. The minimal inhibitory concentration (MIC) of each drug was chosen following WHO's recommendation: 0.2 µg/ml for INH, 40 µg/ml for RIF, 4.0µg/ml for STR, 2.0 µg/ml for EMB, 30 µg/ml for KAN, 40 µg/ml for CAP and 40 µg/ml for OFX. The interpretation of the resistance was determined according to the proportion method principle based on the number of colonies observed [105, 106] .

2.3.2. Xpert MTB/RIF testing

The Xpert MTB/RIF assay is almost fully automated cartridge-based system, utilizing real-time PCR technology to both diagnose TB and detect rifampicin resistance in less than 2 hours. The assay uses molecular beacon technology [107, 108] to detect DNA sequences amplified in a hemi-nested real time-PCR assay. Five different nucleic acid hybridization probes are used in the same multiplex reaction [109, 110]. Each probe is complementary to a different

target sequence within the *rpoB* gene of rifampicin-susceptible *M. tuberculosis* and is labeled with a differently colored fluorophore. Together, these overlapping probes span the entire 81 base pairs core region of the *rpoB* gene. *M. tuberculosis* is identified when at least two of the five probes give positive signals with a cycle threshold (CT) of ≤ 38 cycles [111, 112]. The system reports resistance, when the difference between the first (early CT) and the last (late CT) *M. tuberculosis*-specific beacon (Δ CT) was >3.5 cycles (e.g: the first probe CT is >34.5 cycles and the last probe has a CT of >38 cycles). If Δ CT is ≤ 3.5 cycles, the system reports sensitivity. A semi-quantitative estimate of the concentration of bacilli is defined by the CT range (high, <16 ; medium, 16–22; low, 22–28; very low, >28).

During DRS (2016-2017), Xpert MTB/RIF was performed in parallel with AFB smear microscopic. Specimens analyzed by Xpert MTB/RIF were not treated with CPC. Xpert MTB/RIF testing was performed following Cepheid instructions. Two volumes of the “Sample Reagent” were added to one volume of sputum. The sample was shaken vigorously 10-20 times. After 10 minutes of incubation at room temperature, the sample was shaken again 10-20 times then left at room temperature for another 5 minutes. The sample was then transferred into the cartridge and ready to be loaded in the Xpert MTB/RIF module (Figure 2.2) [113].

***rpoB* GENE 81 bp RIF RESISTANCE DETERMINING REGION**

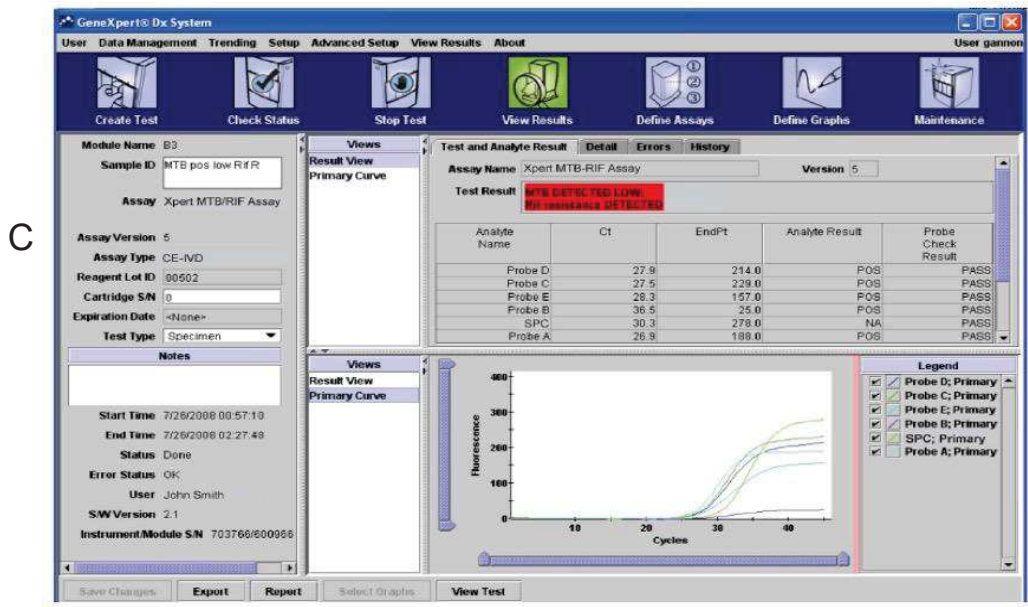
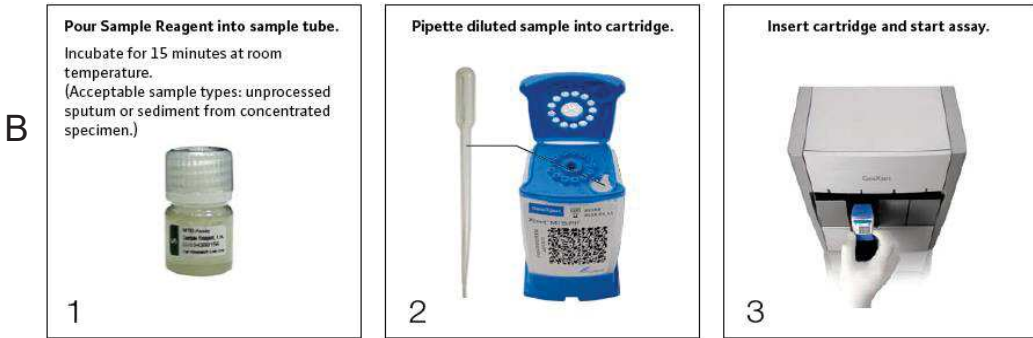


Figure 2. 2 Xpert MTB/RIF testing

(Source: Xpert MTB/RIF Brochure – Cepheid)

- A. Five molecular probes overlapping the entire 81 bp core region of the *rpoB* gene
- B. The three easy steps of Xpert MTB/RIF testing
- C. GeneXpert Dx System—Privileged User View Results window, MTB Detected Low, RIF resistance detected

2.3.3. DNA preparation

Genomic DNA of study samples were obtained either by heat treatment (using water) or by DNA extraction by GenoLyse kit (Hain Lifescience). For heat treatment, the subcultures were scraped from media slopes and resuspended in 300µL of molecular biology-grade water, heated for 20 min at 95°C, centrifuged for 5 minutes at 13000 g. Then the supernatant containing DNA was transferred into a new tube and stored at -80 °c for further molecular analysis. For the use of GenoLyse kit, the subcultures were scraped from media slopes and resuspended in 100 µL of lysis buffer. The samples were then incubated for 5 min at 95°C (lysis under alkaline conditions). Then we added 100 µl of neutralization buffer, vortex sample, spin down and the supernatants were transferred to a new tube and stored at -80 °c.

2.3.4. GenoType® Mycobacteria Series (GenoType® MTBDR*plus*, MTBDR*s/l* and Mycobacterium CM)

The GenoType® MTBDR*plus* ver.1, MTBDR*s/l* ver.1 and Mycobacterium CM tests (Hain Lifescience GmbH) are DNA STRIP®based technologies and permit the molecular genetic identification of different mycobacteria and resistance to anti-TB drugs. These commercial kits are provided with all necessary reagents.

The GenoType® MTBDR*plus* ver.1 allows identifying *M. tuberculosis* complex and its resistance to RIF and/or INH. The identification of RIF resistance is enable by the most common mutations of *rpoB* gene within the 81 base pairs hot-spot region (codon 505-533, *E. coli* numbering). For detection of high and low level INH resistance the *katG* gene (codon 315) and the promoter region of *inhA* gene (nucleic acid position -8 to -16) were examined respectively (GenoType® MTBDR*plus* ver.1 Handbook).

GenoType MTBDR*s/l* ver.1 simultaneously identifies *M. tuberculosis* complex and its resistance to Fluoroquinolones (FQ; e.g. ofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (AG/CP; injectable drug as kanamycin, amikacin/capreomycin

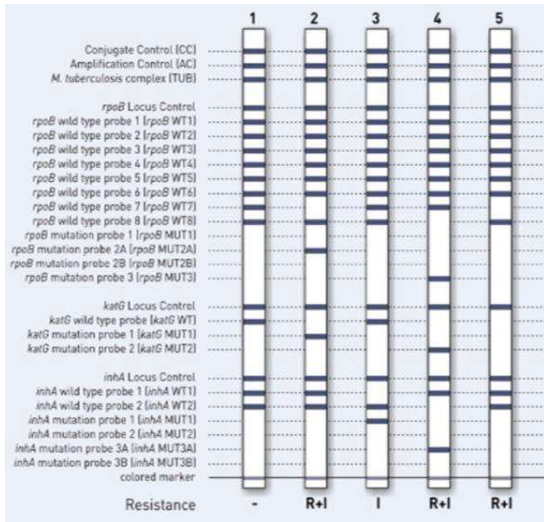
ad viomycin) and/or ethambutol (EMB). The identification of FQ resistance is enable by the most common associated mutations of *gyrA* gene (codon 85-97). For the detection of AG/CP resistance, the *rrs* gene (16S rRNA gene; nucleic acid position 1401, 1402 and 1484) and for the detection of EMB-resistance, the *embB* gene (codon 306) were included in the kit (GenoType MTBDRs/ ver.1 Handbook).

GenoType Mycobacterium CM (Common Mycobacteria) ver.1 test permits the identification of the following mycobacterial species: *M. avium ssp.*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. peregrinum*, *M. marinum/M. ulcerans*, the *M. tuberculosis* complex and *M. xenopi* (GenoType Mycobacterium CM Handbook).

The MTBDR*plus*, MTBDRs/ and Mycobacterium CM were performed on clinical isolates and carried out according to the manufacturer's instructions. The whole process of the tests was divided into 4 steps: 1). DNA extraction (heat treatment or GenoLyse kit), 2). Multiplex amplification with biotinylated primers, 3). Reverse hybridization (chemical denaturation of amplification products, hybridization of single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of streptavidine/alkaline phosphatase (AP) conjugate and AP and an AP mediated staining reaction) and 4). Evaluation and interpretation of results.

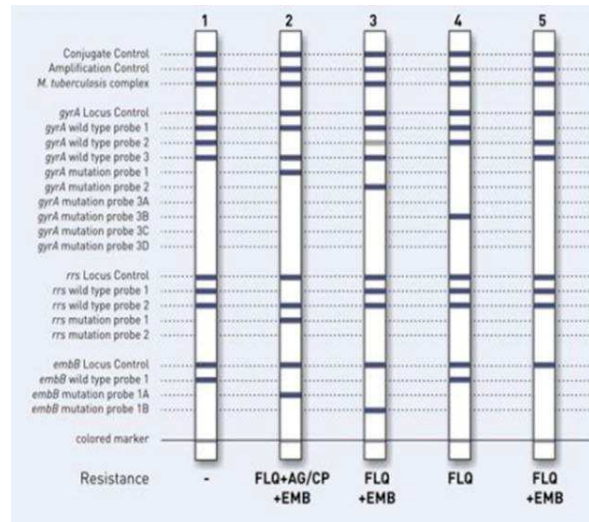
The MTBDR*plus* and MTBDRs/ were firstly evaluated by the presence of three control bands (conjugate control, amplification control and *M. tuberculosis* complex control band) and each locus control (Figure 2.3). Positive results for all wild type probes of a gene and absence of positive signal for mutation probes suggest strain sensitivity for the considered antibiotic. The absence of signal for at least one of the wild type probes (with or without the presence of mutation probes), hence indicates resistance of tested strain to the considered antibiotic (Figure 2.3).

MTBDR_{plus} ver 1.



R: Rifampicin, I: Isoniazid

MTBDR_s ver 1



FLQ: Fluoroquinolones, AG/CP: Aminoglycosides/Cyclic peptides, EMB: Ethambutol

Figure 2. 3 MTBDR_{plus} ver.1 and MTBDR_s ver.1,example of banding patterns for sensitive and resistant samples

For evaluation and interpretation of Mycobacterium CM result, the three control bands (Conjugate Control (CC), Universal Control (UC) and Genus Control (GC)) must be present. Determine species with the help of the interpretation chart (Figure 2.4)

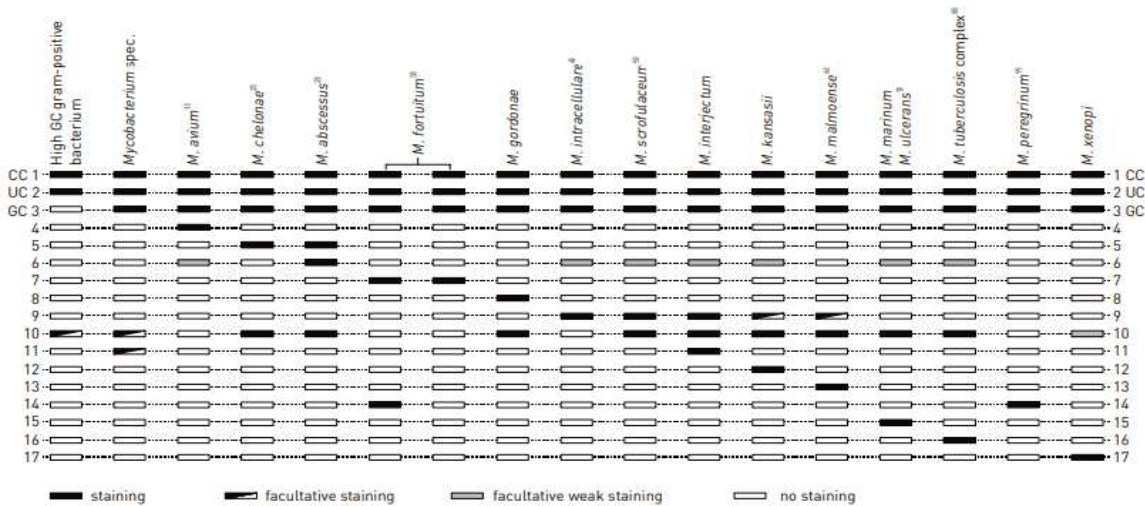


Figure 2. 4 GenoType Mycobacterium interpretation chart

(Source: GenoType Mycobacterium CM hand book)

Band No. 1 (CC): Conjugate Control, Band No. 2 (UC): Universal Control, Band No. 3 (GC): Genus Control

- 1) Species may possibly be further differentiated with the GenoType Mycobacterium AS kit.
- 2) In case the GC band is not developed, the present strain can also be *M. abscessus*.
- 3) Due to variations in the probe region *M. fortuitum* is divided into two groups.
- 4) *M. "paraffinicum"* and *M. parascrofulaceum* show the same banding pattern as *M. scrofulaceum*.
- 5) *M. nebraskense* shows the same banding pattern. *M. haemophilum* can be identified by the GenoType Mycobacterium AS kit.
- 6) *M. ulcerans* can be identified by the GenoType Mycobacterium AS kit.
- 7) For further differentiation use the GenoType MTBC kit.

2.3.5. Spoligotyping

Spoligotyping is an amplification-based genotyping method that assesses the genetic diversity of direct repeat (DR) locus [114]. The DR locus contains multiple 36-base pair (bp) DRs that are separated by 37 to 41 bp unique spacer sequences (Figure 2.5) [115]. The 43 spacers are commonly used for genotyping [114]. Classical spoligotyping is performed by reverse line blot hybridization of biotinylated PCR products to a membrane with 43 covalently bound synthetic oligonucleotides representing the different spacers selected from *M. tuberculosis* H37Rv (spacers 1–19, 22–32, and 37–43) and *M. bovis* BCG (spacers 20–21 and 33–36) (Figure 2.5) [115]. The presence and absence of each spacer is specific for each individual and used for genotyping.

In this study, the classical 43-spacer format of spoligotyping was performed as previously described [114, 116]. DNA samples of the *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG strains were included as positive controls. Molecular biology-grade water was used as a negative control. The spoligotypes (presence and absence of spacers) were then recorded in 43-digit binary format and compared with those recorded in the SpolDB4 database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) to identify the Spoligotype International Type (SIT) and family [62]. For the spoligotypes that matched the SITs, but could not be related to any family (i.e., unknown), and for the spoligotypes that were not present in the SpolDB4 database (e.g., orphan), the SPOTCLUST program, which was built from the spolDB3 database (http://tbinsight.cs.rpi.edu/run_spotclust.html) [117], was used to search for *M. tuberculosis* family similarity. In the SPOTCLUST analyses, the family assignment was retained when the probability was $\geq 90\%$. Nevertheless, the final designation of families and subfamilies was also based on the MIRU-VNTR data (see below).

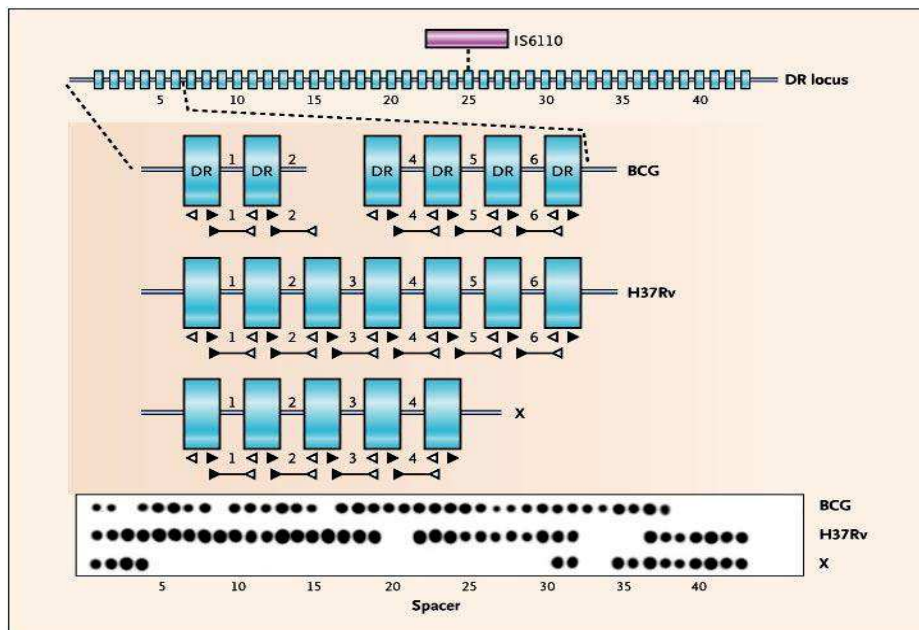


Figure 2. 5 Direct repeat locus and Schema of spoligotyping

The top section shows the 43 direct repeats (rectangles) and spacers (horizontal lines) used in spoligotyping and a copy of IS6110 is inserted within a 36-bp direct repeat in the middle of the DR locus. The middle section shows the products of PCR amplification of spacers 1 through 6 of *M. bovis* BCG, *M. tuberculosis* strain H37Rv, and *M. tuberculosis* hypothetical strain X, with the use of primers (white and black arrowheads) at each end of the DR locus. The bottom section shows the spoligotypes of the three strains.

(Source: Barnes and Cave 2003)

2.3.6. MIRU-VNTR typing

Variable number tandem repeat (VNTR) loci contain tandemly repeated sequences that are dispersed by thousands of copies and found in almost all higher eukaryote genomes [118]. Their repeat numbers are highly variable in many loci and therefore are called “variable number tandem repeat” loci [119, 120]. Small repetitive DNA sequences with different unique characters were found in *M. tuberculosis* and other mycobacterial genomes [111, 121–125]. A novel minisatellite-like structure in the *M. tuberculosis* genome composed of 40- to 100-bp repetitive sequences were identified in 1997 by Supply et al. [126] and named them “mycobacterial interspersed repetitive units” (MIRU). MIRUs are dispersed within intergenic regions and located

in 41 locations throughout the genome of *M. tuberculosis* H37Rv. Among those 41 locations, 12 show polymorphisms in copy number of non-related *M. tuberculosis* isolates (Figure 2.6) [127].

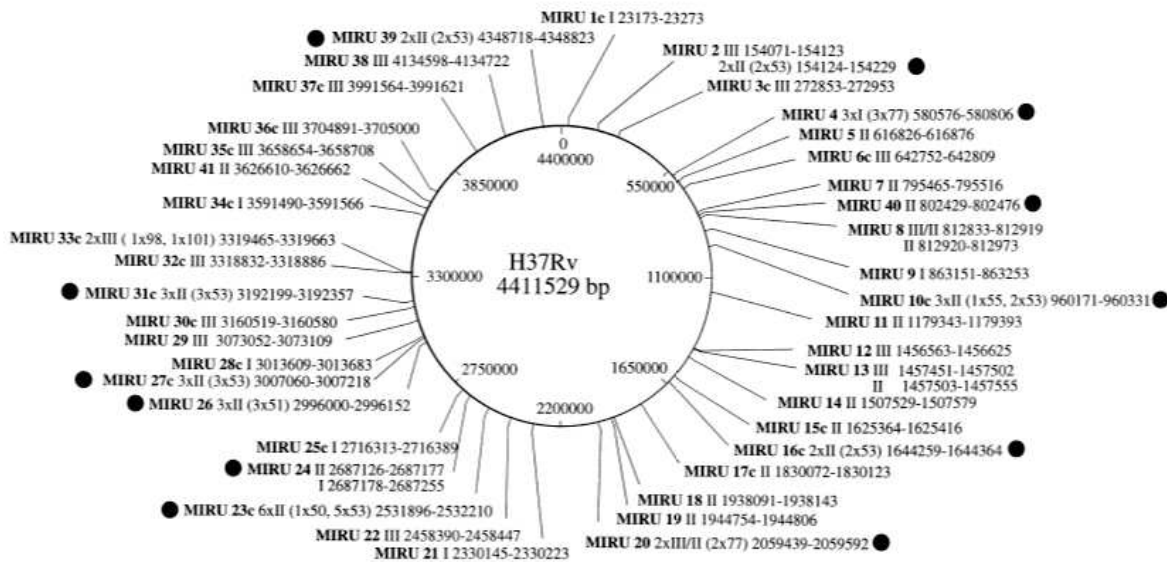


Figure 2. 6 Tandem repeat variability

Position of the 41 MIRU loci on the *M. tuberculosis* H37Rv chromosome. Arabic numbers in bold specify the respective MIRU locus numbers. The “c” designates that the corresponding MIRUs are in the reversed orientation to that defined by Cole et al. 1998. Roman numbers give the type of MIRU (type I, II or III). The exact positions of the MIRU loci are given in arabic numbers after the type numbers. The 12 loci containing variable numbers of MIRUs among the 31 analysed strains are indicated by black dots. (Source: Supply et al. 2000)

To date, standardized sets of 12 or 15 or 24 MIRUs-VNTR can be used to type *M. tuberculosis* strain. MIRU-VNTR typing provides the number and size of the repeats for each independent MIRU locus, after DNA amplification by polymerase-chain-reaction (PCR) assay followed by gel electrophoresis (Figure 2.7). The discriminatory power of MIRU-VNTR genotyping is almost as great as that of IS6110 based genotyping [128, 129] and technically simpler than IS6110-based genotyping.

In our study, MIRU-VNTR typing was performed as previously described [130, 131] and the full set of 24 MIRU-VNTR loci was used for isolate characterization. The patterns obtained

for the 24 loci were used to create a 24-digit allelic profile for each isolate. The MIRU-VNTR typing results were analyzed using MIRU-VNTRplus (<http://www.miru-vntrplus.org>), a freely accessible web-based program [132]. A Neighbor-Joining (NJ) tree based on categorical distances was built by combining the spoligotyping and MIRU-VNTR results. The final designation of family/subfamily was revised using the MIRU-VNTRplus website ([MIRU-VNTRplus.org](http://www.miru-vntrplus.org)) based on the family results for each isolate and the MIRU-VNTR/spoligotyping phylogenetic tree.

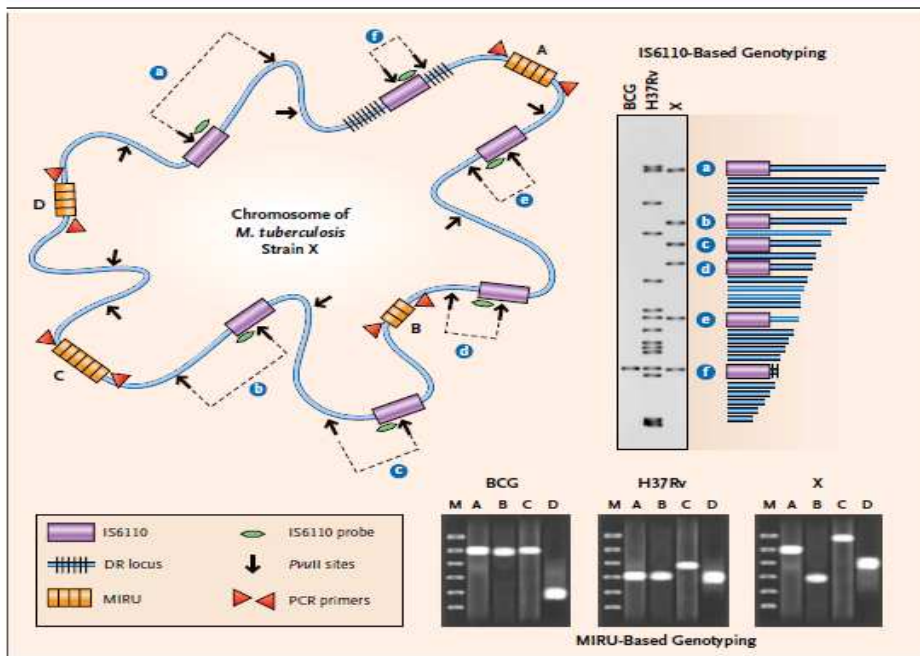


Figure 2. 7 Chromosome of *M. tuberculosis* hypothetical strain X and Genotyping of *M. bovis* BCG, the *M. tuberculosis* laboratory strain H37Rv, and Strain X on the Basis of IS6110 insertion sequences and Mycobacterial Interspersed Repetitive Units (MIRUs).

The top left-hand panel shows the chromosome of hypothetical strain X, as shown by the arrows. The top right-hand panel shows the results of IS6110-based genotyping. The three bottom panels show the results of MIRU-based genotyping.

(Source: Barnes and Cave 2003)

2.3.7. Sanger sequencing

The main genes associated with resistance to first line anti-TB drugs (FLDs) and second line anti-TB drugs (SLDs) were amplified by PCR and sequenced. For the FLD resistance, the following genes and gene fragments were studied: *katG* gene, *inhA* gene coding and the *inhA* promoter (INH resistance); *rpoB* gene (RIF resistance); *rpsL* gene and *rrs*-F1 fragment (STR resistance); *embB* gene (EMB resistance); *pncA* gene and its promoter (PZA resistance). For the SLDs resistance, the following genes and gene fragments were analyzed: *gyrA* and *gyrB* genes (FQ resistance), *rrs*-F2 fragment (SLID resistance). The list of primers (Table 2.1), PCR conditions (Table 2.2) and DNA sequencing are previously described [133, 134]. Each sequence was treated independently using the Bioedit software (version 7.1.10). The consensus sequence was generated. Multi-sequence alignment was then performed. Point mutations were identified by comparison with the sequence of the *M. tuberculosis* H37Rv reference strain available in GenBank (NC.000962.3). To describe the resistance-associated mutations in *rpoB* gene, a numbering system based on the *Escherichia coli* sequence annotation has been used.

Table 2. 1 Primers used for DNA amplification and sequencing of genes involved in anti-TB drug resistance

Drug(s)	Gene/gene promoter	Primer sequence	Annealing T °C	Length (bp)	Target region	
RIF	<i>rpoB</i> - F1	F- <i>rpoB</i> 1: 5'-GTCGACGCTGACCGAAGAAG-3'	62	1148	Clusters I (including RRDR), II, III	
		R- <i>rpoB</i> 1: 5'-TCTCGCCGTCGTCAGTACAG-3'				
INH	<i>katG</i>	F- <i>katG</i> 1: 5'-CCAACCTCTGGAAGGAATGC-3'	58	1168	Full length gene	
		R- <i>katG</i> 1: 5'-AGAGGTCAGTGCCAGCAT-3'				
		F- <i>katG</i> 2: 5'-ACGAGTGGGAGCTGACGAA-3'	60	1217		
		R- <i>katG</i> 2: 5'-AACCCGAATCAGCGCACGT-3'				
	<i>inhA</i>	F- <i>inhA</i> -promoter: 5'-GCGACATACCTGCTGCGCAA-3	60	300		Promoter region
		R- <i>inhA</i> pro: 5'-ATCCCCGGTTTCTCCGGT-3'				
F- <i>inhA</i> : 5'-GACACAACACAAGGACGCA-3'		59	1006	Full length gene		
R- <i>inhA</i> : 5'-TGCCATTGATCGGTGATACC-3'						
STR	<i>rrs</i> -F1	F- <i>rrs</i> -F1: 5'-GAGAGTTTGATCCTGGCTCAG-3'	58	972	Loops 530 & 915	
		R- <i>rrs</i> -F1: 5'-CCAGGTAAGTTCTTCGCGTTG-3'				
	<i>rpsL</i>	F- <i>rpsL</i> : 5'-GCGCCCAAGATAGAAAG-3'	57	440	Full length gene	
		R- <i>rpsL</i> : 5'-CAACTGCGATCCGTAGA-3'				
(KAN, AMK & CAP)	<i>rrs</i> -F2	F- <i>rrs</i> -F2: 5'- GCGCAGATATCAGGAGG-3'	58	918	1400-1500 region	
		R- <i>rrs</i> -F2: 5'- CGCCCACTACAGACAAG-3'				
EMB	<i>embB</i>	F- <i>embB</i> : 5'-TGACCGACGCCGTGGTGATA-3'	62	1312	ERDR & flanking sequences	
		R- <i>embB</i> : 5'-GCCATGAAACCGCCACGAT-3'				
FQ	<i>gyrA</i> and <i>gyrB</i>	F- <i>gyrAB</i> : 5'-GCAACACCGAGGTCAAATCG-3'	62	1296	QRDRs of <i>gyrA</i> & <i>gyrB</i>	
		R- <i>gyrAB</i> : 5'-CTCAGCATCTCCATCGCCAA-3'				
PZA	<i>pncA</i>	F- <i>pncA</i> : 5'- GCTTGCGGCGAGCGCTCCA-3'	62	709	<i>pncA</i> and its promoter	
		R- <i>pncA</i> : 5'-TCGCGATCGTCGCGCGTC-3'				

Table 2. 2 PCR cycle and temperature conditions with HotStarTaq

No	No of cycle	Step	Temperature (°C)	Time
1	1	Taq activation	95	15 min
2	35	Denaturation	95	1 min
3		Annealing*	58-62	1 min
4		Elongation1*	72	2 min
5	1	Elongation2	72	5 min

2.4. Data analysis

Patients' information (the anonymity of the patients was maintained) was registered and cross-checked by research team before being imported to Stata (v12.1, Stata Corporation, USA) for statistical analyses. Median and interquartile were calculated for age of patients. Comparisons between proportions (e.g. Gender, Age group, Strata, Regions, *M. tuberculosis* families, Drug resistant patterns) were performed using chi-square analysis and Fisher's exact test; when the sample size was lower than 5. Statistical significance was defined as a P value of <0.05. A genetic cluster was defined as two or more isolates with identical genotype by 43-spacer spoligotyping and 24-locus MIRU-VNTR typing. Recent transmission was estimated by calculating the clustering rate as follows: $CR = (nc-c)/n$, where CR is the clustering rate, nc is the total number of clustered isolates, c is the total number of clusters, and n is the total number of isolates [135]. A Neighbor-Joining (NJ) tree based on categorical distances was built by combining the spoligotyping and MIRU-VNTR results, using MIRU-VNTRplus (<http://www.miru-vntrplus.org>), a freely accessible web-based program [132]. The performances of molecular tests (Xpert MTB/RIF, GenoType MTBDRplus/MTBDRsl and DNA sequencing) were compared to that of a conventional DST for the detection of anti-TB drug resistance. The sensitivity, specificity, predictive positive value (PPV) and Negative predictive value (NPV) was calculated using online tool (https://www.medcalc.org/calc/diagnostic_test.php)

Chapter 3 RESULTS AND DISCUSSIONS

3.1. Result1 (Paper 1): First insights into the genetic characteristics and drug resistance of *Mycobacterium tuberculosis* population collected during the first National Tuberculosis Prevalence Survey of Lao PDR (2010–2011)

1 First insights into the genetic characteristics and drug resistance of

2 *Mycobacterium tuberculosis* population collected during the first National

3 Tuberculosis Prevalence Survey of Lao PDR (2010–2011)

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22 23 **Abstract**

24 **Background:** In Lao People's Democratic Republic (PDR), tuberculosis (TB) prevalence was
25 estimated at 540/100,000 in 2011. Nevertheless, little is known about the genetic characteristics
26 and anti-TB drug resistance of the *Mycobacterium tuberculosis* population. The main objective
27 of this work was to study the genetic characteristics and drug resistance of *M. tuberculosis*

28 population collected during the first National TB Prevalence Survey of Lao PDR (2010–2011) in
29 order to better understand the TB epidemiology in this country. Two hundred and twenty two
30 isolates were analyzed with the GenoType MTBDR_{plus} test for *M. tuberculosis* identification
31 and drug resistance detection. Then, 206 of the 222 isolates were characterized by
32 spoligotyping and MIRU-VNTR typing.

33 **Results:** Among the 222 *M. tuberculosis* isolates, 11 were mono-resistant to isoniazid and 2
34 were resistant to isoniazid and rifampicin (MDR-TB), using the GenoType MTBDR_{plus} test.
35 Among the 202 genetically characterized isolates, the East African-Indian (EAI) family was
36 predominant (76.7%) followed by the Beijing (14.4%) and T (5.5%) families. EAI isolates came
37 from all the country provinces, whereas Beijing isolates were found mainly in the northern and
38 central provinces. A higher proportion of Beijing isolates was observed in people younger than
39 35 years compared to EAI. Moreover, the percentage of drug resistance was higher among
40 Beijing (17.2%) than EAI (5.2%) isolates, and the two MDR-TB isolates belonged to the Beijing
41 family. Combined analysis of the MIRU-VNTR and spoligotyping results (n=202 isolates)
42 revealed an estimated clustering rate of 11% and the occurrence of mini-outbreaks of drug-
43 resistant TB caused by Beijing genotypes.

44 **Conclusions:** The EAI family (the ancient and endemic family in Asia) is predominant in Lao
45 PDR whereas the prevalence of Beijing, the most harmful *M. tuberculosis* family for humans, is
46 still low, differently from neighboring countries. However, its involvement in recent transmission,
47 its association with drug resistance, and its presence in young patients suggest that the Beijing
48 family could change TB epidemiological pattern in Lao PDR. Therefore, efficient TB control and
49 surveillance systems must be maintained and reinforced to prevent the emergence of highly
50 transmissible and drug-resistant strains in Lao PDR, as observed in neighboring countries.

51 **Key words:** Molecular epidemiology, *Mycobacterium tuberculosis* family, Drug-resistant
52 tuberculosis, Lao PDR.

53 **Background**

54 Tuberculosis (TB) remains a major public health problem. Although the number of TB
55 deaths fell by 22% between 2000 and 2015, TB still was one of the top 10 causes of death
56 worldwide in 2015 (World Health Organization 2016), with an estimated 10.4 million of new TB
57 cases worldwide. Six countries account for 60% of all new cases (India, Indonesia, China,
58 Nigeria, Pakistan and South Africa) and more than half of these cases were in Asia. The
59 emergence of drug-resistance is a global issue for TB control. In 2015, the number of new
60 cases of multidrug-resistant TB (MDR-TB) was estimated at 480 000, with an additional 100,000
61 people with rifampicin (RIF)-resistant TB who are eligible for MDR-TB treatment. India, China
62 and the Russian Federation accounted for 45% of all these cases. In Southeast Asian countries,
63 TB is one of the top ten communicable diseases with an increasing emergence of MDR-TB and
64 extensively drug-resistant TB (XDR-TB) (Coker et al. 2011).

65 Lao People's democratic Republic (PDR) (population of 6.8 million in 2015) is not among
66 the high TB burden countries. However, this landlocked country is surrounded by China,
67 Myanmar, Cambodia, Vietnam and Thailand that are among the 30 high TB burden countries in
68 the world. The National Tuberculosis Control Program (NTCP) of Lao PDR started the Directly
69 Observed Treatment Short (DOTS) course strategy in 1995 with the support of the Damien
70 Foundation Belgium (DFB) and WHO. After the first national TB prevalence survey (2010-2011),
71 WHO re-estimated the prevalence of all TB forms at 540/100,000, 1.9 times higher than
72 previous estimates (Law et al. 2015). Moreover, little is known about anti-TB drug resistance.
73 The only available cross-sectional study (n=87 TB isolates) conducted in three hospitals in 2010
74 showed that 8% of isolates were resistant to isoniazid (INH) and 1.2% caused XDR-TB (Iem et
75 al. 2013). Similarly, the *M. tuberculosis* population in Lao PDR is still unknown. Many studies
76 have reported that the Beijing and East African-Indian (EAI) families are predominant in Asian
77 countries (Chen et al. 2016; Ismail et al. 2014; Yu et al. 2013; Phyu et al. 2009; Zhang et al.
78 2011; Nguyen et al. 2012). In Vietnam, the Beijing family is currently invading the country and is

79 more likely to be drug resistant than the EAI family [10, 11](Nguyen et al. 2012, 2016). In this
80 context, the main objective was to study the genetic characteristics and drug resistance of *M.*
81 *tuberculosis* population collected during the first National TB Prevalence Survey of Lao PDR
82 (2010–2011) in order to better understand the *M. tuberculosis* population structure and the TB
83 epidemiology in this country. The specific objectives were: a) to characterize using different
84 molecular methods *M. tuberculosis* isolates collected during the first national survey; b) to
85 describe the spatial distribution of families and drug resistance; c) to explore the link between
86 genetic diversity and demographical data; d) to estimate the clustering rate according to the *M.*
87 *tuberculosis* families and drug resistant patterns

88 **Methods**

89 **1. Study population**

90 The TB isolates used in this study were collected during the first national TB prevalence
91 survey in Lao PDR (July 2010–December 2011). The survey design and sample size were
92 determined according to the WHO recommendations and has been described in Law et al.
93 (2015). During this survey that covered the 17 provinces of Lao PDR (organized in three main
94 regions, North (1-9), Center (10-13) and South (14-17), see Figure 1) , at least one sputum
95 specimen was collected from 6,290 (99.1%) of the 6,346 participants suspected to have TB on
96 the basis of clinical data (chronic cough and/or hemoptysis and/or chest X-ray abnormalities).
97 Finally, TB was confirmed in 237 participants, according to the study case definition (Law et al.
98 2015). Among these 237 patients, 94 had at least one smear-positive sputum and culture-
99 confirmed *M. tuberculosis* (definite cases), 13 had at least one smear-positive sputum and chest
100 X-ray (CXR) findings suggestive of TB with negative culture (probable cases), and 130 had
101 smear-negative but culture-positive specimens. In summary, the presence of *M. tuberculosis*
102 was confirmed by culture in 224 isolates and 222 isolates of these isolates (corresponding to
103 222 different patients) could be included in this study. The collected sputum specimens were

104 decontaminated with 4% sodium hydroxide and then they were inoculated on two slopes of solid
105 Kudoh-modified Ogawa medium without centrifugation (Hans L. Rieder, Armand Van Deun, Kai
106 Man Kam, et al. 2007). All subcultures were sent by the National Tuberculosis Reference
107 Laboratory (NRL) to the Center of Infectiology Lao-Christophe Mérieux (CILM) for species
108 identification and genetic characterization. Colonies were scraped from the medium slopes and
109 resuspended in 300µL of distilled water, heated at 95°C for 20 min, and centrifuged at 13000 g
110 for 5 min. Then, the DNA-containing supernatant was transferred into a new tube and stored at -
111 80°C.

112 The patients' demographic, epidemiologic, and clinical data were collected using a
113 questionnaire, including residence, sex, age, TB history, TB symptoms, and CXR findings.

114 **2. *Mycobacterium tuberculosis* complex identification and drug resistance testing**

115 The GenoType® MTBDR*plus* test (Hain Lifescience GmbH), a DNA STRIP®-based
116 technology, was used according to the manufacturer's instructions to identify the *M. tuberculosis*
117 complex and resistance to RIF and/or INH ("GenoTypeMTBDRplusver1.Pdf," n.d.).

118 **3. Spacer Oligonucleotide Typing (Spoligotyping)**

119 Spoligotyping (the classical 43-spacer format) was performed as previously described
120 (Filliol et al. 2003; Kamerbeek et al. 1997). DNA samples of the *M. tuberculosis* H37Rv and
121 *Mycobacterium bovis* BCG strains were included as positive controls. Molecular biology-grade
122 water was used as a negative control. The spoligotypes were then recorded in 43-digit binary
123 format and compared with those recorded in the SpolDB4 database ([http://www.pasteur-
124 guadeloupe.fr:8081/ SITVIT_ONLINE/](http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/)) to identify the Spoligotype International Type (SIT) and
125 family (Demay et al. 2012). For the spoligotypes that matched the SITs, but could not be related
126 to any family (i.e., unknown), and for the spoligotypes that were not present in the SpolDB4
127 database (i.e., orphan), the SPOTCLUST program, which was built from the spolDB3 database
128 (http://tbinsight.cs.rpi.edu/run_spotclust.html) (Vitol et al. 2006), was used to search for *M.*

129 *tuberculosis* family similarity. In the SPOTCLUST analyses, the family assignment was retained
130 when the probability was $\geq 90\%$. Nevertheless, the final designation of families and subfamilies
131 was also based on the MIRU-VNTR data (see below).

132 **4. Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU- 133 VNTR) typing**

134 MIRU-VNTR typing was performed as previously described (Supply et al. 2006; Gauthier et
135 al. 2015) and the full set of 24 MIRU-VNTR loci was used for isolate characterization. The
136 patterns obtained for the 24 loci were used to create a 24-digit allelic profile for each isolate.
137 The MIRU-VNTR typing results were analyzed using MIRU-VNTRplus ([http://www.miru-
139 vnrplus.org](http://www.miru-
138 vnrplus.org)), a freely accessible web-based program (Allix-Béguet et al. 2008). A Neighbor-
140 Joining (NJ) tree based on categorical distances was built by combining the spoligotyping and
141 MIRU-VNTR results.

142 The final designation of family/subfamily was revised using the MIRU-VNTRplus website (MIRU-
143 VNTRplus.org) based on the family results for each isolate and the MIRU-VNTR/spoligotyping
144 phylogenetic tree.

144 **5. Data analysis**

145 A cluster was defined as two or more isolates with identical genotype by spoligotyping and
146 MIRU-VNTR typing. Recent transmission was estimated by calculating the clustering rate as
147 follows: $CR = (nc - c)/n$, where CR is the clustering rate, nc is the total number of clustered
148 isolates, c is the total number of clusters, and n is the total number of isolates (van Deutekom et
149 al. 2004). The patients' age was shown as median and interquartile range (IQR). Associations
150 between *M. tuberculosis* families, patient data and overall drug resistance status were assessed
151 using the Chi-square or Fisher's exact test, when the sample size was lower than 5. The
152 statistical analysis was not performed for RIF and INH resistance independently due to the small

153 number of resistant isolates. A P-value <0.05 was considered statistically significant. Statistical
154 analyses were done using Stata (v12.1, Stata Corporation, USA).

155 **Ethics approval and consent to participate**

156 This retrospective genotyping study was approved by the National Ethics Committee of
157 Health Research of Lao PDR. A written informed consent was obtained from all study
158 participants during the national tuberculosis prevalence survey (2010-2011).

159 **Results**

160 **1. *M. tuberculosis* complex identification and epidemiological data**

161 The GenoType® MTBDR \textit{plus} test allowed confirming that the 222 isolates included in the
162 study belonged to the *M. tuberculosis* complex. The patients' median age was 56 years (IQR:
163 40-68), with a men to women ratio of 2:1. Patients were mainly from rural areas (83.3% vs
164 16.7% from urban areas), and the number of *M. tuberculosis* isolates across the 17 provinces
165 varied from 0 to 34 (mean number= 13) (Table S1).

166 **2. Characterization of anti-TB drug resistance (GenoType® MTBDR \textit{plus} test)**

167 Analysis of the RIF and INH resistance profile of 222 isolates with the GenoType®
168 MTBDR \textit{plus} test showed that 209 isolates (94.1%) were sensitive to both drugs, 11 (5%) were
169 resistant only to INH, and 2 (0.9%) were resistant to both INH and RIF (MDR-TB). Among the
170 13 INH-resistant isolates, 10 (76.9%) had mutations in the *katG* gene (S315T in all isolates) and
171 3 (23.1%) had mutations in *inhA* promoter region (C15T in two isolates and T8C in one). The
172 two RIF-resistant isolates carried the D516V mutation in *rpoB* gene.

173 **3. Identification of the *M. tuberculosis* families/subfamilies**

174 Spoligotyping and 24-locus MIRU-VNTR typing were performed on 206 of the 222 isolates
175 (Table 1, Figure S1). The *M. tuberculosis* family/subfamily identifications were determined using

176 SITVITWEB (SpolDB4 database), SPOTCLUST and MIRU-VNTRplus. The patterns of four
177 isolates reflected either clonal variants (with double alleles at a single MIRU-VNTR locus) or
178 mixed infections (with double alleles at two MIRU-VNTR loci) (Shamputa et al. 2004) (Table
179 S1). These four isolates were removed from the analysis to avoid incorrect designation. The
180 other 202 isolates had 58 different spoligotype profiles among which 41 spoligotypes were
181 unique and 17 patterns allowed the clustering of 161 isolates. Each cluster contained 2 to 40
182 isolates (average = 9). Moreover, 165 isolates (81.68% of 202) were assigned to 29 SITs and
183 seven families present in the SpolDB4 database; two (1.0%) were unknown; and 35 (17.3%)
184 were orphans. The 35 orphan and the two unknown isolates were then compared using
185 SPOTCLUST. Finally, isolates could be classified in seven *M. tuberculosis* families and ten
186 subfamilies (Table 1). EAI was the predominant family (76.7%, n=155 isolates), followed by
187 Beijing (14.4%, n=29) and T (5.5%, n=11). Five isolates (2.5%) belonged to other families, such
188 as Haarlem (H), Central Asian Strain (CAS), Latin American-Mediterranean (LAM), and Manu.
189 Only one orphan and one unknown isolate could not be identified. Within the EAI family, the
190 most frequent subfamily was EAI5 (53.0%, n=107), followed by EAI1-SOM (8.9%, n=18) and
191 EAI2-Nonthaburi (6.4%, n=13) (Table 1). The subfamily EAI4-VNM, which is found specifically
192 in Vietnam, was poorly represented in our sampled strains (4.5%, n=9) (Table 1). In the
193 southern provinces (N. 14-17) where only the EAI family was represented (Figure 1), the EAI5
194 subfamily was the most common (65.4%, n=34), followed by EAI1-SOM (19.2%, n=10),
195 whereas EAI4-VNM was absent. Unlike the EAI family, which was present in all regions of Lao
196 PDR, the Beijing family was predominantly observed in the northern (58.6%, n=17) and central
197 provinces (41.4%, n=12), and was absent in the southern provinces (Figure 1).

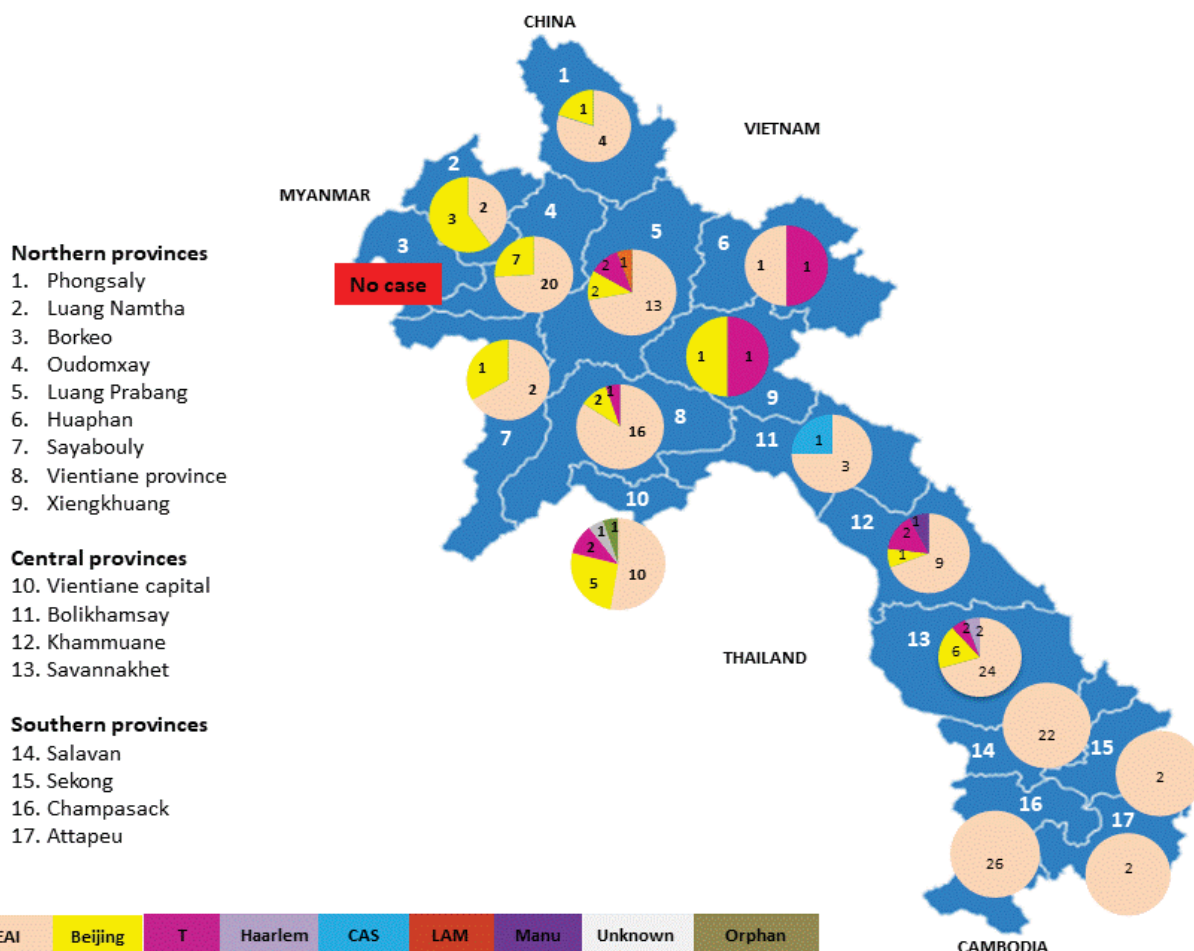
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*Spoligotype defined by SPOTCLUST (probability ≥ 0.9)
^a One isolate with double allele on ETRA and one isolate with double allele on QUB26 were removed from the analysis
^b One isolate with double allele on ETRA was removed from the analysis
^c One isolate with hybridization for all 43 spacers + double alleles on ETRA and Mtub29 was removed from the analysis



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Figure 1. Distribution of *M. tuberculosis* families in the different provinces of Lao PDR (PDF)

The numbers on the map (1 to 17) correspond to the provinces divided in three regions (North, Center, and South). The numbers in the pie charts indicate the number of isolates found in each province. Each *M. tuberculosis* family is represented by a different color (see color code in figure)

4. The distribution of the *M. tuberculosis* EAI and Beijing families varies according to age, geographical origin and drug-resistance

The *M. tuberculosis* family (EAI or Beijing) distribution in the three age groups (15-34, 35-64, and ≥ 65 years of age) was significantly different ($p=0.002$, Table 3). Specifically, the percentage

221 of Beijing family was higher in the “15-34” group compared to EAI (34.5%, 10/29 vs 10.3%,
 222 16/155), and the percentage of EAI family higher in the “35-64” group compared to Beijing
 223 (54.8%, 85/155 vs 34.5%, 10/29). Their geographical distribution also was significantly different
 224 ($p=0.001$, Table 3). In the North and Center, the percentage of Beijing isolates was higher than
 225 that of EAI isolates (58.6% and 41.4% vs 37.4% and 29.0% respectively), whereas the Beijing
 226 family was not observed in the South. Similarly, drug-resistance was higher in the Beijing than
 227 EAI family ($p=0.03$): 17.2% (5/29) of Beijing isolates were resistant to RIF and/or INH compared
 228 with 5.2% (8/155) of EAI isolates. Conversely, the proportion of Beijing and EAI isolates was not
 229 significantly different when patients were divided according to sex and strata (urban versus
 230 rural) (Table 3).

231 **Table 2. Characteristics of the patients infected with EAI (76.7%) or Beijing isolates (14.4%)**

Characteristics	Patients infected with EAI, n=155 (%)	Patients infected with Beijing, n=29 (%)	p-value
Age group (years)			
15-34	16 (10.3)	10 (34.5)	0.002
35-64	85 (54.8)	10 (34.5)	
≥65	54 (34.8)	9 (31.0)	
Sex			
Men	105 (67.7)	15 (51.7)	0.09
Women	50 (32.3)	14 (48.3)	
Strata			
Rural	134 (86.5)	22(75.9)	0.14
Urban	21 (13.5)	7(24.1)	
Regions			
North	58 (37.4)	17(58.6)	<0.001
Centre	45 (29.0)	12(41.4)	
South	52 (33.6)	0	
Anti-Drug resistance status ^a			
Sensitive ^b	147 (94.8)	24(82.8)	0.03
Resistant ^c	8 (5.2)	5^d (17.2)	

232 ^a Tested with the MTBDR*plus* test for Rifampicin (RIF) and isoniazid (INH) resistance.

233 ^b Sensitive to INH and RIF

234 ^c Isolates were considered resistant when they were INH and/or RIF-resistant.

235 ^d Contains two isolates resistant to both INH and RIF (MDR-TB).

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240 **5. 24-locus MIRU-VNTR typing and cluster analysis**

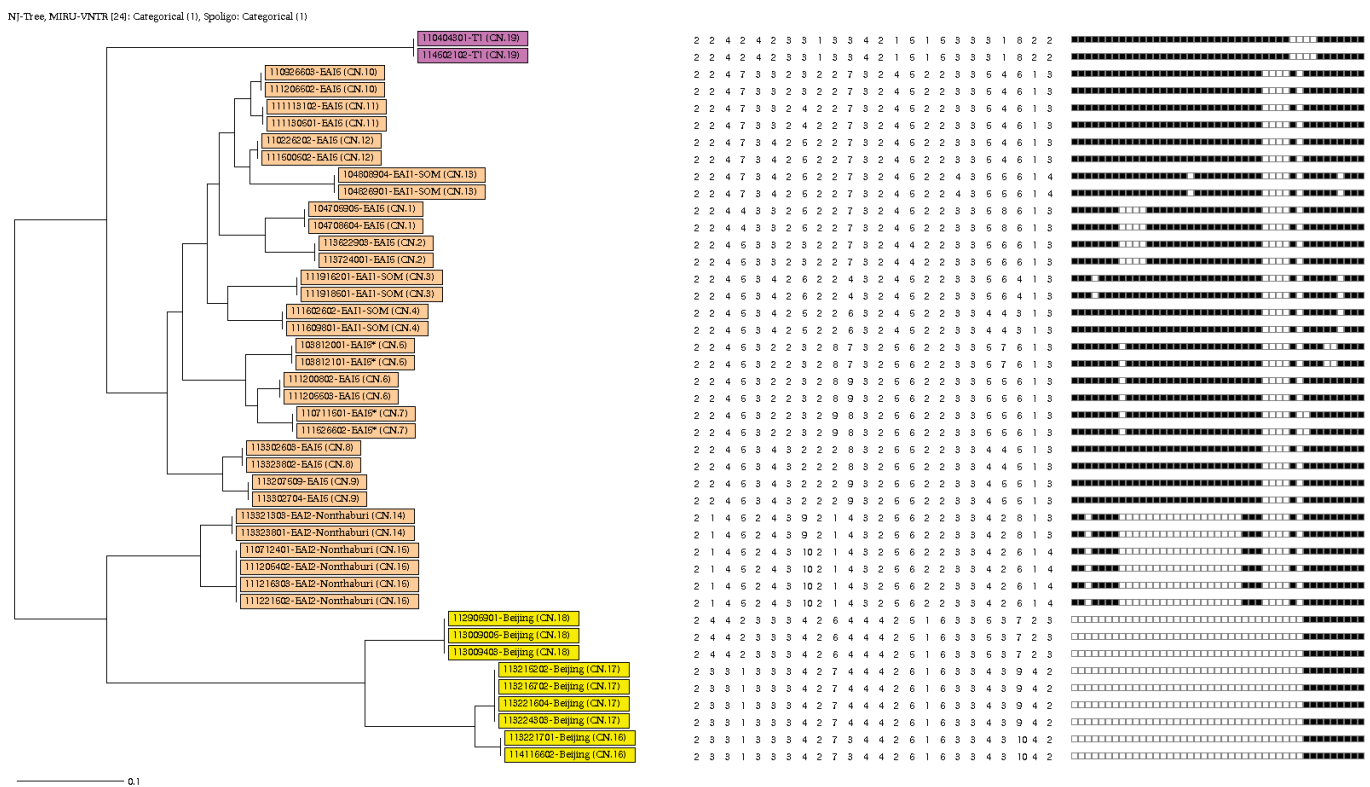
241 **5.1. 24-locus MIRU-VNTR patterns**

242 The 206 isolates that underwent spoligotyping were also typed by 24-locus MIRU-VNTR
243 typing. In 182 isolates (88.4%), all 24 loci could be amplified, whereas in 24 (11.7%) at least one
244 locus could not be amplified (repeated three times). ETRA was the most frequently non-
245 amplified locus (9/206 isolates), followed by QUB4156 (6/206) and QUB11b (5/206). These
246 results were treated as missing data. The four isolates with double alleles (three had double
247 alleles at only one locus and one at two loci (Table S1)) were removed from the global analysis.
248 Thus, the analyses were performed on 202 isolates. By using the results of the 24-locus MIRU-
249 VNTR technique alone, the 202 isolates generated 173 profiles (152 unique profiles and 21
250 clusters). The 21 clusters contained 50 isolates (2-4 isolates per cluster; average: 2.4). Two
251 clusters included four isolates, four clusters contained three isolates, and 15 were composed by
252 two isolates.

253 **5.2. Phylogenetic tree and cluster analysis**

254 The NJ tree built by combining the MIRU-VNTR and spoligotyping data for the 202 isolates
255 clearly differentiated the Beijing clade from the other families (Figure S1). Nineteen clusters
256 including 43 isolates (2 to 4 isolates per cluster; average: 2.3 isolates per cluster) were showed
257 (see Figure 2 and Table S1). The EAI, Beijing and T families were present in these clusters,
258 accounted for 32, 9 and 2 isolates respectively (Table 3) and were grouped in 15, 3 and 1
259 cluster respectively. 13 out of 15 EAI clusters and all 3 Beijing clusters could be geographically
260 linked (isolates were either from patients living in the same village or district or provinces) (see
261 Figure 2 and Table S1). Regarding drug resistant isolates, only one cluster of Beijing family
262 (CN.18, Figure 2) contained three INH-resistant isolates.

263 Finally, these data allowed calculating the overall clustering rate (11.9%) and the clustering
264 rate for the Beijing, EAI and T families (Table 3).



265

266 **Figure 1 Neighbor-joining tree based on the MIRU-VNTR and spoligotyping data for 43 clustered isolates**

267 From left to right: i) Neighbor-joining tree based on the 24-locus MIRU-VNTR and spoligotyping data for
 268 the 43 isolates grouped in 19 clusters (built using the MIRU-VNTRplus analysis tool; ii) Number of
 269 repetitions of each VNTR according to the nomenclature by Supply et al (2006); and iii) 43-spacer
 270 spoligotypes: black spots represent the presence and white spot represent the absence of 1-43 spacers
 271 (according to the numbering by Van Embden et al. 2000). Yellow squares, Beijing clusters; orange
 272 squares, EAI clusters; dark pink, T clusters.

273

274 **Table 3. Estimation of the clustering rate for the EAI, Beijing and T families**

Characteristics	EAI	Beijing	T
Total number of isolates	155	29	11
Unique isolates	123	20	9
Clustered isolates	32	9	2
N. of clusters	15	3	1
Clustering rate	11.0%	20.7%	9.1%

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278 **Discussion**

279 ***M. tuberculosis* families in Lao PDR**

280 This is the first study on the genetic structure of the *M. tuberculosis* population in Lao
281 PDR. First of all, a high proportion of orphan and unknown *M. tuberculosis* isolates (18.3%) was
282 detected in our sample, probably because of the lack of previous genetic data. Indeed, in
283 countries where many genetic studies have been already performed, the proportion of orphan
284 isolates is lower, for instance 9.5% in Vietnam [10](Nguyen et al. 2012), and 8.2% in China
285 (Dong et al. 2010). Conversely, the proportion of isolates belonging to minor families (T, H,
286 CAS, LAM, and MANU) was lower in Lao PDR than in Vietnam and Myanmar (7.9% vs 23%
287 and 15%, respectively) (Phyu et al. 2009; Nguyen et al. 2012). Moreover, only one isolate
288 belonged to the CAS family, which is totally absent in Cambodia and Vietnam (Zhang et al.
289 2011; Nguyen et al. 2012). This result is in agreement with the reported low prevalence of CAS
290 isolates in Southeast Asia, differently from South-Central Asia (56.5% in Pakistan, 26% in India)
291 (Ali et al. 2014; Gutierrez et al. 2006).

292 Our findings indicate that the *M. tuberculosis* population in Lao PDR is mainly composed
293 of strains belonging to the EAI (76.7%) and Beijing (14.4%) families, similarly to neighboring
294 countries but in different proportions. Indeed, in Cambodia and Myanmar, the EAI family is
295 predominant (60% and 48.4% respectively), but the Beijing family also is highly prevalent (30%,
296 and 31.9%) (Phyu et al. 2009; Zhang et al. 2011). In Vietnam, the Beijing and EAI families
297 represent 38.5%/each of the *M. tuberculosis* population (Beijing isolates were found particularly
298 in urban areas with high population density, such as Hanoi and Ho Chi Minh) (Nguyen et al.
299 2012). Conversely, in China, the Beijing family represents 74.1% of the *M. tuberculosis*
300 population and was detected in all studied provinces, whereas only 0.03% of isolates belongs to
301 the EAI family (only in Fujian province) (Dong et al. 2010). The low proportion of Beijing isolates
302 found in our study could be explained by the low population density (27 people per km²) in Lao

303 PDR and the fact that 67% of the Lao population live in rural areas (Lao PDR-Population and
304 Housing Census 2015). Moreover, the distribution of the *M. tuberculosis* families was
305 heterogeneous in the different provinces of Lao PDR. EAI family isolates were from all over the
306 country, whereas Beijing isolates came mainly from the northern and central provinces (see
307 Figure 1). In most of the biggest provinces (Luang Prabang, Vientiane Capital, Savannakhet),
308 isolates belonged to different *M. tuberculosis* families, except in Champasack province where all
309 isolates were identified as EAI (Figure 1). Concerning the EAI subfamilies, the proportion of
310 EAI5 was two times higher in Lao PDR (69.0 %) than in Cambodia (28.8%) and in Vietnam
311 (30.6%). On the other hand, EAI4-VNM, which was mainly identified in Vietnam (65.9%), was
312 less frequent (4.5%) and found only in the central provinces. These data suggest that EAI5 is
313 the most ancient *M. tuberculosis* family circulating in Lao PDR. The long history of social-
314 economic exchange with neighboring countries has undoubtedly favored the spread of specific
315 genotypes in the country. The “4th Population and Housing Census” (PHC) of 2015 estimated
316 the global number of migrants at 42,000 (Lao PDR-Population and Housing Census 2015). Most
317 of them came from Thailand (37%), Vietnam (26%), China (23%), Myanmar (6%) and
318 Cambodia (1%). Currently, Vientiane Capital hosts the largest proportion of migrants, and this
319 could explain the high diversity of *M. tuberculosis* families (n=5) observed in this province
320 compared with most of the other provinces (0 to 4 families) (Figure 1 and Table S1). Migrants
321 from China and Myanmar live mostly in northern provinces, those from Thailand are mainly in
322 the central part of the country, and migrants from Vietnam are found in the center and in
323 Attapeu province in the South (Lao PDR-Population and Housing Census 2015). The number of
324 migrants from Cambodia (1%) is very low compared with those from other neighboring countries
325 and they are distributed all over the country. These data could partly explain the distribution of
326 the Beijing and EAI4-VNM subfamilies in Lao PDR and raise the question of the risk of a
327 progressive invasion by Beijing strains, as previously observed in Vietnam (Nguyen et al. 2012).

328 **Genetic diversity and transmission of *M. tuberculosis* families in Lao PDR**

329 To explore the genetic diversity of *M. tuberculosis* population in Lao PDR, 202 isolates
330 were characterized by spoligotyping and MIRU-VNTR typing. The results revealed 178
331 genotypes, a result similar to the one reported for Cambodia (91 patterns in 105 isolates) and
332 higher than that for Vietnam (153 genotypes for 221 isolates) (Zhang et al. 2011; Nguyen et al.
333 2012). As expected, the EAI family was more diverse than the Beijing family (138 genotypes for
334 155 isolates vs 23 genotypes for 29 isolates). The 19 clusters grouped 43 isolates that belonged
335 only to the three main families (EAI, Beijing and T). The overall clustering rate was 11.9%,
336 reflecting a non-negligible level of recent transmission compared with high TB burden countries,
337 such as Vietnam (16.3%) (Nguyen et al. 2012) and China (18.4%) (Yang et al. 2015). Moreover,
338 the Beijing family clustering rate was higher than the clustering rates of the other families
339 (20.7% for Beijing vs 11.0% for EAI vs 9.1% for T), suggesting a higher involvement of the
340 Beijing family in recent transmission cases, as demonstrated in many studies (Nguyen et al.
341 2012; Wang et al. 2011; Iwamoto et al. 2012; Niemann et al. 2010). Nevertheless, it is worth
342 noting that the combination of 24 Loci MIRU-VNTR and spoligotyping can lack discrimination
343 (only the whole genome sequencing can give us the real genotype of each isolate) making
344 possible that some clusters include slightly different genotypes. This lack of discrimination can
345 lead to a global overestimated clustering rate in our study. However, the large difference
346 observed between the families (20.7% for Beijing vs 11.0% for EAI vs 9.1% for T) supports the
347 hypothesis that Beijing, as demonstrated in many studies, is more involved in recent
348 transmission than the other families in Laos. EAI isolate predominance, higher diversity and
349 lower clustering rate compared with the Beijing family reinforce the hypothesis that the EAI
350 family (specifically the EAI5 sub-family) is the more ancient *M. tuberculosis* family in Lao PDR.
351 Most isolates in clusters (16 of the 19 clusters, and 37 of the 43 clustered isolates) were
352 geographically linked, reflecting the occurrence of recent transmissions. Clusters were mainly
353 observed in the northern and southern provinces, and mostly in rural area. Surprisingly, no

354 cluster was observed in the capital city. This could be explained by the global low population
355 density in cities and the higher patients' recruitment in rural areas than in urban areas in our
356 study.

357 **Epidemiological consideration and drug resistant TB**

358 The proportion of the two main families was significantly different in function of the age
359 group, region of origin and drug-resistant status. The proportion of isolates belonging to the EAI
360 family was higher in the 35-64 age group, as observed in Cambodia, Vietnam and Myanmar,
361 reflecting the endemic circulation of EAI in this part of the world. On the other hand, in Lao PDR
362 the proportion of Beijing isolates in the 15-34 and 35-64 age groups was similar, whereas in
363 Vietnam the proportion of Beijing isolates decreases with age (Nguyen et al. 2012).
364 Finally, despite the low prevalence of drug resistance in Lao PDR, the Beijing family was more
365 represented among drug-resistant isolates, as previously reported in Cambodia, Vietnam, and
366 China (Zhang et al. 2011; Nguyen et al. 2012; Pang et al. 2012). The Beijing isolates in clusters
367 were geographically linked and one of the three Beijing clusters included drug-resistant isolates
368 (see Figure 2 and TableS1). These findings underline the risk of Beijing strain expansion in Lao
369 PDR and consequently the increasing risk of primary drug resistance in recent transmission.

370 **Conclusion**

371 This study provides the first genetic insights into the *M. tuberculosis* population in Lao
372 PDR. The presence of the main families detected in neighboring countries, particularly the EAI
373 and Beijing families, and the 11% of recent transmission rate show that TB represents a
374 challenge in Lao PDR. Although, the EAI family is predominant, the diversity of families
375 observed in big cities (Vientiane, Luang Prabang, Khammuane and Savannhaket) highlights the
376 risk of transmission of other families than EAI in the country. Although the Beijing family
377 prevalence is still low, its presence mainly in the northern and central provinces, its association
378 with drug resistance and its involvement in recent transmission (clustering rate = 20% based on

379 the combination of spoligotyping and 24 loci MIRU-VNTR) indicate that this family may change
380 TB epidemiological pattern in Lao PDR. This underlines the need to continue and reinforce the
381 effort to maintain an efficient TB control and surveillance system in order to prevent the
382 emergence of highly transmissible and drug-resistant strains in Lao PDR, as observed in
383 neighboring countries.

384

385 **Declarations**

386 **Consent for publication:** Not applicable

387 **Availability of data and materials**

388 The dataset supporting the conclusions of this article is included within the article and its
389 additional file.

390 **Competing interests**

391 The authors declare that they have no competing interests.

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397 **Authors' contributions**

398 Design of the study: ALB, TVAN, SS. Supervision of the study: ALB, TVAN, JLB, PP, GPB.
399 Technical transfers: MG, JLB, TVAN, QHN, TTV. Sample collection and experiments: VI, SS,
400 SS, IK, MG, VA. Collection of patient’s information: PV, DI, VI. Data analysis: SS, ALB, PC.
401 Paper writing: SS, ALB. Paper writing contribution: JLB; MG, QHN, VI.

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543 **Supporting Information**

544 **Figure S1. Neighbor joining tree based on the MIRU-VNTR and spoligotyping data showing the genetic**
545 **relationships of 202 *M. tuberculosis* isolates from Lao PDR (PDF)**

546 From left to right: i) Neighbor joining tree based on the 24-locus MIRU-VNTR and spoligotyping data for
547 the 202 isolates built using the MIRU-VNTRplus analysis tool; ii) Number of repetitions of each VNTR
548 according to the nomenclature by Supply et al. 2006); and iii) 43-spacer spoligotypes: black spots indicate
549 the presence and white spot the absence of the 1-43 spacers (according to the numbering by Van
550 Embden et al. 2000). Yellow squares, Beijing clusters; orange squares, EAI clusters; dark pink, T clusters.

551 **Table S1. Complete data (clinical, epidemiological, demographic and genetic data) for the 222 *Mycobacterium***
552 ***tuberculosis* isolates included in this study (xlsx)**

553 **The data was stored in google drive, please follow the link bellow:**

554 <https://drive.google.com/drive/folders/1Y8t5t-nAeXeyVC14JLqFEzHGibdypNtL>

555

3.2. Result 2 (Paper 2): Genetic characterization of *Mycobacterium tuberculosis* from presumptive MDR-TB in Lao PDR

Genetic characterization of *Mycobacterium tuberculosis* from presumptive MDR-TB in Lao PDR

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Abstract

Background: In Laos, presumptive MDR-TB cases were routinely screened; however no molecular information is available. The aim of this study is to genetically characterize the presumptive MDR-TB (resistant to at least Rifampicin and Isoniazid) cases in Laos in order to determine the causative species, the drug resistance patterns and the associated genetic determinants.

Methods: 155 isolates correspond to 155 presumptive MDR-TB cases were collected during 2010-2014; Genotype MTBDR*plus* tests, DNA sequencing of the main drug resistant-associated genes, Spoligotyping and MIRU-VNTR typing were performed

Results: Patients were mainly collected from relapses (53.7%) and failure/late smear conversion (23.5%) cases. MTBDR*plus* confirmed 139 (89.7 %) MTBc and 16 (10.3 %) NonMTBc. Of 139 MTBc, 96 (69.1 %) were susceptible and 43 (30.9 %) were resistant (seven

31 rifampicin resistant, 19 isoniazid resistant and 17 MDR). DNA sequencing of 42 available
32 isolates revealed the mutations associated with anti-TB drugs allowing the identification of
33 multigenic patterns and the probable drug resistant profiles (nine mono drug resistant, 11 poly
34 drug resistant, 10 MDR, nine QDR (Quadruple resistance to first line drugs) and three pre-XDR
35 (MDR plus resistance either to one fluoroquinolone or to one second line injectable drug).
36 Beijing, EAI and other families of *M. tuberculosis* were observed among all presumptive MDR-
37 TB (41 %, 38 % and 21 % respectively). Beijing was significantly higher than EAI and other
38 families among drug resistant isolates ($p=0.005$) and only Beijing was observed among pre-
39 XDR cases. The proportion of failures/late smear conversions was also higher than relapses
40 among drug resistant isolates ($p=0.01$).

41 **Conclusions:** DNA sequencing revealed a large panel of mutations associated with drug
42 resistance reflecting various patterns from mono-resistance to pre-XDR. The results show that
43 the screening of resistance to both FLD and SLD by molecular method could be extremely
44 useful to rapidly determine the optimal treatment regimen. As expected, Beijing family is
45 associated with drug resistance and this is the future concern in terms of MDR-TB increase risk
46 in Lao PDR. Efforts for accurate and rapid detection of drug resistance is urgently needed for all
47 TB patients in order to prescribe appropriate treatment and limit the transmission of drug
48 resistant TB in Laos.

49

50 **Key words:** Presumptive MDR-TB, *Mycobacterium tuberculosis*, Genetic characterisation, Drug-
51 resistant tuberculosis, Lao PDR.

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56 **Background**

57 Tuberculosis (TB) is the ninth leading cause of death worldwide due to a single agent,
58 ranking above HIV/AIDS (Human immunodeficiency virus/Acquired immunodeficiency
59 syndrome). The emergence/re-emergence of drug resistant TB aggravates the situation
60 worldwide and challenges the prospect of ending TB by 2035. Even though the number of
61 multidrug-resistant TB (MDR-TB, Box 1) and rifampicin resistant TB (RR-TB, Box 1) is only 5%
62 of TB incidence, the number of deaths among MDR/RR-TB is almost threefold higher than
63 among susceptible TB (40% vs 16%) . In addition, about 6.2% of MDR-TB cases are
64 extensively drug-resistant (XDR-TB, Box 1). The treatment success rate for these forms is only
65 30% (World Health Organization 2017). Globally, 4.1% of new cases and 19% of previously
66 treated TB cases was estimated to be RR or MDR-TB. Many studies have identified the factors
67 associated with MDR-TB, including social-demographic, clinic and genetic factors. The common
68 risk factors for MDR-TB are having TB history, contact with confirmed TB patients, TB/HIV co-
69 infection (Paudel 2017; Marahatta et al. 2012; Faustini, Hall, and Perucci 2006; Ahmad et al.
70 2012; Mulisa et al. 2015; Balabanova et al. 2012). Furthermore, young people and males are
71 more likely to have MDR-TB (Paudel 2017; Faustini, Hall, and Perucci 2006; Ahmad et al.
72 2012). Another key factor is the poor adherence to anti-TB drug treatment (Paudel 2017).
73 Regarding *M. tuberculosis* families, Beijing family was reported to be associated with MDR-TB
74 in many parts of the world, especially in Asian countries (Drobniewski et al. 2005; Mokrousov et
75 al. 2018; Krüüner et al. 2001; Tracevska et al. 2003; Glynn et al. 2002; Bifani et al. 1999; J.
76 Zhang et al. 2011; Pang et al. 2012; Phyu et al. 2009; Lisdawati et al. 2015; Cheunoy et al.
77 2009; Anh et al. 2000; An et al. 2009; Buu et al. 2009; Nguyen et al. 2016).

78 Regarding the drug resistance detection, the culture-based drug susceptibility testing
79 (DST) is the gold standard for MDR-TB diagnosis; however, the method is labor and time
80 consuming and requires high biosafety level. Nowadays, molecular-based tests like Xpert

81 MTB/RIF (Cepheid, Sunnyvale, CA, United States) and Line Probe assays (Hain Lifescience
82 GmbH, Nehren, Germany) are recommended by World Health Organisation (WHO) in order to
83 obtain rapid results for the TB/MDR-TB diagnosis. Besides, the sequencing and analysis of
84 specific gene regions of *M. tuberculosis* known to be associated with anti-TB drug resistance
85 are a valuable tool for drug resistance detection and surveillance, providing new opportunities to
86 monitor drug resistance in TB in resource-poor countries (Zignol et al. 2018).

87 In Lao PDR, the Xpert MTB/RIF is used to screen TB and RR-TB among new and previously
88 treated cases in all provinces of Laos. The culture-based DST for the first line and main second-
89 line anti-TB drugs is only available at the National reference laboratory (NRL); the line probe
90 assays, MTBDR_{plus} and MTBDR_{s/l} (Hain Lifescience GmbH, Nehren, Germany) are available
91 at the Center for Infectiology Lao-Christophe Mérieux (CILM). These molecular tests identify the
92 *M. tuberculosis* complex, the rifampicin (RIF) and isoniazid (INH) resistances, the resistance to
93 any fluoroquinolones (FQ) and second line injectable drugs (capreomycin (CAP), kanamycin
94 (KAN) and amikacin (AMK)).

95 The first XDR-TB case was detected in 2010 in a multicentric study conducted in three
96 regional hospitals. This study showed that 7 (8.0%) out of 87 *M. tuberculosis* isolates were INH
97 mono resistant and 1 was XDR-TB (Iem et al. 2013). The data from the first national TB
98 prevalence survey of Lao PDR (2010–2011) showed 11 (5 %) out of 222 *M. tuberculosis*
99 isolates were INH mono resistant, and 2 (0.9 %) were MDR-TB (unpublished data). The number
100 of MDR-TB patients notified and enrolled in treatment increased from 14 in 2013 to 25 in 2014.
101 Each year, around 130 presumptive MDR-TB cases are reported but no molecular information
102 and drug resistance are available.

103 The aim of this study is to genetically characterize the presumptive MDR-TB cases in Lao
104 PDR in order to determine the causative species, the drug resistance patterns and the
105 associated genetic determinants.

106 **Materials and Methods**

107 **1. Study settings and population**

108 During 2010-2014, the specimens of presumptive MDR-TB were collected and sent by
109 health facilities of NTCP network to NRL located in Vientiane capital to perform the cultures.
110 Fresh subcultures were then sent to CILM for molecular testing to identify *M. tuberculosis*
111 complex (MTBc) and its resistance to RIF and/or INH by the line probe assay (MTBDR_{plus},
112 Hain Lifescience GmbH, Nehren, Germany). Each isolate was sent with the patient's request
113 form, including socio-demographic and clinical data. The study population included only patients
114 aged ≥ 15 years old, who met the 12 presumptive MDR-TB criteria as defined in the guidelines
115 of NTCP established in 2010 (NTCP, Lao PDR 2010). In this study, the types of presumptive
116 MDR-TB cases were grouped into 1). Relapse after treated with FLD/SLD; 2). Failure/late
117 smear conversion; 3). Return after loss to follow up; 4). New patient with TB-HIV co-infection;
118 5). New patient in contact with proven RR /MDR-TB

119

Box 2. Definitions of drug resistance terms used in this paper

120

Mono-resistance: resistance to only one anti-TB drug (First Line Drugs (FLDs) or Second Line Drugs (SLDs))

121

Poly-resistance: resistance to more than one anti-TB drug (FLD and/or SLD) other than both isoniazid (INH) and rifampicin (RIF)

122

Multidrug resistance (MDR): resistance to at least both INH and RIF

123

Quadruple drug resistance (QDR): MDR plus resistance to at least 2 more FLDs.

124

Pre-Extensive drug resistance (pre-XDR): MDR plus resistance to any fluoroquinolone (FQ) or to any second-line injectable drugs (SLIDs)

125

Extensive drug resistance (XDR): MDR resistance plus resistance to any FQ and at least one of the three SLIDS, capreomycin (CAP), kanamycin (KAN) and amikacin (AMK)

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132 **2. Molecular characterization of *Mycobacterium tuberculosis***

133 ➤ **GenoType® MTBDR*plus* and GenoType® Mycobacterium CM kits**

134 The GenoType® MTBDR*plus* ver.1.0 test permitted to identify *M. tuberculosis* complex
135 and its resistance to R and/or H. The GenoType® Mycobacterium CM ver.1.0 test permitted the
136 identification of the common NonTuberculous mycobacteria (NTM).

137 The subcultures sent by NRL were scraped from media slopes and resuspended in
138 300µL of distilled water, heated for 20 min at 95°C, centrifuged for 5 minutes at 13000 g. Then
139 the supernatant containing DNA was transferred into a new tube and stored at -80 °c. The
140 GenoType® MTBDR*plus* ver.1.0 and the GenoType® Mycobacterium CM ver.1.0 tests, a DNA
141 STRIP® based technology, were performed according to the instructions of the manufacturer
142 (Hain Lifescience GmbH).

143 ➤ **43-spacer oligotyping (Spoligotyping)**

144 Spoligotyping was performed as previously described (Filliol et al. 2003; Kamerbeek et
145 al. 1997). DNA of H37Rv strain and *M. bovis* BCG were included as positive controls. Biological
146 molecular grade water was used as a negative control. The spoligotypes were then recorded in
147 43-digit binary format and compared with those recorded in the SpolDB4 database
148 (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) in order to identify the SIT
149 (Spoligotype International Type) and the families (Demay et al. 2012). For the spoligotypes
150 which matched to Spoligotype International Types (SITs) but could not be related to any family
151 (unknown) and for the spoligotypes which were absent from SpolDB4 (orphan), SPOTCLUST
152 (http://tbinsight.cs.rpi.edu/run_spotclust.html) (Vitol et al. 2006) was used to search *M.*
153 *tuberculosis* family similarity. For SPOTCLUST analyses, we considered the family assignment
154 when the probability was equal or higher than 90 %. Nevertheless, the final designation of
155 families and subfamilies was also based on the MIRU-VNTR data (see below).

156

157 ➤ **24-locus Mycobacterial Interspersed Repetitive Unit–Variable Number Tandem-**
158 **Repeat (MIRU-VNTR) typing**

159 The full set of 24 MIRU-VNTR loci was used to characterize the isolates. The MIRU-
160 VNTR typing was performed as previously described (Supply et al. 2006; Gauthier et al. 2015).
161 The patterns obtained for the 24 loci were used to create a 24 digit allelic profile for each isolate.
162 The results of the MIRU-VNTR typing method were analyzed using MIRU-VNTRplus
163 (<http://www.miru-vntrplus.org>), a freely accessible web based program (Allix-Béguet et al.
164 2008). The final designation of family/subfamily was revised using the MIRU-VNTRplus website
165 ([MIRU-VNTRplus.org](http://www.miru-vntrplus.org)) based on the family results for each isolate and the MIRU-
166 VNTR/spoligotyping phylogenetic tree.

167 ➤ **Detection of drug resistance associated mutations by Sanger sequencing.**

168 The main genes associated with resistance to first line anti-TB drugs (FLDs) and second
169 line anti-TB drugs (SLDs) were amplified by PCR and sequenced. For the FLD resistance, the
170 following genes and gene fragments were studied: *katG* gene, *inhA* coding region and the *inhA*-
171 promoter (INH resistance); *rpoB* gene (RIF resistance); *rpsL* gene and *rrs-F1* fragment
172 (Streptomycin (STR) resistance); *embB* gene (Ethambutol (EMB) resistance); *pncA* gene and its
173 promoter (pyrazinamide (PZA) resistance). Regarding SLD resistance, the following genes and
174 gene fragments were analyzed: *gyrA* and *gyrB* genes (Fluoroquinolone (FQ) resistance), *rrs-F2*
175 fragment (second line injectable drug (SLID) resistance). The list of primers, PCR conditions
176 and DNA sequencing were previously described (Nguyen et al. 2015; Nguyen et al. 2017). Each
177 sequence was treated independently using the Bioedit software (version7.1.10) and the
178 consensus sequence was generated. Multi-sequence alignment was then performed. Point
179 mutations were identified by comparison with the sequence of the *M. tuberculosis* H37Rv
180 reference strain available in GenBank (NC.000962.3). To describe the resistance-associated

181 mutations in *rpoB* gene, a numbering system based on the *Escherichia coli* sequence
182 annotation has been used.

183 **3. Data analysis**

184 Patients' information (the anonymity of the patients was maintained) was registered and
185 cross-checked by CILM research team before being imported to Stata (v12.1, Stata
186 Corporation, USA) for statistical analyses. Median and interquartile were calculated for age of
187 patients. Among presumptive MDR-TB cases, the distribution of susceptible and drug resistant
188 isolates were studied according to different variables such as gender, age, region of residence
189 (North, Center, South), presumptive MDR-TB criteria (failure, relapse, etc.) and *M. tuberculosis*
190 families using Chi-square or Fisher's exact test (when the number of observations was lower
191 than 5). The *p*-value less than 0.05 was considered to be statistically significant. A Neighbor-
192 Joining (NJ) tree based on categorical distances was built by combining the spoligotyping and
193 MIRU-VNTR results, using MIRU-VNTR*plus* (<http://www.miru-vntrplus.org>), a freely accessible
194 web-based program (Allix-Béguec et al. 2008)

195 **Ethics statement**

196 This retrospective genotyping study was approved by the National Ethics Committee of Health
197 Research of Lao PDR.

198 **Results**

199 **1. Socio-demographic characteristics of presumptive MDR-TB**

200 A total of 155 presumptive MDR-TB culture positive isolates collected between 2010 and
201 2014 were included in the study. The median age of the patients was 52 years-old (IQR: 36-60).
202 The number of patients increased with age (except for the age range > to 65 years old) (Table
203 1). There were more men than women (69.7% vs 30.3%), with a men/women ratio of 2.3. More
204 than 50 % of patients were from the center part of Laos (n=80, 51.6 %), where the NRL and

205 CILM are located. The number of patients was variable according to the presumptive MDR-TB
 206 criteria with a majority of relapses (53.7 %) and failures (23.5 %) (Table 1)

207 **Table 1 Socio-demographic and characterization of presumptive MDR-TB patients**

Characteristics of presumptive MDR-TB	N (%)	
Median age (N=154 ^a)	52 (IQR: 36-60)	
Age groups (in year)	15-24	12 (7.8)
	25-34	20 (13.0)
	35-44	27 (17.5)
	45-54	28 (18.2)
	55-64	36 (23.4)
	≥65	31 (20.1)
Gender (N=155)	Female	47 (30.3)
	Male	108 (69.7)
Regions of residence (N=155)	North	60 (38.7)
	Centre	80 (51.6)
	South	15 (9.7)
Presumptive MDR-TB type (N=149 ^b)	Relapse after treated with FLD/SLD	80 (53.7)
	Failure/late smear conversion	35 (23.5)
	Return after loss to follow up	12 (8.1)
	New patient with TB-HIV co-infection	21 (14.1)
	New patient in contact with proven RR /MDR case	1 (0.7)

^a one missing data ; ^b six missing data

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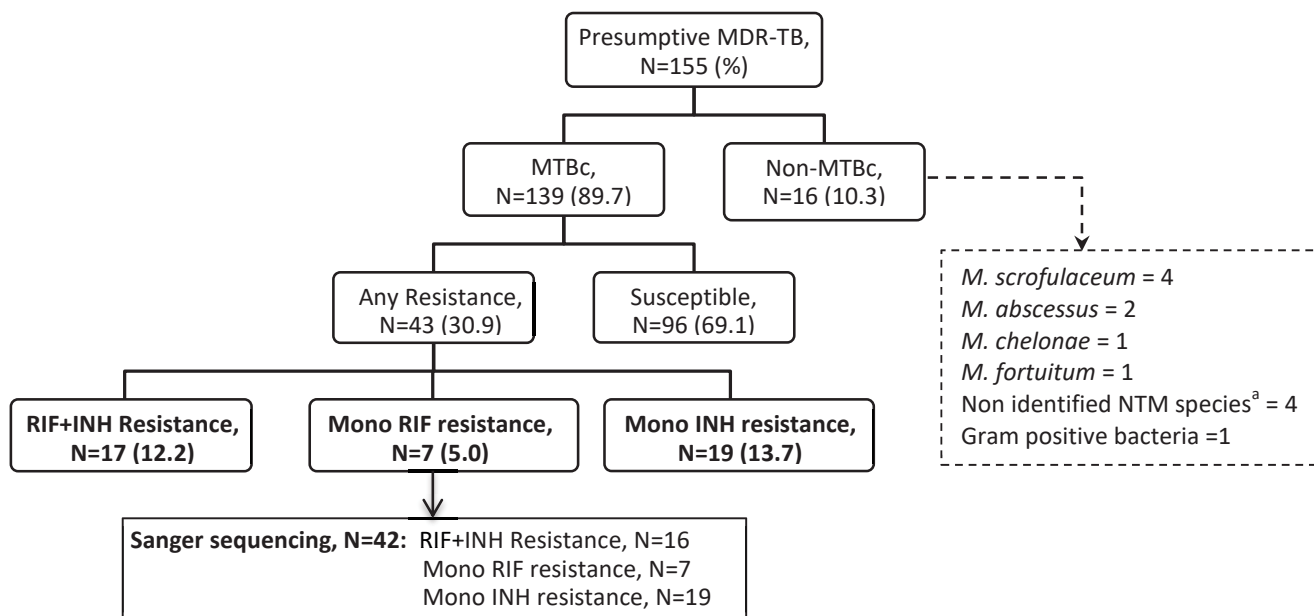
211 **2. Identification of Mycobacteria species and resistance to rifampicin and isoniazid by**

212 **GenoType® Mycobacterium CM and MTBDRplus**

213 Among the 155 isolates, the MTBDRplus test identified 139 (89.7 %) *M. tuberculosis*
 214 complex (MTBc) isolates. The 16 (10.3 %) remaining isolates did not belong to MTBc. The 16
 215 non MTBc isolates were more observed among failures/late smear conversions (n=5, 31.3 %),
 216 relapses (n=5, 31.3%) and HIV infection (n=3, 18.8 %). Thirteen non MTBc isolates could be
 217 tested by the GenoType® Mycobacterium CM test. Four different Non tuberculous mycobacteria
 218 (NTM) species were identified, *M. scrofulaceum* (N=4), *M. abscessus* (N=2), *M. chelonae* (N=1)
 219 and *M. fortuitum* (N=1). Four NTM isolates could not be identified and one isolate was
 220 determined as Gram positive bacteria (Figure 1).

221 Among the 139 confirmed MTBc, 96 (69.1 %) were susceptible and 43 (30.9 %) were
 222 resistant to at least one anti-TB drug (RIF and/or INH). Among the 43 drug resistant isolates, 17
 223 (12.2 %) were MDR, seven (5.0 %) were mono-resistant to RIF and 19 (13.7 %) were mono-
 224 resistant to INH (Figure 1). Of 43 resistant isolates, 25 (58.1 %) were relapse cases, 14 (32.6
 225 %) failure/late smear conversion cases, two (4.7 %) return after loss to follow up cases, one (2.3
 226 %) TB-HIV co-infected and one (2.3 %) missing data. Of 17 MDR-TB cases, nine were relapse
 227 cases; seven failure/late smear conversions and one missing data (Table 2). According to
 228 MTBDR*plus*, 23 (54.8 %) isolates had mutations in *rpoB* gene, 29 (67.4 %) isolates had
 229 mutations only in *katG*, six (14.0 %) isolates had mutations only in *inhA*-promoter and one (2.3
 230 %) isolate had mutations in both *katG* and *inhA*-promoter (Table S 2.2).

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237 **Figure 1 Identification of mycobacteria and resistance to isoniazid (INH) and/or rifampicin (RIF) among**
 238 **presumptive MDR-TB cases**

239 ^a The GenoType® Mycobacterium CM could not identify the NTM species for 4 isolates

240
241

242 **Table 2 Distribution of drug susceptible and drug resistant isolates among presumptive MDR-TB**

Presumptive MDR-TB	Total, N=139 (%)	Susceptible, N= 96 (%)	Resistant, N=43 (%)		
			MDR-TB, N=17	Mono RIF, N=7	Mono INH, N=19
Relapse after treated with FLD/SLD	75 (54.0)	50 (52.1)	9 (52.9)	5 (71.4)	11 (57.9)
Failure/late smear conversion	30 (21.6)	16 (16.7)	7 (41.2)	1 (14.3)	6 (31.6)
Return after loss to follow up	11 (7.9)	9 (9.4)	0	0	2 (10.5)
New patient with TB-HIV	18 (13.0)	17 (17.7)	0	1 (14.3)	0
New patient in contact with proven RR/MDR	1 (0.7)	1 (1.0)	0	0	0
Missing data	4 (2.9)	3 (3.1)	1 (5.9)	0	0

243

244 **3. Sanger sequencing of drug resistance genes or regions**

245 The Sanger sequencing of specific genes or regions involved in drug resistance to FLDs
 246 and SLDs could be performed on 42 out of the 43 resistant isolates determined by the
 247 MDRTB*plus* test. Based on MTBDR*plus* test, 16 out of 42 isolates were MDR, seven were
 248 mono RIF resistant, and 19 were mono INH resistant. The sequencing was able to detect
 249 mutations in all the 42 drug resistant isolates. Two more *rpoB* mutant isolates and four more
 250 *katG* mutant isolates (Tables 2 and S 2.1) were detected by sequencing leading to a
 251 concordance between MTBDR*plus* and the sequencing results of 92 % for *rpoB* (RIF
 252 resistance) and 90 % for *katG* and *inhA* genes and *inhA*-promoter together (INH resistance).

253 Of 42 isolates, 25 (59.5 %) had mutations in *rpoB* gene, the majority (n=23, 92 %) of
 254 them were found only within the 81-bp rifampicin resistance determining region (RRDR). The
 255 most common mutations were Ser531Leu (n=6, 24 %); His526Tyr (n=5, 20 %) and His526Arg
 256 (n=5, 20 %), these mutations have generally been reported as conferring high level resistance
 257 to RIF (Lee et al. 2005; Campbell et al. 2011). One isolate had combination of two mutations,
 258 one mutation located in the RRDR (Met515Leu) and one located outside the RRDR (Ile572Phe)
 259 (Table S 2.1). The *rpoB* mutant (ID: MR20; see Tables S1 and S2) detected only by sequencing
 260 showed Leu511Pro mutation. One isolate revealed a mutation out of the RRDR (Met655Thr)
 261 combined with an insertion at the codon 514 (TGCCAA, CysGln) (Table S 2.1).

262 Thirty-nine out of the 42 isolates (92.9 %) revealed non-synonymous mutations or
263 deletion or insertion of bases in *katG* and/or in *inhA*-promoter and/or in *inhA*-coding gene. A
264 total of 33 (84.6 %) isolates had mutations in *katG* gene, 29 out of 33 (87.9%) carried a mutation
265 at codon 315 (the most prevalent associated-INH resistant codon). The most frequent mutation
266 was Ser315Thr (n=25, 75.8 %). This mutation was found alone or in combination with a
267 mutation in *inhA*-promoter or in *inhA* coding gene (Table S 2.1). One non-synonymous mutation
268 not known to be associated to INH resistance was detected at the codon Pro100Thr. Another
269 mutation, Asp189Gly, recently associated with INH resistance (Brossier et al. 2016) was
270 observed. One isolate showed an insertion of T at the codon 630 leading to a frameshift. This
271 frameshift mutation was previously found to be likely associated with INH resistance (Kandler et
272 al. 2018). One isolate showed a C deletion at the codon 32 leading to a frameshift in the
273 sequence; no information is available regarding INH resistance for this mutation. Mutations in
274 *inhA*-promoter were identified in seven (17.9 %) isolates. The position (-) 15CT was the most
275 prevalent (n=6). Three (7.7 %) isolates had mutations in *inhA* coding region, all these mutations
276 were found in combination either with *katG* mutation or with *inhA*-promoter mutation (Table S
277 2.1). In total, four isolates (10.3%) showed a combination of mutations in different genes: one
278 isolate carried *katG* and *inhA*-promoter mutations (Ser315Thr/-15(CT)), one carried *inhA*-
279 promoter and *inhA* coding region (-15(CT)/Ile21Val), two carried mutations in *katG* and *inhA*-
280 coding gene (Ser315Thr/Asp335Asn and Ser315Thr/Ile144Val) (Tables S 2.1). It is worth noting
281 that we did not include the mutation Arg463Leu of *katG* gene, described as phylogenetic marker
282 and not as drug resistant determinant (Sreevatsan et al. 1997; Torres et al. 2015). This mutation
283 was observed in 38 out of 42 isolates.

284 Regarding *embB* gene linked to EMB resistance, 14 (33.3%) isolates revealed seven
285 different non-synonymous mutations (Table S 2.1). The most frequent mutations were at codon
286 306 (Met306Val, n=4, Met306Ile, n=3), followed by the mutation at codon 360 (Val360Met, n=3).
287 Twelve out of 42 isolates carried the Glu378Ala mutation. This mutation was not included in the

288 analysis because it was considered as phylogenetic marker and was not associated with EMB
289 resistance (Köser et al. 2014).

290 *PncA* mutations linked to PZA resistance were found only in coding region of four (9.5
291 %) isolates, including non-synonymous mutations (Cys72Arg and Phe106Val) and base deletion
292 (C deletion at codon 19 and A deletion at codon 160) (Table S 2.1).

293 Mutations in *gyrA* gene (FQ resistance) were observed also in four (9.5 %) isolates.
294 These mutations detected in codons 88, 90, 91 and 94 were previously associated with drug
295 resistance (Table S 2.1). We excluded the Glu21Gln and Ser95Thr mutations (found in 41/42
296 and 40/42 isolates respectively), described as lineage genetic markers (Table S 2.2) (Farhat et
297 al. 2016; Miotto, Cirillo, and Migliori 2015). No mutation was found in *gyrB* (FQ resistance) and
298 in *rrs-F2* (SLID resistance).

299 Regarding mutations associated to STR resistance, a total of 26 (61.9%) of 42 isolates
300 revealed non-synonymous mutations either in *rrs* F1 and/or *rpsL*. Mutations in *rrs-F1* were
301 observed in 5 (11.9%) isolates with the most common mutation at 517(CT), while *rpsL*
302 mutations were found in 22 (52.4 %) isolates, with the most common mutation, Lys43Arg (Table
303 S 2.1).

304 Finally, according to the non-synonymous mutations patterns (including nucleotide
305 deletion and insertion), we could determine a multi-genic pattern for each isolate (Table 3). Nine
306 isolates (21.4%) showed mutations linked to resistance to only one anti-TB drug in agreement
307 with a mono-resistance pattern (Box 1); 11 (26.2 %) showed drug resistance-associated
308 mutations in genes or DNA region in agreement with a poly-drug resistance pattern (Box 1); 10
309 (23.0%) showed at least mutations linked to resistance to RIF and INH corresponding to MDR
310 pattern; 9 (21.4 %) showed mutations linked to resistance to four FLDs in agreement with a
311 QDR pattern (Box 1) and 3 (7.1 %) revealed mutations linked to RIF, INH and FQ resistances,
312 suggesting pre-XDR. Finally, among the 42 isolates either mono RIF resistant or mono INH

313 resistant or MDR detected by MTBDR_{plus}, 28 (66.6 %) isolates showed additional mutations
 314 conferring resistances to EMB, PZA, FQ or STR (Tables 3 and S 2.1).

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316 **Table 3 Probable anti-TB drug resistant patterns based on sequencing data among the 42 drug**
 317 **resistant isolates determined by GenoType® MTBDR_{plus} and according to the *M. tuberculosis* family.**

Drug resistance patterns	Total, N=42 (%)	Beijing, N=25 (%)	EAI, N=14 (%)	Others, N=3 (%)
Mono-Resistance	9 (21.4)	4(16.0)	5 (35.7)	0
H	6 (14.3)	2 (8.0)	4 (28.6)	0
R	3 (7.1)	2 (8.0)	1 (7.1)	0
Poly-Drug resistance	11 (26.2)	9 (36.0)	1 (7.1)	1 (33.3)
HS	8 ^a (19.1)	6 (24.0)	1(7.1)	1 (33.3)
HES	2 (4.8)	2 (8.0)	0	0
HFS	1 (2.4)	1 (4.0)	0	0
MDR	10 (23.8)	3 (12.0)	6 (42.7)	1 (33.3)
RH	5 ^b (11.9)	1 (4.0)	3 (21.4)	1 (33.)
RHE	2 ^c (4.8)	0	2 (14.3)	0
RHS	3 (7.1)	2 (8.0)	1 (7.1)	0
QDR	9 (21.4)	6 (24.0)	2 (14.3)	1 (33.3)
RHES	7 ^d (16.7)	4 (16.0)	2 (14.3)	1 (33.3)
RHZS	1 (2.4)	1 (4.0)	0	0
RHZES	1 (2.4)	1 (4.0)	0	0
Pre-XDR	3 (3.7)	3 (12.0)	0	0
RHEFS	1 (2.4)	1 (4.0)	0	0
RHZFS	1 (2.4)	1 (4.0)	0	0
RHZEFS	1 (2.4)	1 (4.0)	0	0

318

319 ^aone isolate had a combination of *rrs*-F2/*rpsL* (274GA/ Lys43Arg)

320 ^bone isolate had a combination of *katG/inhA*-coding (Ser315Thr/Ile144Val) and one *inhA*-promoter/*inhA*-
 321 coding ((-15)CT/ Ile21Val)

322 ^cone mutant *rpoB* (Leu511Pro) detected by sequencing, this isolate had a combination of *katG/inhA*-
 323 coding (Ser315Thr/Asp335Asn)

324 ^done insertion (TGCCAA) in *rpoB* detected by sequencing

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338 4. Spoligotyping characterization

339 Spoligotyping was performed on 139 *M. tuberculosis* isolates. The *M. tuberculosis*
340 family/subfamily identifications were determined using SITVITWEB (SpolDB4 database),
341 SPOTCLUST and MIRU-VNTRplus. A total of 43 different patterns were observed including 12
342 clusters (108 isolates) and 31 unique patterns (Table 4). Each cluster contained 2 to 53 isolates
343 (average 9 isolates per cluster). One hundred and nineteen (85.6%) isolates could be assigned
344 to 24 existing SITs and five families in SpolDB4; two (1.4%) were unknown (existing SITs in
345 SpolDB4 but could not be related to any family); and 18 (12.9%) were orphans (absent from
346 SpolDB4). The two unknown and 18 orphans were then compared with SPOTCLUST (SpolDB3)
347 database. Finally, 133 (95.7%) isolates represented five families, consisting of Beijing, East
348 African Indian (EAI), T, Haarlem (H) and Manu, and 7 (5.0%) isolates remained orphans or
349 unknowns (Table 4, 5). Among the 139 presumptive MDR-TB, Beijing was the predominant
350 family (41.0 %, n=57), followed by EAI (38.1 %, n=53) and other families (20.9 %, n=29) (Table
351 5). Moreover, Beijing was more prevalent among the 43 drug resistant isolates (determined by
352 MTBDRplus) than EAI and other families (60.5 %, 32.2 % and 9.3 % respectively) (Table 5) and
353 this was significantly different ($p=0.005$). The poly-drug resistant and QDR patterns were more
354 prevalent in Beijing isolates and pre-XDR isolates belonged only to Beijing family (Table 3).

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371 **Table 5 Families and subfamilies of *M. tuberculosis* identified among presumptive MDR-TB cases, drug**
 372 **susceptible and drug resistant isolates.**

Families and subfamilies	Total isolates, N=139 (col. %)	Susceptible, N=96 (col. %)	Resistant, N=43 (col. %)
Beijing	57 (41.0)	31 (32.3)	26 (60.5)
EAI	53 (38.1)	40 (41.7)	13 (32.2)
EAI5	32 (23.0)	25 (26.0)	7 (16.3)
EAI1-Som	7 (5.0)	3 (3.1)	4 (9.3)
EAI2-Nonthaburi	6 (4.3)	5 (5.2)	1 (2.3)
EAI4-VNM	4 (2.9)	4 (4.2)	0
EAI6-BGD1	3 (2.2)	3 (3.1)	0
EAI2-Manila	1 (0.7)	0	1 (2.3)
Other families	29 (20.9)	25 (26.0)	4 (9.39)
T1	14 (10.1)	14 (14.6)	0
T2	2 (1.4)	1 (1.0)	1 (2.3)
T3	2 (1.4)	2 (2.1)	0
H1	1 (0.7)	0	1 (2.3)
H3	2 (1.4)	1 (1.0)	1 (2.3)
Manu-Ancestor	1 (0.7)	1(1.0)	0
orphan/unknown	7 (5.0)	6 (6.3)	1 (2.3)

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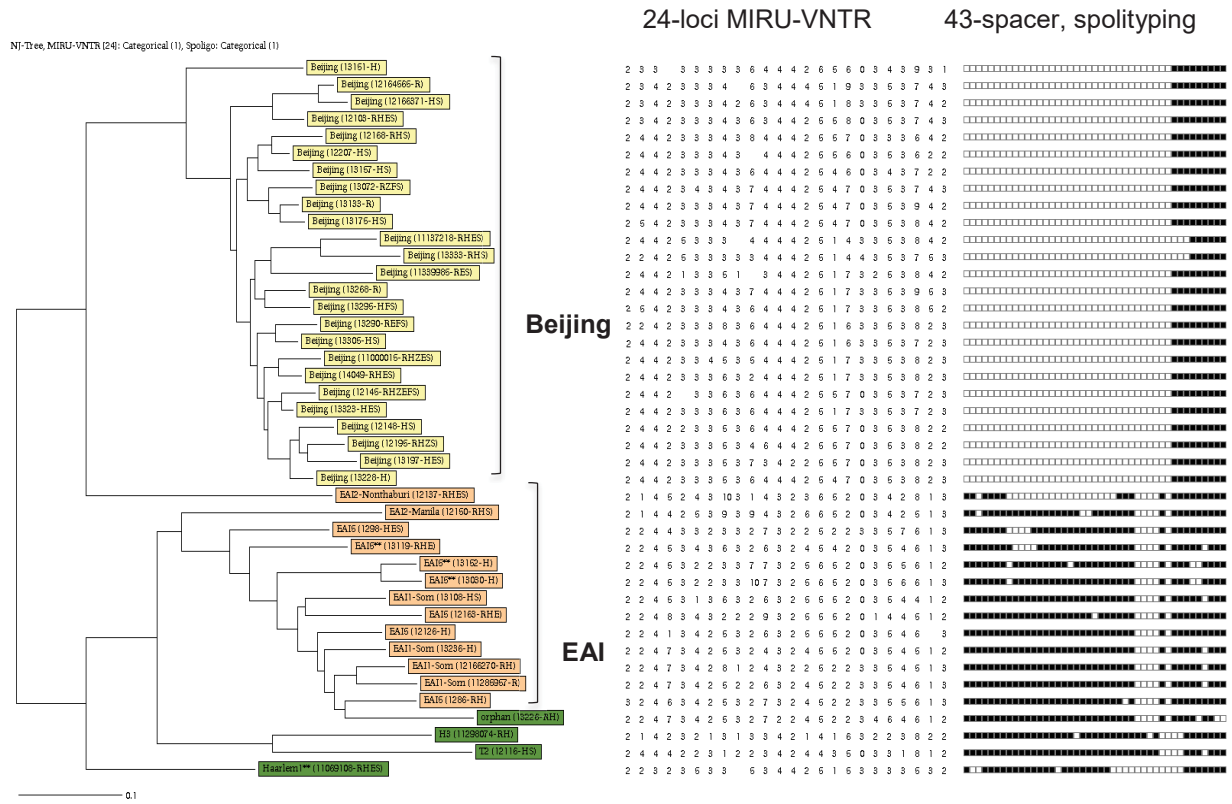
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375 **5. 24-locus MIRU-VNTR typing**

376 In order to explore the transmission, the 42 drug resistant isolates were typed by 24-locus
 377 MIRU-VNTR typing, resulting in 42 different patterns (Figure 2). The figure 2 illustrates the
 378 absence of clusters in this sample and the clear differentiation between Beijing and EAI families.
 379 The tree highlights the higher frequency of Beijing in overall drug resistant, poly-drug resistant
 380 and QDR. The pre-XDR isolates were only in Beijing clade.

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384 **Figure 2 MIRU-VNTR and spoligotyping profiles among 42 resistant isolates**

385 From left to right: i) Neighbor-joining tree based on the 24-loci MIRU-VNTR and 43-spacer spoligotyping
 386 data for the 42 isolates; ii) Number of repetitions of each VNTR according to the nomenclature by Supply
 387 et al (2006) (Supply et al. 2006) and iii) 43-spacer spoligotypes: black spots represent the presence and
 388 white spot represent the absence of 1-43 spacers (according to the numbering by Van Embden et al.
 389 2000) (Embden et al. 2000). Yellow squares = Beijing isolates; orange squares = EAI; Green square =
 390 others (T, H, Orphan

388 Each isolate is represented by the family, the isolate code and the resistant based on sequencing : H =
 389 isoniazid, R = rifampicin, E = ethambutol, S = streptomycin, F = fluoroquinolone.

391 **6. Stratification of social-demographic data, type of presumptive MDR-TB and genotypic**
 392 **data in overall drug resistant patterns**

393 The different proportions of gender, age, regions of residence, types of presumptive
 394 MDR-TB and *M. tuberculosis* families were assessed among susceptible and resistant isolates
 395 (defined by MTBDRplus). Age of patients was also grouped according to independent (15-64
 396 years old) and dependent age group (from 65 years old) and for better distribution, the
 397 independent age group therefore was subdivided into 2 groups 15 to 34 and 35 to 64 years old.

398 For the types of presumptive MDR-TB, contact with proven RR/MDR case was not included in
 399 the analysis due to only one observed case. The analysis showed that there were significant
 400 different proportions of drug resistant isolates according to the types of presumptive MDR-TB
 401 and the *M. tuberculosis* families ($p=0.01$ and $p=0.005$ respectively) (Table 6). Among different
 402 types of presumptive MDR-TB, the proportion of failure/late smear conversion cases having
 403 drug resistant isolates (46.7 %) was significantly higher than relapses, returns after loss follow
 404 up and TB-HIV cases (46.7 % > 33.3 % > 18.2 % > 5.6 %). However, there were no significant
 405 different proportions of drug resistant isolates within gender, age group or regions of residence.

406 **Table 6 Characteristic of patients infected with susceptible and resistant *M. tuberculosis* isolates**

Characteristics of presumptive MDR-TB		Total presumptive MDR-TB, N=139	Susceptible, N=96 (69.1%)	Resistant, N=43 (30.9%)	p-value
Gender (N=139)	Female	38	27 (71.0)	11 (29.0)	0.7
	Male	101	69 (68.3)	32 (31.7)	
Age (N=138)	15-34	31	24 (77.4)	7 (22.6)	0.2
	35-64	81	56 (69.1)	25 (30.9)	
	>=65	26	15 (57.7)	11 (42.3)	
Regions (N=139)	North	52	33 (63.5)	19 (36.5)	0.5
	Center	73	53 (72.6)	20 (27.4)	
	South	14	10 (71.4)	4 (28.6)	
Types of presumptive MDR-TB					
Relapse after treated with FLDs/SLDs		75	50 (66.7)	25 (33.3)	0.01
Failure/late smear conversion		30	16 (53.3)	14 (46.7)	
Return after loss follow up		11	9 (81.8)	2 (18.2)	
New patient with TB-HIV		18	17 (94.4)	1 (5.6)	
<i>M. tuberculosis</i> families					
Beijing		57	31 (54.4)	26 (45.7)	0.005
EAI		53	40 (75.5)	13 (24.5)	
others		29	25 (86.2)	4 (13.8)	

407
 408 Regarding the *M. tuberculosis* families, the distribution was significantly different in the
 409 three age groups ($p=0.01$, Table 7). Specifically, the percentage of Beijing family was higher in
 410 the “15-34” and “35-64” group compared to EAI (65.2 %, 56.3 % vs 34.8 %, 43.7 %
 411 respectively), and the percentage of EAI family higher in the “> 64” group compared to Beijing

412 (73.9 % vs 26.1 %). However, their gender and geographical distribution were not significantly
 413 different (Table 7). Regarding presumptive MDR-TB types, the distribution of Beijing family was
 414 higher among failure/late smear conversion cases than EAI, the proportion was significantly
 415 different ($p=0.02$) (75.0 % vs 25.0 %) (Table 7)

416 **Table 7 Characteristics of the patients infected with EAI or Beijing isolates**

Characteristics	Total, N=110	Infected with EAI, n=53 (48.2%)	Infected with Beijing, n=57 (51.8%)	p-value
Age group (years)				
15-34	23	8 (34.8)	15 (65.2)	0.01
35-64	64	28 (43.7)	36 (56.3)	
≥65	23	17 (73.9)	6 (26.1)	
Sex				
Men	80	42 (52.5)	38 (47.5)	0.1
Women	31	12 (38.7)	19 (61.3)	
Regions				
North	39	16 (41.0)	23 (59.0)	0.3
Centre	60	30 (50.0)	30 (50.0)	
South	12	8 (66.7)	4 (33.3)	
Presumptive MDR-TB^a				
Relapse after treated with FLDs/SLDs	61	33 (54.1)	28 (45.9)	0.02
Failure/late smear conversion	24	6 (25.0)	18 (75.0)	
Return after loss follow up	8	5 (62.5)	3 (37.5)	
New patient with TB-HIV	13	9 (69.2)	4 (30.7)	

417 ^a four missing data of presumptive MDR-TB type and one case of new patient in contact with proven
 418 RR/MDR was not included

419

420 Discussion

421 Identification of drug resistant TB and Non-tuberculous mycobacteria among 422 presumptive MDR-TB by MTBDRplus

423 Beside *M. tuberculosis*, the Non-tuberculous mycobacteria (NTM) were identified in 12 (8.6
 424 %) presumptive MD-TB cases, which is higher than in China (3.4 %) (Shao et al. 2015).
 425 Moreover, the four species (*M. scrofulaceum*, *M. abscessus*, *M. chelonae* and *M. fortuitum*)

426 found in our study were reported to be the cause of pulmonary infections (Griffith et al. 2012;
427 Stout, Koh, and Yew 2016). This finding underlines the need to also detect the NTM species in
428 patients with TB-like symptoms but with MTBc negative results in order to give the appropriate
429 treatment.

430 According to MTBDR_{plus} results, mono RIF resistance rate (5.0 %) was higher than the one
431 in Cambodia (2.4 %), but lower than in India and Ethiopia (13.0 % and 10.7 % respectively).
432 Conversely, the mono INH resistance rate (13.7 %) was higher compared to Cambodia, India
433 and Ethiopia (7.3 %, 8.0 % and 7.3% respectively) (Khann et al. 2013; Pradhan et al. 2013;
434 Tesfay et al. 2016). Among INH resistant, 30 (69.8 %) isolates had mutations in *katG* gene
435 (codon 315), which is known to be associated with a high level of INH resistance (Jagielski et al.
436 2014; ; Lempens et al. 2018) and high level of INH resistance cannot be overcome by the use of
437 high-dose INH in the treatment (Domínguez et al. 2016). This is an important concern for TB
438 treatment in Laos, since INH resistance is not screened in routine. The patients with mono INH
439 resistance will be treated by the standard regimen (2HREZ/4HR), these patients are at risk for
440 treatment failure, relapse or acquisition of additional drug resistances, especially the acquisition
441 of additional resistance to RIF due to inadequate treatment of INH resistant TB
442 (“FAQ_TB_policy_recommendations_guidelines.Pdf” n.d.). At last, the rate of MDR-TB (12.2 %)
443 is lower than those obtained in Asian and African countries like Cambodia, India and Ethiopia
444 (26.0 %, 53.0 % and 54.6 % respectively) (Khann et al. 2013; Pradhan et al. 2013; Tesfay et al.
445 2016).

446 **Genetic determinants associated with anti-TB drug resistance**

447 The specific genes or DNA regions selected in the study are known to be associated
448 with anti-TB drug resistance (Dookie et al. 2018). It is worth noting that the sequencing and
449 MTBDR_{plus} data showed an agreement of 92 % for the detection of RIF resistance and of 90 %
450 for INH resistance.

451 According to the sequencing results, the mutations in *rpoB* were mainly located in the
452 RRDR known to be associated with RIF resistance (Hirano, Abe, and Takahashi 1999; Zaw,
453 Emran, and Lin 2018; Bahrmand et al. 2009). Besides, one mutation outside RRDR (Ile572Phe)
454 and one insertion (TGCCAA, CysGln) at codon 514 represented 8 % of all *rpoB* mutations. This
455 mutation and insertion were recently identified in MDR isolates (Hirano, Abe, and Takahashi
456 1999; Zaw, Emran, and Lin 2018; Bahrmand et al. 2009; Takawira et al. 2017; Nguyen 2017).
457 Although MTBDR*plus* was designed to cover RRDR, one mutant isolate (Leu511Pro) detected
458 by sequencing was misdiagnosed by MTBDR*plus*. The Leu511Pro mutation was described as a
459 mutation linked to low-level resistance to RIF (Jo et al. 2017; Ocheretina et al. 2014), however,
460 this isolate was identified from patient with relapse after treatment.

461 INH resistance is mainly mediated by mutations in *katG* or *inhA* genes or within the
462 promoter region of *inhA* (Dookie et al. 2018). Around 80 % of *M. tuberculosis* isolates with INH
463 resistance revealed mutations in codon 315 of the *katG* gene or position -15 in the *inhA*-
464 promoter (43 % to 94 % of *katG*315; 19 % of -15 *inhA*-promoter) (Seifert et al. 2015). Our
465 results showed similar proportions of *katG* and *inhA*-promoter mutations (87.9 % and 18.2 %
466 respectively). However, other uncommon mutations associated with low level and high level of
467 INH resistance were detected (Asp189Gly and G630 leading to a Frameshift) (Brossier et al.
468 2016; Kandler et al. 2018). A combination of mutations in *inhA*-promoter and in *inhA*-coding (-
469 15(CT)/Ile21Val), previously described to be associated with high level of INH resistance and
470 cross-resistance to ethionamide and it was found in one isolate of our samples (Machado et al.
471 2013).

472 The conventional culture-based DST for EMB resistance is problematic. Indeed, poor
473 and variable agreements between MGIT 960, agar proportion methods or Bactec 460 were
474 observed (Horne et al. 2013). This could be due to the narrow range between the MICs of
475 susceptible and resistant isolates of *M. tuberculosis*. Indeed, the MICs of some isolates were
476 only weakly higher than the critical concentration and could result in false-susceptible results

477 (Horne et al. 2013; Madison et al. 2002; Campbell et al. 2011). On molecular point of view, the
478 mutations in *embB* at codon 306 are the most commonly detected point mutations of EMB
479 resistant strains (from 30% to 70%) (Y. Zhang and Yew 2009; Z. Zhang et al. 2014). However,
480 diversity of mutations in *embB* associated with EMB resistance was reported, consisting of 16
481 unique mutation within 11 different codons, two mutations Met306Val and Met306Ile accounted
482 for 77 % (53 % and 24 % respectively), other mutations included Met306Leu, Tyr319Cys,
483 Asp328Tyr, Arg351His, Asp354Ala, Gly406Asp, etc. (Campbell et al. 2011). In our study, the
484 two mutations at Met306Val and Met306Ile were also prevalent, accounting for 50 % (29 % and
485 21 % respectively), another 50 % carried five different mutations (Val360Met, Asp354Ala,
486 Pro404Ser, Gly406Asp, Gln497Lys) (Table S 2.1). These mutations were identified among EMB
487 resistant in previous studies (Q. H. Nguyen 2016; Plinke et al. 2010; Campbell et al. 2011)

488 The conventional DST for the detection of PZA resistance is also challenging and
489 problematic due to the poor growth of *M. tuberculosis* under the acidic conditions (pH 5.5 to 6.0)
490 required for optimal drug activity (Mackanness 1956; McDERMOTT and Tompsett 1954). On a
491 molecular point of view, the acquisition of mutations in *pncA* gene is the main mechanism
492 associated with PZA resistance. It is worth noting that mutations in *pncA* are very diverse and
493 widely dispersed throughout the gene (Morlock et al. 2000). In our analysis, two isolates
494 carrying mutations in *pncA* (Cys72Arg and Phe106Val) and two had base deletion (C deletion at
495 codon 19 and A deletion at codon 160, leading to frameshift). The Cys72Arg and the deletion of
496 C at codon 19 were identified among PZA resistant isolates in recent studies (Wade et al. 2004;
497 Q. H. Nguyen et al. 2017). The deletion of A at codon 160 has not been specifically previously
498 described to be associated with PZA resistance, but mutation occurring at codon 160
499 (Thr160Lys/ACG160AAG) was already found to be associated with PZA resistance (Cuevas-
500 Córdoba et al. 2013). At last, the Phe106Val has not been previously described to be
501 associated with PZA resistance but the Phe106Ser mutation was recently described (Maningi et
502 al. 2018) . Thus all the isolates with mutations could not be firmly associated with PZA

503 resistance. Further studies will be necessary to explore the link between the A deletion at codon
504 160 and the Phe106Val mutation and the PZA resistance. Since the culture-based DST of EMB
505 and PZA are still challenging, the DNA sequencing could bring an indubitable advantage.

506 Fluoroquinolones (FQs) are important bacterial antibiotics currently used as second line
507 treatment for MDR-TB. FQ resistance in *M. tuberculosis* is mainly due to the acquisition of
508 mutations within the quinolone resistance-determining region (QRDR) of the *gyrA* gene (codons
509 74 to 113), globally accounting for nearly 90 % of FQ resistance in *M. tuberculosis*. Codons 90,
510 91, and 94 are the most mutated sites (Miotto, Cirillo, and Migliori 2015; Maruri et al. 2012; Lau
511 et al. 2011). Our study identified four mutations at codons 88, 90, 91 and 94 of the *gyrA* gene.
512 The presence of these mutations led to one poly-drug resistant (HFS) and three pre-XDR TB
513 isolates (RHEFS, RHZFS and RHZEFS, see Table 3). The pre-XDR TB accounted for 2.2 % of
514 all presumptive MDR-TB. This underlines that it is extremely necessary to look for pre-XDR in
515 presumptive MDR isolates in Laos in order to prescribe the appropriate treatment regimen

516 Regarding STR resistance, the number of resistant isolates with mutations associated
517 with STR resistance was quite high in our sample (61.9 %) and only two mutations were
518 observed (Lys43Arg and Lys88Thr). These patterns fully justify that STR is no longer used in
519 Laos according to WHO recommendation.

520 Among the 42 drug resistant isolates, no mutation was observed in *rrs*-F2 (resistance to
521 injectable drugs) reflecting the absence of XDR-TB in presumptive MDR-TB cases under study.
522 This shows that XDR is still little detected and thus it is urgent to develop efficient TB and DR-
523 TB detection in Lao PDR to preclude the emergence and spread of XDR strains.

524 In total, the sequencing revealed 66.6 % (28/42) of drug resistant isolates detected by
525 the MTBDR_{plus} with additional mutations conferring resistance to EMB, PZA, FQ or STR. The
526 patterns of drug resistance include mono-resistance, poly-resistance, MDR, QDR and pre-XDR.
527 The determination of these patterns is critical for the prescription of appropriate treatment.

528 **Characteristics of *M. tuberculosis* population among presumptive MDR-TB and drug**
529 **resistant TB**

530 Spoligotyping identified Beijing and EAI as the two most predominant *M. tuberculosis*
531 families among presumptive MDR-TB. It is worth noting that the proportion of Beijing was higher
532 than EAI (41 % vs 38 %), although EAI is predominant in the global *M. tuberculosis* population
533 of Laos (Somphavong et al. unpublished data). Indeed, the previous population-based study
534 performed on the samples collected in the framework of the first National TB Prevalence Survey
535 (TBPS) showed a low proportion of Beijing compared to EAI (14 % vs 76 %). In addition, both
536 studies showed that Beijing is significantly more detected than EAI among drug resistant
537 isolates (TBPS: 17 % DR-Beijing vs 5 % DR-EAI ($p=0.03$); Presumptive MDR-TB: 46 % DR-
538 Beijing vs 25 % DR-EAI ($p=0.005$)). This result indicates that Beijing could be a factor
539 associated with drug resistance and highly drug resistance (Beijing family was observed among
540 pre-XDR isolates) and thus is more frequently observed in presumptive MDR-TB cases in Lao
541 PDR. This finding is in agreement with previous studies worldwide, especially in Asian countries
542 (Cheunoy et al. 2009; An et al. 2009; Cox et al. 2005)

543 Regarding the type of presumptive MDR-TB cases, the proportion of failure/late smear
544 conversion cases was significantly higher than relapses, returns after loss follow up and TB-HIV
545 among drug resistant isolates (46.7 % > 33.3 % > 18.2 % > 5.6 % respectively) ($p=0.01$). This
546 group has thus a higher potential to develop drug resistant TB than the other groups and
547 required full investigation for identifying disease etiology. It is essential to notice that the
548 proportion of Beijing was also higher than EAI among failure/late smear conversion cases
549 ($p=0.02$). Moreover, Beijing appears as a factor of failures and relapses in TB patients as
550 previously observed in many settings (Ramazanzadeh and Sayhemiri 2014).

551 Nevertheless, these DR isolates do not seem to spread in the country. Indeed, out of the
552 42 DR isolates, MIRU-VNTR typing generated 42 unique patterns, indicating the absence of
553 recent transmission among the resistant isolates among the presumptive MDR-TB samples.

554 Nevertheless, since the sample is not exhaustive, this result cannot be extended at the national
555 level. Further studies are needed to explore the molecular epidemiology of the MDR *M.*
556 *tuberculosis* isolates.

557 **Conclusion**

558 The DNA sequencing provides crucial information on mutations associated with drug
559 resistance among presumptive MDR-TB cases in Laos. The results revealed various
560 mutations reflecting different patterns of resistance from mono-resistance to pre-XDR. This
561 information is essential to help the prescription of appropriate treatment. Regarding the *M.*
562 *tuberculosis* families, as expected our data showed that Beijing is significantly associated
563 with drug resistance and more particularly with highly drug resistance patterns (QDR and
564 pre-XDR). Every effort for accurate and rapid detection of causative species and drug
565 resistance patterns is urgently needed for all TB patients in Laos. Molecular methods could
566 be promising and reliable tools for drug resistance detection in order to limit the emergence
567 and spread of MDR, pre-XDR and XDR-TB in the country.

568

569 **Competing interests**

570 The authors declare that they have no competing interests

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907
908

909 **Supplementary tables**910 **Table S 2.1 Frequencies of each mutation among the 42 drug resistant isolates.**

Anti-TB Drug/Gene (N. of mutated isolates, %)	Observed mutations	Total, N (%)
Rifampicin^a		
<i>rpoB</i> (n=25/42, 59.5%)		N=25 (%)
	Ser531Leu	6 (24.0)
	His526Arg	5 (20.0)
	His526Tyr	5 (20.0)
	Leu511Pro	2 (8.0)
	Asp516Val	2 (8.0)
	His526Asp	2 (8.0)
	His526Leu	1 (4.0)
	Met515Leu & Ile572Phe	1 (4.0)
	insert TGCCAA (CysGln) at 514 & Met655Thr	1 (4.0)
Isoniazid^b (n=39, 92.9)		
<i>katG</i> (n=33/42, 78.6 %)		N=33 (%)
	Ser315Thr	24 (72.7)
	Ser315Asn	3 (9.1)
	Ser315Arg & Glu582Lys	1 (3.0)
	Ser315Thr	1 (3.0)
	Asp189Gly	1 (3.0)
	Pro100Thr	1 (3.0)
	C deletion at codon 32 → Frameshift	1 (3.0)
	T insertion at codon 630 → Frameshift	1 (3.0)
<i>inhA</i> -promoter (n=7/42, 16.7%)		N=7 (%)
	-15 (CT)	6 (85.7)
	-17 (GT)	1 (14.3)
<i>inhA</i> coding region (n=3/42, 7.1%)		N=3 (%)
	Ile21Val	1 (33.3)
	Asp335Asn	1 (33.3)
	Ile144Val	1 (33.3)
Ethambutol		
<i>embB</i> (n=14/42, 33.3%)		N=14 (%)
	Met306Val	4 (28.6)
	Met306Ile	3 (21.5)
	Val360Met	3 (21.5)
	Asp354Ala	1 (7.1)
	Pro404Ser	1 (7.1)
	Gly406Asp	1 (7.1)
	Gln497Lys	1 (7.1)

Pyrazinamide		
<i>pncA</i> (n=4/42, 9.5%)		N=4 (%)
	C deletion at codon 19 → Frameshift	1 (25.0)
	A deletion at codon 160 → Frameshift	1 (25.0)
	Cys72Arg	1 (25.0)
	Phe106Val	1 (25.0)
Fluoroquinolone		
<i>gyrA</i> (n=4/42, 9.5%)		N=4 (%)
	Gly88Ala	1 (25.0)
	Ala90Val	1 (25.0)
	Ser91Pro	1 (25.0)
	Asp94Gly	1 (25.0)
<i>gyrB</i> (n=0/42)		
	-	-
Injectable drugs (AG/CP)		
<i>rrs-F2</i> (n=0/42)		
	-	-
Streptomycin ^c (n=26/42, 61.9%)		
<i>rrs-F1</i> (n=5/42, 11.9%)		N=5 (%)
	517(CT)	2 (40.0)
	514(AC)	1 (20.0)
	514(AT)	1 (20.0)
	274(GA)	1 ^d (20.0)
<i>rpsL</i> (n=22/42, 52.4%)		N=22 (%)
	Lys43Arg	16 (72.7)
	Lys88Thr	6 (27.3)

911 ^a One isolate was not detected by MTBDR_{plus} (Leu511Pro)

912 ^b One isolate showed a combination of mutations in *katG/inhA*-promoter (Ser315Thr/(-)15CT); two isolates
913 showed a combination in *katG/inhA*-coding (Ser315Thr/Asp335Asn and Ser315Thr/ Ile144Val); and One
914 isolate showed a combination in *inhA*-promoter/*inhA*-coding ((-15)CT/ Ile21Val)

915 ^c One isolate showed a combination in *rrs-F1/ rpsL* (274GA/ Lys43Arg)

916

917 **Table S 2.2. Complete data (clinical, epidemiological, demographic and genetic data) for the 155**
918 **isolates from presumptive MDR-TB in Lao PDR (2010-2014) (xlsx)**

919 **The data was stored in google drive, please follow the link bellow:**

920 <https://drive.google.com/drive/folders/1tiqNwVjTM5OQAF0lJn-rxxu-R678QPIa>

921

922

3.3. Result 3: Molecular analysis of drug resistance in *Mycobacterium tuberculosis* population collected during the first national anti-tuberculosis Drug Resistance Survey in Lao PDR (2016-2017)

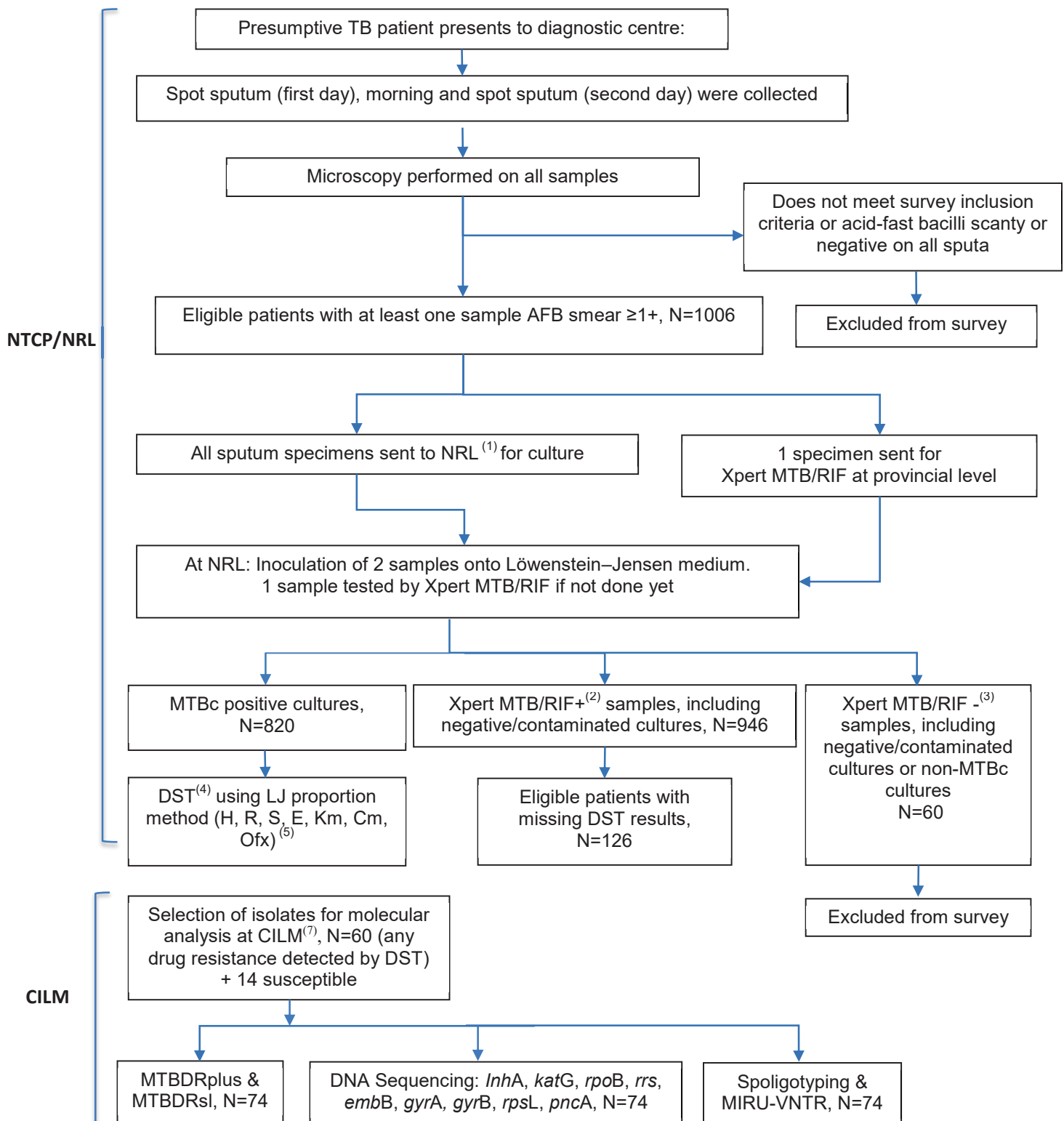
Sampling

The figure 3.1 presents the algorithm of sampling during drug resistance survey (2016-2017) and the selected samples for molecular analysis

Methods

The methods used on these samples are described in “Material and Methods” section (see page 49)

Figure 3. 1 Algorithm of the study



(1)NRL: national reference laboratory; (2)Xpert MTB/RIF+: MTB detected
 (3)Xpert MTB/RIF- : MTB not detected; (4) DST: drug susceptibility testing
 (5) H: isoniazid, R: rifampicin, S: streptomycin, E: ethambutol, Km: kanamycin, Cm: capreomycin, Ofx: ofloxacin, (6) CILM: Center for Infectiology Lao-Christophe Merieux

Results

1. Characteristics of patients

1006 patients eligible with at least one sample AFB smear positive were included in the study. The median age of patients was 50 years old (IQR: 36-61). The proportion of patients aged below 45 years old was lower than patients aged above 45 years old. The male/female ratio was 2.1. The majority of cases were new cases (94.2 %, n=948), with previously treated cases found in a small proportion (5.8 %, n=58). Out of the 1006 eligible patients, the cultures were positive for 820 (82 %) patients, of which the total number of isolates with resistance to at least one anti-TB drug (any drug resistance) was 75. The sixty available drug resistant isolates out of the 75 (80.0 %) were subjected to molecular analysis as well as 14 randomly selected susceptible isolates. The median age of these 74 cases was 45 years old (IQR: 30-58). Patients aged between 25-34 and 45-54 were more observed than others groups of age (23 % and 27 % respectively). The man to women ratio was 2.4. The majority of cases were new cases (93.2 %, n=69), while the previously retreated cases accounted for 6.8 % (n=5). About half of study cases were from center part of the country (Table 3.1).

Table 3. 1 Characteristics of patients included in the drug resistance survey and molecular analysis

Characteristics	AFB smear+ eligible, N=1006	<i>M. tuberculosis</i> culture+, N=820	Any drug resistance, N=75	Molecular analysis, N=74
Median age	50 (IQR: 36-61)	50 (IQR: 36-61)	48 (IQR: 36-60)	45 (IQR: 30-58)
Age group, years				
0-24	87 (8.7)	67 (8.2)	3 (4.0)	4 (5.4)
25-34	130 (12.9)	106 (12.9)	15 (20.0)	17 (23.0)
35-44	161 (16.0)	136 (16.6)	9 (12.0)	13 (17.6)
45-54	208 (20.7)	171 (20.9)	23 (30.7)	20 (27.0)
55-64	212 (21.1)	177 (21.6)	12 (16.0)	8 (10.8)
≥65	208 (20.7)	163 (19.9)	13 (17.3)	12 (16.2)
Gender				
Male	685 (68.1)	558 (68.1)	56 (74.7)	52 (70.3)
Female	321 (31.9)	262 (32.0)	19 (25.3)	22 (29.7)
Region of residence				
North	240 (23.9)	190 (23.2)	26 (34.7)	23 (31.1)
Center	510 (50.7)	433 (52.8)	41 (54.7)	40 (54.1)
South	256 (25.5)	197 (24.0)	8 (10.7)	11 (14.9)
TB patient category				
New cases	948 (94.2)	776 (94.6)	70 (93.3)	69 (93.2)
Previously cases	58 (5.8)	44 (5.4)	5 (6.7)	5 (6.8)

2. Drug resistant patterns from the drug resistance survey

Of 1006 patients included in the survey, 820 patients had DST results of all drugs. Based on the DST results, the prevalence of any resistance to FLD and SLD was 9.1 % (95 % CI: 7.3-11.3), which represents 9.0 % (95 % CI: 7.1-11.1) and 11.4 % (95 % CI: 3.8-24.6) among new cases and previously treated cases, respectively. The prevalence of any resistance to FLD was 8.7 % (95 % CI: 6.8-10.8) and any resistance to SLD was 0.9 % (95 % CI: 0.3-1.8). The prevalence of mono drug resistance, poly drug resistance and multi drug resistance (Box 1, p.12) were 6.8 % (95 % CI: 5.2-8.8), 1.7 % (95 % CI: 0.9-2.8) and 0.6 % (95 % CI: 0.2-1.4) respectively (Table 3. 2). Five MDR-TB cases were identified, of which two cases had additional resistance to STR (RHS); three cases had additional resistance to STR and EMB (RHSE), referring to QDR (Box 1, p.12). The MDR-TB prevalence among new cases was 0.6 % (95 %

CI: 0.2-1.5), whereas the MDR-TB was not identified among previously retreated cases due to negative culture results. However, based on Xpert MTB/RIF results, the rate of RR-TB among new cases was 1.2 % (95 % CI: 0.5-2.0) and among previously retreated cases was 4.1 % (95 % CI: 0-9.6) (DRS report, June 2018). Regarding FQ and SLIDS, the mono OFX resistance and mono CAP resistance were observed (Table 3. 2). However, no resistance to SLIDs or FQs was detected among MDR-TB patients.

Table 3. 2 Drug resistance patterns to FLD and SLD based on drug susceptibility testing

Drug-resistance pattern	New cases, N = 776 (% [95% CI])	Previously treated, N = 44 (% [95% CI])	Total, N = 820 (% [95% CI])
Susceptible to all drugs	706 (91.0 [88.7-92.9])	39 (88.6 [75.4-96.2])	745 (90.9 [88.7-92.7])
Any resistance to FLD and SLD	70 (9.0 [7.1-11.1])	5 (11.4 [3.8-24.6])	75 (9.1 [7.3-11.3])
Any resistance to FLD (R, H, E, S)	64 (8.2 [6.4-10.4])	5 (11.4 [3.8-24.6])	71 (8.7 [6.8-10.8])
Any resistance to FQ and SLID (Ofx, Km, Cm)	7 (0.9 [0.4-1.8])	0	7 (0.9 [0.3-1.8])
Mono drug resistance	54 (7.0 [5.3-9.0])	2 (4.5 [0.6-15.5])	56 (6.8 [5.2-8.8])
R	5 (0.6 [0.2-1.5])	0	5 (0.6 [0.2-1.4])
H	24 (3.1 [2.0-4.6])	1 (2.3 [0.1-12.0])	25 (3.0 [2.0 -4.5])
S	19 (2.4 [1.5-3.8])	1 (2.3 [0.1-12.0])	20 (2.4 [1.5-3.7])
Ofx	3 (0.4 [0.1-1.1])	0	3 (0.4 [0.1-1.1])
Cm	3 (0.4 [0.1-1.1])	0	3 (0.4 [0.1-1.1])
Poly drug resistance	11 (1.4 [0.7-2.5])	3 (6.8 [1.4-18.7])	14 (1.7 [0.9-2.8])
RS	2 (0.3 [0.03-0.9])	1 (2.3 [0.1-12.0])	3 (0.4 [0.08-1.1])
HE	0	2 (4.5[0.6-15.5])	2 (0.2 [0.03-0.9])
HS	8 (1.0 [0.4-2.0])	0	8 (1.0 [0.4-1.9])
SKmCm	1 (0.1 [0.003-0.7])	0	1 (0.1 [0.003-0.7])
MDR/QDR	5 (0.6 [0.2-1.5])	0	5 (0.6 [0.2-1.4])
RHS	2 (0.3 [0.03-0.9])	0	2 (0.2 [0.03-0.9])
RHSE	3 (0.4 [0.1-1.1])	0	3 (0.4 [0.08-1.1])

* FLD = First line drug; SLD = Second line drug; R = Rifampicin, H = isoniazid; E = ethambutol; S = Streptomycin; FQ = fluoroquinolone; SLID, Second line injectable drug; Ofx = Ofloxacin; Km = Kanamycin; Cm= Capreomycin.

3. Mutations in genes or regions of *M. tuberculosis* associated with anti-TB drug resistance

The Sanger sequencing of specific genes or regions involved in drug resistance to FLDs and SLDs could be performed on 74 isolates (60 any drug resistant and 14 susceptible). However, the sequencing result could not be obtained for some isolates, due to the absence of amplification or poor quality of sequence. The results of these isolates were notified as “Invalid” (Table 3.3).

Rifampicin resistance and *rpoB* gene: Based on available sequencing results, the majority (n=8/9, 88.9 %) of RIF resistant isolates harbored at least one mutation within the 81-bp Rifampicin resistance-determining region (RRDR), while one out of 9 (11.1 %) did not harbor any mutation in *rpoB* region under study. The most common mutations in *rpoB* were His526Tyr, His526Asp and Ser531Leu (27.3%, 18.2% and 18.2% respectively) (Table 3.3). Among RIF susceptible isolates, one out of 54 (1.8%) isolates with valid sequencing result harbored His526Ser (CAC526TCC) mutation (ID: 635, Table S 3.1). A total of five isolates harbored the Met736Thr mutation outside the RRDR. This mutation was found in combination with His526Tyr (His526Tyr/Met736Thr) in one RIF resistant isolate and alone in four susceptible isolates (Table 3.3).

Isoniazid resistance and *katG* gene and *inhA* (promoter and coding region): Of 29 INH resistant isolates, 16 (55.2%) isolates had mutation only in *katG* gene (single and double mutations) (Table 3. 3), five (17.2%) isolates had mutation only in *inhA*-promoter, one (3.4 %) had mutation only in *inhA* coding and one (3.4%) had triple mutations in *KatG* (Ala480Ser) & *inhA*-promoter (-15CT) and *inhA*-coding region (Ile21Val). No mutation was found in one resistant isolate (wild type) and five isolates had invalid results (Table 3. 3 and Table S 3.1). The most common mutations were Ser315Thr (48.3%) in *katG* and (-)15CT (13.8%) in *inhA*-promoter. Among 45 INH susceptible isolates, a total of eight (17.8%)

isolates had mutations in either *katG* or *inhA* (both promoter and coding region) (Table 3. 3), including one isolate with Pro232Ser of *katG*; four isolates with (-15CT) of *inhA*-promoter and three isolates with Ile144Val of *inhA*-coding region. It is worth noting that we did not include the mutation Arg463Leu of *katG* gene, described as a phylogenetic marker and not as a drug resistant determinant [136, 137] . This mutation was observed in 51 out of 58 isolates with valid sequencing results.

Ethambutol resistance and *embB*: In our study, two isolates were EMB resistant; one had Asp354Ala and one had Met306Ile mutations in *embB* gene. The sequencing detected Val360Met mutation in three EMB susceptible isolates whereas, this mutation was reported to be associated with EMB resistant [138]. Twenty seven out of 56 isolates carried the Glu378Ala mutation. This mutation previously defined as phylogenetic marker was not included in the analysis [139].

Streptomycin resistance and *rpsL* and *rrs-F1* fragment: Among 31 STR resistant isolates, mutations in *rpsL* were more observed than in *rrs-F1* (72.2 % vs 6.5 %). The most common mutation in *rpsL* was Lys43Arg (54.8 %) and in *rrs-F1* fragment was (-)517CT. No mutation was detected for three STR resistant isolates neither in *rpsL* nor *rrs-F1*. The Val8Phe mutation of *rpsL* and (-)87AG mutation of *rrs-F1* were identified, however they are found only in STR susceptible isolates.

Ofloxacin resistance and *gyrA*, *gyrB*: Of three OFX resistant isolates, two had Ala90Val mutation in *gyrA*, while no mutation was detected neither in *gyrA* nor *gyrB* for one OFX resistant isolate. Among the susceptible isolates, there was no mutation observed neither in *gyrA* nor in *gyrB*. We excluded the Glu21Gln and Ser95Thr mutations (found in 57/60 and 56/60 isolates respectively), since they were described as lineage genetic markers [140, 141]

Injectable drug resistance (Kanamycin (KAN), Capreomycin (CAP)) and *rrs-F2*: There was one KAN resistant isolate for which the sequencing analysis was invalid due to

the poor quality of sequencing result. Among the four CAP resistant isolates, sequencing results showed no mutation in *rrs*-F2 for three isolates while result was invalid for the remaining isolate.

Pyrazinamide resistance and *pncA*: DST was not performed for detection of PZA resistance. We evaluated the resistance to PZA by sequencing of *pncA* gene. In our study, five unique mutations were observed ((-)¹³GT and (-)⁷TG) in the promoter, Pro54ser, Ile5Thr and Cys72Stop codon in the coding gene).

Table 3. 3 Cumulative frequency of all mutations among the 60 drug resistant isolates and the 14 susceptible isolates.

Drugs/Genes	DNA Sequencing	DST using LJ proportion method		
	Mutations	N. of resistant	N. of susceptible	Total
Rifampicin <i>rpoB</i>		N=11 (%)	N=63 (%)	N=74 (%)
	His526Tyr	2 (18.2)	0	2 (2.7)
	His526Tyr & Met736Thr	1 (9.1)	0	1 (1.4)
	His526Asp	2 (18.2)	0	2 (2.7)
	Ser531Leu	2 (18.2)	0	2 (2.7)
	Leu533Pro	1 (9.1)	0	1 (1.4)
	His526Ser	0	1 (1.6)	1 (1.4)
	Met736Thr	0	4 (6.4)	4 (5.4)
	Wild type	1 (9.1)	49 (77.8)	50 (67.6)
	Invalid	2 (18.2)	9 (14.28)	11 (14.9)
Isoniazid <i>katG</i> only		N=29 (%)	N=45 (%)	N=74 (%)
	Ser315Thr	14 (48.3)	0	14 (18.9)
	Ser315Asn	1 (3.5)	0	1 (1.4)
	Ser315Asn & Ile317Val	1 (3.5)	0	1 (1.4)
	Ala480Ser ^a	1 (3.5)	0	1 (1.4)
	Pro232Ser	0	1 (2.2)	1 (1.4)
	Wild type	5 ^b (17.2)	36 (80.0)	41 (55.4)
	Invalid	7 (24.1)	8 (17.8)	15 (20.0)
<i>inhA</i> promoter	(-) ¹⁵ CT	4 (13.8)	4 (8.9)	8 (10.8)
	(-) ¹⁷ GT	1 (3.5)	0	1 (1.4)
	(-) ³⁴ CG	1 (3.0)	0	1 (1.4)
	Wild type	23 ^c (79.3)	41 (91.1)	64 (86.5)
	Invalid	0	0	0
<i>inhA</i> coding region	Ile21Thr	1 (3.5)	0	1 (1.4)
	Ile21Val	1 (3.5)	0	1 (1.4)
	Ile144Val	0	3(6.7)	3 (4.1)
	Wild type	24 ^d (82.8)	40 (88.9)	64 (86.5)
	Invalid	3 (10.3)	2 (4.4)	5 (6.8)

Ethambutol		N=2 (%)	N=72 (%)	N=74 (%)
<i>embB</i>	Asp354Ala	1 (50.0)	0	1 (1.4)
	Met306Ile	1 (50.0)	0	1 (1.4)
	Val360Met	0	3 (4.2)	3 (4.1)
	Wild type	0	51 (70.38)	51 (68.9)
	Invalid	0	18 (25.0)	18 (24.3)
Streptomycin		N= 31 (%)	N=43 (%)	N=74 (%)
<i>rpsL</i>	Lys43Arg	16 (51.6)	0	16 (21.6)
	Lys43Argv& Thr39Thr	1 (3.2)	0	1 (1.4)
	Lys88Arg	6 (19.4)	0	6 (8.1)
	Val8Phe	0	1 (2.3)	1 (1.4)
	Wild type	6 ^e (19.4)	42 (97.7)	48 (64.9)
	Invalid	2 (6.4)	0	2 (2.7)
<i>rrs-F1</i>	(-)-517CT	2 (6.5)	0	2 (2.7)
	(-)-87AG	0	1 (2.3)	1 (1.4)
	Wild type	23 ^f (74.2)	36 (83.7)	59 (79.7)
	Invalid	6 (19.4)	6 (14.0)	12 (16.2)
Ofloxacin		N= 3 (%)	N= 71 (%)	N=74 (%)
<i>gyrA</i>	Ala90Val	2 (66.7)	0	2 (2.7)
	Wild type	1 (33.3)	59 (83.1)	60 (81.1)
	Invalid	0	12 (16.9)	12 (16.2)
<i>gyrB</i>	Wild type	3 ^g (100)	58 (81.7)	61 (82.4)
	Invalid	0	13 (18.3)	13 (17.6)
Kanamycin (KAN)		N=1 (%)	N=73	N=74 (%)
<i>rrs-F2</i>	Wild type	0	67 (91.8)	67 (90.5)
	Invalid	1 (100)	6 (8.2)	7 (9.5)
Capreomycin (CAP)		N=4 (%)	N=70 (%)	N=74 (%)
<i>rrs-F2</i>	Wild type	3 (75.0)	64 (91.4)	67 (90.5)
	Invalid	1 (25.0)	6 (8.6)	7 (9.5)
Pyrazinamide*		NA	NA	N=74 (%)
	Wild type	NA	NA	61 (82.4)
	(-)-7TG	NA	NA	1 (1.4)
	(-)-13GT	NA	NA	1 (1.4)
	Ile5Thr	NA	NA	1 (1.4)
	Pro54ser	NA	NA	1 (1.4)
	Cys72Stop codon	NA	NA	1 (1.4)
	Invalid	NA	NA	8 (10.8)

* DST was not performed for detection of PZA resistance

^a combination of *KatG* (Ala480Ser) & *inhA*-promoter (-15CT) & *inhA*-coding region (Ile21Val)

^b three isolates had mutation in *inhA*-promoter, one had mutation in *inhA*-coding and one isolate with no mutation

^c sixteen isolates had mutations in *katG* only, one had mutation in *inhA*-coding gene only. One isolate had no mutation and five isolates had wild type for *inhA*-promoter but invalid results of *katG* and *inhA*-coding gene.

^d sixteen isolates had mutations in *katG* only, five had mutation in *inhA*-promoter only. One isolate had no mutation and two isolates had wild type for *inhA*-promoter and coding gene but had invalid results for *katG*.

^e two isolates had mutations in *rrs-F1*, three had no mutation, one had wild type for *rpsL* but invalid results for *rrs-F1*

^f twenty isolates had mutations in *rpsL*, three had no mutation

^g two isolates had mutation in *gyrA*, one had no mutation

4. Performance of different molecular methods for detection of drug resistant TB

All the 74 samples included in our analysis had Xpert MTB/RIF results; the MTBDR*plus* ver.1 and MTBDR*s/* ver.1 tests were obtained for 73 isolates. Regarding the sequences, it was variable according to the gene or region under study (Table 3. 4). The performance of Xpert MTB/RIF, MTBDR*plus*/MTBDR*s/*, and sequencing for detection of anti-TB drug resistance were assessed using the culture based phenotypic DST results as reference. The sensitivity, specificity, predictive positive value (PPV) and Negative predictive value (NPV) were calculated. The value of likelihood ratio positive (LR+) and likelihood ratio negative (LR-) were also presented (Table 3. 4). Based on DST results, 60 out of 74 isolates were resistant to at least one anti TB drug (including mono drug resistant, poly drug resistant, MDR and QDR), 14 were susceptible to all the seven tested drugs (RIF, INH, EMB, OFX, KAN, CAP and STR).

Performance of the Xpert MTB/RIF assay for the detection of RIF resistance: Based on phenotypic DST result, 11 isolates were resistant to RIF and 63 were susceptible. Xpert MTB/RIF assay detected RIF resistance in 9/11 of RIF resistant isolates and in 1/63 of RIF susceptible isolates. Xpert MTB/RIF assay demonstrated a sensitivity of 81.8 % and specificity of 98.4 %, with a PPV of 90 % and a NPV of 96.9 % (Table 3. 4).

Performance of MTBDR*plus* and MTBDR*s/* for the detection of resistance to RIF, INH, FQ, SLIDS (KAN, CAP) and EMB: MTBDR*plus* ver.1 and MTBDR*s/* ver.1 results were available for 73 isolates. The MTBDR*plus* test had a sensitivity and specificity of 63.6 % and 95.2 % for the detection of RIF resistance and 60.7 % and 86.7 % for INH resistance respectively. The MTBDR*s/* test had a sensitivity and specificity of 100 % and 100 % for detection of OFX resistance, 100 % and 100 % for detection of KAN resistance, 25.0 % and 100 % for detection of CAP and 50.0 % and 100 % for detection of EMB resistance respectively (Table 3. 4).

Performance of DNA sequencing for the detection of resistance to RIF, INH, EMB, FQ, SLIDS (KAN, CAP) and STR: Seventy-four isolates were submitted to Sanger sequencing of genes/regions associated with FLD and SLD resistance. We obtained variable numbers of interpretable sequences in function of the genes or regions, only isolates with interpretable sequences and DST data were included in the analysis. The performance of sequencing for detection of RIF resistance, EMB resistance and SLIDS (KAN, CAP) was evaluated by analyzing the presence of mutations in the single gene of *rpoB*, *embB* and *rrs-F2* respectively. The performance of sequencing for detection of INH resistance was evaluated by the presence of mutations in *katG*, *inhA*-promoter and *inhA*-coding gene together. For detection of OFX resistance, the mutations in both *gyrA* and *gyrB* were considered and for detection of STR resistance, mutations in both *rpsL* and *rrs-F1* were considered (Table 3.4). The sequencing revealed a sensitivity and a specificity of 88.9 % and 90.7 % for the detection of RIF resistance; 95.5 % and 78.4 % for the detection of INH resistance; 100 % and 94 % for the detection of EMB resistance; 66.7 % and 100 % for the detection of OFX resistance; 89.3 % and 94.6 % for the detection of STR resistance. The only one KAN resistant isolate had invalid result of sequencing; there was no mutation in *rrs-F1* found neither in CAP resistant nor in CAP susceptible, Therefore, the performance of sequencing for detection of the resistance to KAN and CAP could not assessed.

Table 3. 4 Performance of Xpert MTB/RIF; MTBDRplus/MTBDRsl and Sequencing for the detection of FLD and SLD resistance compared to DST results

Molecular methods (gene/region analyzed)	Drugs	DST results, No.		Performance, % (95% CI)					
		R	S	Sensitivity	Specificity	PPV	NPV	LR+	LR-
Xpert MTB/RIF (<i>rpoB</i>)	RIF-R	9	1	81.8	98.4	90	96.9	51.6	0.9
	RIF-S	2	62	(48.2-97.7)	(91.5-100)	(55.8-98.5)	(89.8- 99.1)		
MTBDRplus (<i>rpoB</i>)	RIF-R	7	3	63.6	95.2	70	93.67	13.2	0.4
	RIF-S	4	59	(30.8-89.1)	(86.5-99.0)	(41.5-88.5)	(87.1-97.0)		
MTBDRplus (<i>katG+inhA-promoter</i>)	INH-R	17	6	60.7	86.7	73.9	78	4.6	0.5
	INH-S	11	39	(40.6-78.5)	(73.2-95.0)	(56.0-86.3)	(68.8-85.1)		
MTBDRsl (<i>embB</i>)	EMB-R	1	1	50.0	100	100	98.6	NA	0.5
	EMB-S	1	70	(1.3-98.7)	(94.9-100)	NA	(94.6-99.6)		
MTBDRsl (<i>gyrA</i>)	OFX-R	3	0	100	100	100	100	NA	0
	OFX-S	0	70	(29.2-100)	(94.9-100)				
MTBDRsl (<i>rrs-F2</i>)	KAN-R	1	0	100	100	100	100	NA	0
	KAN-S	0	72	(2.5-100)	(95.0-100)	NA			
MTBDRsl (<i>rrs-F2</i>)	CAP-R	1	0	25	100	100	95.8	NA	0.8
	CAP-S	3	69	(0.6-80.6)	(94.8-100)	NA	(92.9-97.6)		
Sequencing (<i>rpoB</i>)	RIF-R	8	5	88.9	90.7	61.5	98	9.6	0.1
	RIF-S	1	49	(51.8-99.7)	(79.7-96.9)	(40.2-79.2)	(88.5-99.7)		
Sequencing (<i>katG+inhA-promoter+inhA-codong</i>)	INH-R	21	8	95.5	78.4	72.4	96.7	4.4	0.06
	INH-S	1	29	(77.2-99.9)	(61.8-90.2)	(58.5-83.0)	(80.9-99.5)		
Sequencing (<i>embB</i>)	EMB-R	2	3	100	94.4	40.0	100	18.0	0
	EMB-S	0	51	(15.8-100)	(84.6-98.8)	(18.2-67.0)			
Sequencing (<i>gyrA+gyrB</i>)	OFX-R	2	0	66.7	100	100	98.4	NA	0.3
	OFX-S	1	61	(9.4-99.2)	(94.1-100)	NA	(92.5-99.7)		
Sequencing (<i>rrs-F2</i>)	KAN-R	0	0	NA	100	NA	100	NA	1
	KAN-S	0	67	NA	NA	NA	NA		
Sequencing (<i>rrs-F2</i>)	CAP-R	0	0	0	100	NA	95.5	NA	1
	CAP-S	3	64	NA	NA		(95.5-95.5)		
Sequencing (<i>rpsL+rrs-F1</i>)	STR-R	25	2	89.3	94.6	92.6	92.1	16.5	0.1
	STR-S	3	35	(71.8-97.7)	(81.8-99.3)	(76.3-98.0)	(80.0-97.2)		

5. Identification of *M. tuberculosis* population by Spoligotyping and MIRU-VNTR

➤ Spoligotyping patterns

Spoligotyping and 24-locus MIRU-VNTR typing were performed on 74 isolates. The patterns of 11 (14.9%) isolates reflected either clonal variants (with double alleles at a single MIRU-VNTR locus) or mixed infections (with double alleles at least at two MIRU-VNTR loci) [142] (Table 3. 5 and Table S 3.1). These 11 isolates were removed from the family definition analysis to avoid incorrect designation. Thus, the analysis was performed on 63 isolates. Spoligotyping generated 34 different profiles, of which 27 were unique and seven represented clusters (36 isolates). Each cluster contained 2 to 19 isolates (average = 5). Forty-one out of 63 (65.1%) isolates were assigned to 15 SITs and four families reported in the SpolDB4 database; 1 (1.6%) was unknown (the spoligotype matched the SIT but could not be related to any family); and 21 (33.3%) were orphans (not match with any SIT in SpolDB4 database). The 21 orphan and the one unknown isolate were then compared using SPOTCLUST (Table 3. 5). Finally, the 63 isolates could be classified in four *M. tuberculosis* families (EAI, Beijing, T and Haarlem) and 9 subfamilies, three isolates remained orphan and one unknown (Table 3. 5 and Table S 3.1). EAI was the predominant family (47.6 %, n=30), followed by Beijing (34.9 %, n=22). T and H families were present in small proportion (7.9 %, n=5 and 3.2 %, n=2 respectively). Within the EAI family, the most frequent subfamily was EAI5 (41.3 %, n=26), followed by EAI2-Nonthaburi (4.8 %, n=3), only one EAI1-SOM was observed. When we compared the proportions between EAI and Beijing and other families among overall drug resistant and susceptible isolates, Beijing showed significant ($p=0.01$) higher proportion than EAI and other families among drug resistant isolates (95.4 %, 76.7 % and 54.5 % respectively) (Table 3. 6)

Table 3. 6 Drug resistant patterns based on DST according to *M. tuberculosis* families

Drug resistant status	Total, N=63 (col. %)	<i>M. tuberculosis</i> families		
		Beijing, n=22 (col. %)	EAI, n=30 (col. %)	Others, n=11 (col. %)
Susceptible	13 (20.6)	1 (4.6)	7 (23.3)	5 (45.5)
Resistant	50 (79.4)	21 (95.4)	23 (76.7)	6 (54.5)
Mono resistance	39 (61.9)	14 (63.6)	19 (63.3)	6 (54.5)
R	5 (7.9)	3 (13.6)	1 (3.3)	1 (9.1)
H	14 (22.2)	1 (4.6)	12 (40.0)	1 (9.1)
S	15 (23.8)	9 (40.9)	5 (16.7)	1 (9.1)
Ofx	3 (4.7)	1 (4.6)	1 (3.3)	1 (9.1)
Cm	2 (3.2)	0	0	2 (18.2)
Poly-resistance	9 (14.3)	6 (27.3)	3 (10.0)	0
RS	1 (1.6)	1 (4.6)	0	0
HE	1 (1.6)	0	1 (3.3)	0
HS	6 (9.5)	4 (18.2)	2 (6.7)	0
SKmCm	1 (1.6)	1 (4.6)	0	0
MDR/QDR	2 (3.2)	1 (4.6)	1 (3.3)	0
RHS	1 (1.6)	0	1 (3.3)	0
RHSE	1 (1.6)	1 (4.6)	0	0

R = rifampicin, H = isoniazid; E = ethambutol; S = Streptomycin; FQ = fluoroquinolone; Ofx = Ofloxacin; Km = Kanamycin; Cm= Capreomycin; MDR = multidrug resistance; QDR = quadruple drug resistance.

➤ **MIRU-VNTR typing**

By using the 24-locus MIRU-VNTR data alone, the 63 isolates generated only unique profile which corresponded to 63 different patterns. These findings underlined the absence of clusters and thus no case of recent transmission in our sample (Figure 3.2). The tree based on the combination of spoligotyping and MIRU-VNTR globally differentiate correctly Beijing from EAI. One orphan isolate was related with Beijing.

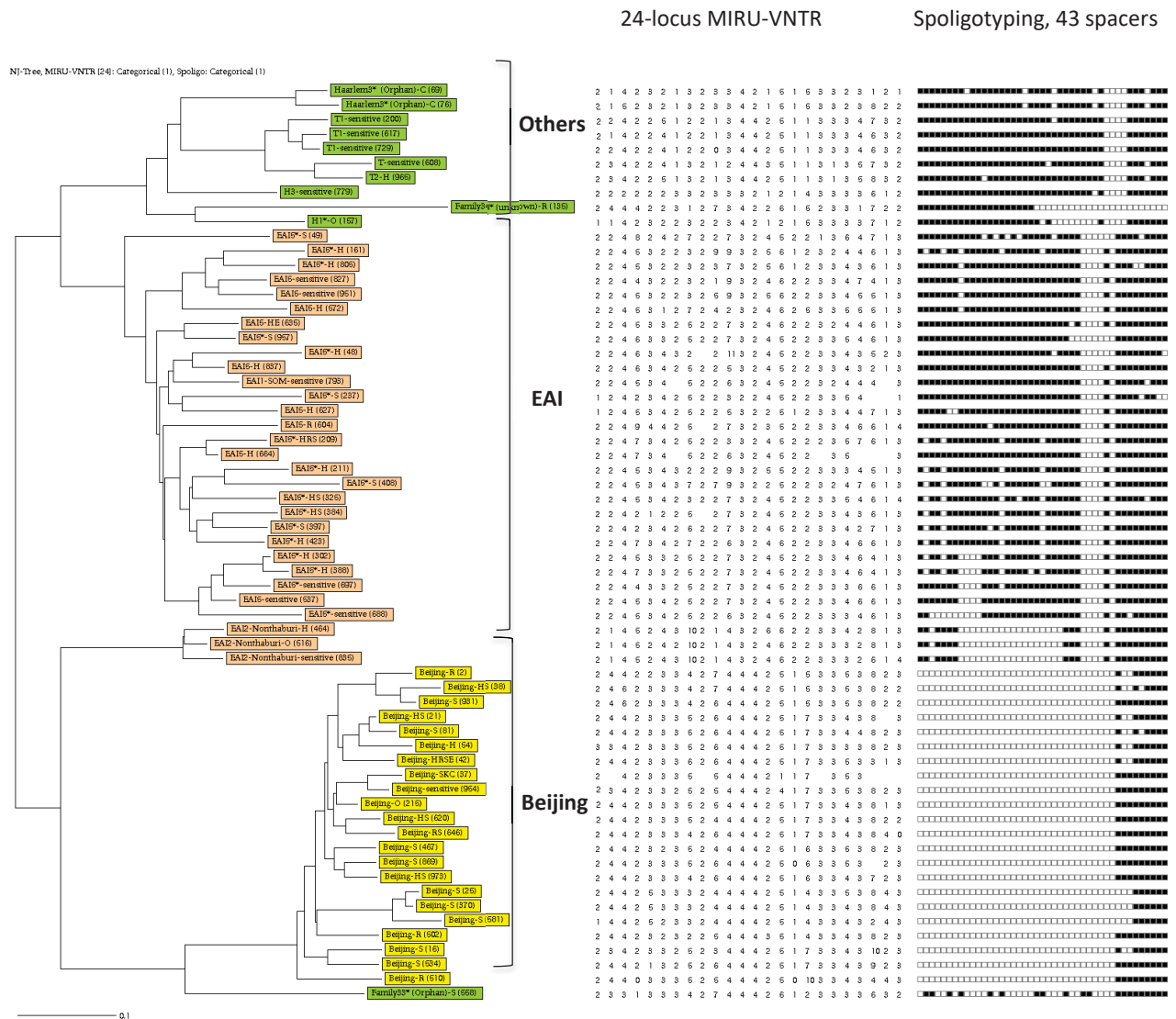


Figure 3. 2 Dendrogram based on MIRU-VNTR and spoligotypes profiles from the 63 isolates

From left to right: i) Neighbor-joining tree based on the 24-locus MIRU-VNTR and spoligotyping data from the 63 isolates included in the analysis; ii) Number of repetitions of each VNTR according to the nomenclature by Supply et al. [130] and iii) 43-spacer spoligotypes: black spots represent the presence and white spot represent the absence of 1-43 spacers (according to the numbering of Van Embden et al.)([143]. Yellow squares = Beijing isolates; orange squares = EAI; green square = other families

6. Stratification of social-demographic data in overall drug resistant and susceptible patterns

The proportions of gender, age group, regions of residence, type of patients and *M. tuberculosis* families were assessed among susceptible and resistant isolates. Ages of patients were grouped according to independent (15-64 years old) and dependent age group (from 65 years old). For better distribution, the independent age group therefore was subdivided into two groups 15 to 34 and 35 to 64 years old. The total number of isolates with successful designation of *M. tuberculosis* families was 63, the rest were not included into the analysis due to the detection of mixed infection. The analysis showed significant different proportions of drug resistant isolates according to the regions of residence and *M. tuberculosis* families ($p=0.03$ and $p=0.01$ respectively). The proportion of drug resistance was higher in the northern part than in the central and southern part, while the proportion of drug resistance were similar in the central and southern part (90.9 % , 66.7 % and 66.7 % respectively). Among *M. tuberculosis* families, the proportion of drug resistance was higher in Beijing family than EAI and other families (95.4 % , 76.7 % and 54.5 % respectively). There was no significant difference of the proportion of drug resistance in gender, age group and type of patients (Table 3.7).

Table 3. 7 Characteristic of patients with resistant and susceptible *M. tuberculosis* isolates

Characteristics of patients		Total, N=74	Susceptible, N=14 (30.9%)	Resistant, N=60 (69.1%)	p-value
Gender	Female	22	5(22.7)	17(77.3)	0.7
	Male	52	9 (17.3)	43 (82.7)	
Age	15-34	21	5 (23.8)	16 (76.2)	0.8
	35-64	33	6 (18.2)	27 (81.8)	
	>=65	20	3 (15.0)	17 (85.0)	
Regions of residences	North	44	4 (9.1)	40 (90.9)	0.03
	Center	24	8 (33.3)	16 (66.7)	
	South	6	2 (33.3)	4 (66.7)	
Type of patients					
	New cases	69	13 (18.8)	56 (81.2)	1
	Previously retreated cases	5	1 (20.0)	4 (80.0)	
<i>M. tuberculosis</i> families^a					
	EAI	30	7 (23.3)	23 (76.7)	0.01
	Beijing	22	1 (4.5)	21 (95.4)	
	Others	11	5 (45.5)	6 (54.5)	

^a a total of 63 isolates with successful family designation

Discussion

Socio-demographic characteristics of the study population

The median age of the 1006 patients with AFB smear positive (50 years old) found in this survey was similar to the one observed in the first National TB prevalence survey of Lao PDR in 2010-2011 [2]. This study showed that males are more infected than females which is also similar to the global observation, especially in Southeast Asia [1]. Regarding TB treatment history, 5.8 % of patients recruited were previously treated cases (including relapses) whereas, in Vietnam the population of retreated cases in the last drug resistance survey represented 15 % [144]. This difference can reflect a problem of case detection among previously treated (including relapses) cases in Lao PDR. In terms of geography, despite the fact that the patients

from the central provinces, including Vientiane capital, represented about half of the study population, the proportion of drug resistant isolates was higher in the northern provinces ($p=0.03$) (Table 3. 7). It is worth noting that there was also a significant difference of drug resistance proportion according to the *M. tuberculosis* family ($p=0.01$). Indeed, the proportion of drug resistance was higher among Beijing isolates than in EAI and other family isolates. This finding suggests that the association of drug resistance with the northern part of Lao PDR is linked to the higher preponderance of Beijing in the North as described in the first National TB prevalence survey (see Chapter 3, Result 1 (Paper 1)).

Prevalence and extent of drug resistant TB in Lao PDR

In this first national anti-tuberculosis drug resistance survey (DRS), the MDR-TB rate based on phenotypic DST is 0.6 % among new cases (no positive culture for retreated cases), and based on Xpert MTB/RIF, the RR-TB rate was 1.2 % among new cases and 4.1 % among previously retreated cases. These levels are particularly lower than the global averages published by WHO estimated at 4.1 % (95 % CI: 2.8–5.3) and 19 % (95 % CI: 9.8–27) respectively. These rates were also lower than the South East Asia region averages, 2.8 % (95 % CI: 2.4–3.1) and 13 % (95 % CI: 10–15) [1]. The overall prevalence of the resistance to FLD and SLD was 9.1 %. The prevalence of resistance to FLD among new and previously treated cases were 8.2 % and 11.4 % respectively. These rates are much lower than those reported in neighboring countries, such as China (34.2 % and 54.5 %), Vietnam (32.7 % and 54.2 %), Cambodia (13.6 % and 20.8 %) and Myanmar (10.0 % and 30.2 %) (Zhao et al. 2012; Nhung et al. 2015; “Report-National-Tuberculosis-Drs-2006-2007.Pdf,”; Ti et al. 2006). The low MDR-TB rate observed in Lao PDR could be explained by a low capacity of TB case detection in the past linked to low use of antibiotics, leading to a high number of missing TB cases but to a slower progression of drug resistance compared to the other neighboring countries. Nevertheless, even if the MDR rate in Laos is still low, it was identified among new cases, indicating the

transmission of MDR in the country. Surprisingly, mono resistance to OFX and CAP were detected, reflecting a wide use of OFX in the country for the treatment of respiratory diseases other than TB. The circulation of these isolates is of high concern because they have a higher potential to evolve towards pre-XDR and XDR. Furthermore, a variety of drug resistant patterns, including mono and poly-drug resistant patterns other than RIF resistance and MDR were observed. These isolates can also have a negative impact on the treatment outcome and might lead to relapse or treatment failure. Indeed, several studies underlined that drug resistant isolates are more prone to acquire other drug resistances compared to susceptible ones (see for review [148])

Mutations associated with first and second line anti-TB drugs

The mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been shown to be strongly associated with RIF-resistant phenotypes in multiple study populations [149–151]. The most common mutated codons found in our study (codon 526 and 531) were similar to the data observed in neighboring countries (China and Vietnam) [152, 153]. In one RIF resistant isolate, no mutation was detected. Similar observations have been reported in previous studies [154, 155], suggesting that RIF resistance associated mutations could be located outside the region under study. One RIF susceptible isolate had His526Ser mutation (ID: 635, Table 3. 3). This mutation is a “disputed” mutation (discordant results by DST) and was rarely detected. It was found inconsistently associated with RIF resistance [156–158]. However, recently a study demonstrated an association of this mutation with very low level of RIF resistance but probably clinically relevant RIF resistance [159, 160]

Among the INH resistant isolates tested, the proportion of mutations in *katG* was predominant (55 %), followed by the mutation in *inhA*-promoter (17 %). In Vietnam, the mutation in *katG* was found in 70 % of INH resistant isolates and mutation in *inhA*-promoter was found in 17 % (Caws et al. 2006). The mutations at codon 315 of *katG* gene were strictly detected in INH

resistant isolates while the (-)15CT mutation of *inhA*-promoter were observed in both INH resistant and susceptible isolates as single mutation or combined with *katG* and *inhA*-coding gene. Overall 17.8 % of INH susceptible isolates had mutations in either *katG* or *inhA*, indicating that the mechanism of INH resistance is not yet clearly determined as previously observed [161].

Among the two EMB resistant isolates, the sequencing allowed to detect the *embB* mutations associated with these two isolates. Nevertheless, the Val360Met mutation in *embB* was detected in three EMB susceptible isolates, whereas this mutation was reported to be associated with EMB resistant [138], however, this mutation need to be confirmed with the a large number of samples. On the other hand, the discordance could be explained by the problem encountered with the conventional culture-based EMB susceptibility testing which could give false-susceptible EMB results [162]. The sequencing of *embB* would substantially improve the diagnostic of EMB resistance. Regarding OFX and the detection of mutations in *gyrA* and *gyrB*, of three OFX resistant isolates, two carried Ala90Val mutation in *gyrA* which is one of the most common resistance-associated mutations to FQ resistance [100, 163]. No mutation was detected for one of the three OFX isolates. This profile could be explained by another mechanisms of FQ resistance [161]. Regarding injectable drug resistant isolates, all the three CAP resistant isolates did not harbor any mutations in *rrs-F2*. The CAP resistance could be due to other mechanism of resistance, as observed in a recent study. The authors demonstrated that resistance to CAP could be also associated with mutations in *tlyA* gene [164]. Include the analysis of the *tlyA* gene could provide a better understanding of CAP resistance mechanism.

The number of STR resistant isolates (n=31) was higher than other drug resistance in our study. The main mutations associated with STR resistance were observed in both *rpsL* and *rrs-F1* and 9.7 % of STR resistant isolates lacked mutations in *rpsL* and *rrs-F1*. In contrast, 4.7 % of STR susceptible isolates had mutations either in *rpsL* or *rrs-F1*. The high rate of STR resistant

isolates observed in this survey justifies stopping the use of this antibiotic in Lao PDR in agreement with WHO recommendations.

The presence of mutations in *pncA* is correlated with PZA resistance [165]. A large panel of *pncA* mutations has been reported [166, 167]. In our study, we found five different mutations which were previously associated with PZA resistance [165–167]. Conventional DST of PZA is challenging and problematic due to the poor growth of *M. tuberculosis* under acidic conditions (pH 5.5 to 6.0) that are required for optimal drug activity [168]. Thus, the good correlation between the presence of *pncA* mutations and PZA resistance makes DNA sequencing very useful for the PZA resistance detection. Nevertheless, a number of mutations still need to be experimentally tested to confirm their link with PZA resistance through the determination of MIC values or the use of functional genomics.

The Performance of different molecular methods for detection of first line drugs and second line drugs

➤ The performance of Xpert MTB/RIF for the detection of RIF resistance:

According to literature, the overall sensitivity of Xpert MTB/RIF assay for detection of MTBc was different based on settings, sample type, subject age, HIV co-infection and smear-positivity [169]. For the detection of RIF resistance, the pooled sensitivity and specificity was 95 % and 98 % respectively [170]. In our study, the sensitivity was found lower than the one observed in previous studies (81.8 % vs 95 %) but the specificity was similar (98.4 % vs 98 %). The Xpert MTB/RIF missed two isolates among RIF resistant, isolates whereas one more RIF resistant was detected (Table 3. 4). The data suggest the presence of *rpoB* mutations outside RRDR or different mechanisms of RIF resistance or a mixture of mycobacterial subpopulations with different susceptibilities to RIF as previously observed [171, 172]. Regarding the last point, it is worth noting that MIRU-VNTR typing revealed occurrence of mixed infections and/or clonal

variants in the two isolates that was not correctly detected by Xpert MTB/RIF (ID: 68 and ID: 526, Table S 3.1).

➤ **The performance of MTBDRplus ver.1 and MTBDRsl ver.1 for the detection of resistance to RIF, INH, EMB, FQs and SLIDs (KAN, CAP):**

By comparison with culture-based DST, the overall sensitivity of the MTBDR*plus* ver.1 for the detection of RIF and INH resistance was 98 % and 89 % respectively and the specificity was 90 % and 91 % respectively [28, 173–175]. In our study, the sensitivity for detection of RIF and INH resistance (63.6 % and 60.7 %) were much lower than those observed in previous studies, whereas the specificity for detection of RIF resistance (95.2 %) was higher. The specificity for detection of INH resistance (86.7 %) was also lower [28, 173–175]. The presence of mix infections or cross-contamination might hamper the result of test [176]. In addition, these disagreements between DST and MTBDR*plus* can be due to the low number of targets included in the MTBDR*plus* test.

Regarding the MTBDR*sl* ver.1, the test showed a sensitivity of 50 %, 100 %, 100 % and 25 % for the detection of EMB, OFX, KAN and CAP resistance respectively, whereas the specificities were 100 % for each. Nevertheless, due to the small number of resistant isolates for each of these drugs, EMB, OFX, KAN and CAP, (2, 3, 1, and 4 respectively), it is impossible to conclude

➤ **The performance of DNA sequencing for detection of resistance to RIF, INH, EMB, FQ, SLIDs (KAN, CAP) and STR:**

The sequencing showed a sensitivity of 88.9 % for detection of RIF resistance; the value was higher than Xpert MTB/RIF (81.8 %) and MTBDR*plus* (63.6 %), while the specificity of 90.7 % was lower than Xpert MTB/RIF (98.4 %) and MTBDR*plus* (95.2 %). The low sensitivity could be explained by the detection of the Met736Thr mutation in *rpoB* among susceptible isolates

(Table 3. 3). This mutation was never described as RIF resistance associated mutation. The sensitivity of sequencing for detection of INH resistance was higher than MTBDR*plus* (95.5 % vs 60.7 %), while the specificity was lower (78.4 % vs 86.7 %). This was due to the detection of (-)15CT (this mutation is known to be associated with low level of INH resistance) in susceptible isolates and the detection of unknown mutations in *katG* and *inhA*-coding gene (Table 3. 3). The small number of resistant isolates of EMB, OFX, KAN and CAP (n=2, 3, 1, and 4 respectively) did not allow to give a strong evaluation of sequencing performance for these drug resistance. Regarding STR resistance, sequencing showed high value of sensitivity and specificity, 89.3 % and 94.6 % respectively.

Finally, from this study, we can state that the targeted gene sequencing can be an extremely powerful tool for drug resistance detection. Nevertheless, a first step of evaluation is necessary to establish the link between each mutation present in Lao PDR and the corresponding drug resistance in order to increase the specificity and the sensibility for each resistance. As example, the *tlyA* should be added in the list of genes and the Met736Thr mutation should not be considered as drug resistance associated mutation. Furthermore, the “disputed” mutations should be used and linked to drug resistance with an estimated probability to orientate the clinical making decision and the treatment prescription.

Conclusion

Globally this study revealed a very low MDR prevalence in Lao PDR with 0.6 % in new cases and the RR-TB 1.2 % among new cases and 4.1 % among previously retreated cases. The situation of Lao PDR is particular since despite a relatively high TB incidence 168/100,000 in this country, the drug resistance is still low. It is thus urgent to increase the TB and drug resistance detection in order to preclude the rapid emergence of highly drug resistance strains in the country as we observed in the neighboring countries. Furthermore, Beijing family is

present in Laos and linked to drug resistance, it is thus essential from now to stop its unavoidable progression that will occur if the TB control is not rapidly improved.

Some main risk factors associated with drug resistance could be identified such as the north part of the country and the Beijing family. Surprisingly, a low number of retreated cases were included in the survey suggesting a basic problem of patient recruitment. This aspect needs to be also rapidly improved in Lao PDR.

Besides, the indispensable need to improve capacity building for a better TB patient detection and care, the drug resistance diagnostic is also a serious concern. This study showed that the sequencing and especially the targeted gene sequencing can really improve the drug resistance diagnostic in terms of time and quality (detection of large drug resistant patterns).

Chapter 4 GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

4.1. General discussion

This is the first study focused on the genetic characteristics and drug resistance of *M. tuberculosis* population in Lao PDR. Several characteristics were investigated, including: the families/sub-families/genotypes of *M. tuberculosis* population circulating in Lao PDR; the genetic structure of *M. tuberculosis* population according to the socio-demographic data; epidemiological patterns including the estimation of recent versus ancient transmission; the patterns of drug resistance occurring in Lao PDR and the prevalence of each pattern including the highly drug resistance ones (MDR, QDR, pre-XDR, XDR); the type and frequency of mutations associated with anti-TB drug resistance; the performance evaluation of the different molecular tests used in Lao PDR compared to the culture-based DST and their usefulness for rapid detection of drug resistance. The main findings obtained in this study and their potential consequences and applications in terms of public health are discussed below.

➤ Diversity of *M. tuberculosis* population circulating in Lao PDR and risk of epidemiological changes of tuberculosis in the country

M. tuberculosis families were described for the first time in Lao PDR using isolates from three different samplings (population based sampling (TBPS 2010-2011), hospital based sampling focused on drug resistance cases (DRS 2016-2017) and presumptive MDR-TB (2010-2014)). The proportions of *M. tuberculosis* families were different according to samplings. Based on the result of TBPS population, the *M. tuberculosis* populations were mainly composed of strains belonging to EAI and Beijing families (76.7% and 14.4% respectively), similarly to neighboring countries but in different proportions. Most of neighboring countries like China,

Vietnam, Cambodia and Myanmar showed lower proportion of EAI than that observed in Laos (0.03%, 38.5%, 60%, and 48.4% respectively) but higher proportion of Beijing (74.1% , 38.5 %, 30 % and 31.9% respectively) [10–12, 177]. As previously described, EAI is one of the most ancient and predominant *M. tuberculosis* family in many Asian countries [55, 178]. This family was associated with older population, lower rates of TB transmission and mildly virulent [11, 179]. In contrast, Beijing family was associated with young people, urban areas [11, 180], greater virulence, drug resistance and highly transmissible [181–183]. The findings in Laos are in favor of the endemicity of EAI and the circulation of Beijing family still at low level unlike Vietnam [184]. This could be explained by the low population density (27 people per km²) of Lao PDR [185]. Nevertheless, the data can be biased since the participants included in the TBPS were mainly from rural areas with a majority of TB cases collected from older age group [2]. EAI family is generally associated to these groups of people as demonstrated in Vietnam but also to the reactivation of latent TB as observed in our study (low rate of recent transmission) [11]

Conversely to population based sampling (TBPS), the Beijing family was more preponderant in hospital based sampling (DRS 2016-2017), and presumptive MDR-TB cases (2010-2014) 35 % and 41 % respectively. This finding highlights the association of Beijing family with drug resistant TB and presumptive MDR-TB cases and thus the risk of increase and spread of this family in the country.

Regarding social-geographic distribution, the TBPS sampling showed higher proportion of Beijing in Northern provinces compared to the other provinces, whereas EAI is distributed all over the country. This finding suggests an introduction of Beijing family in the country from the North (N=0 in the South). This result is confirmed by the origin of the DRS isolates and presumptive MDR-TB cases (data not shown). Furthermore, the EAI family was more detected in age group ≥ 35 years old, whereas Beijing family was also strongly present in younger age group (lower than 35 years old, 34.5% of Beijing isolates). Furthermore, the analysis carried

out in the framework of the TBPS showed 11.9% of recent transmission with a higher clustering rate in Beijing family compared to EAI (11% versus 20.7%, respectively). These findings suggest that the TB epidemiological pattern in Lao PDR can shortly change with a high risk of increase of Beijing family in the country. This hypothesis is supported by numerous studies that showed the high capacity to produce outbreaks and the epidemiogenic properties of the Beijing family [186–188]. This highlights the urgent need to improve the TB detection in the north and to follow the Beijing infection cases to preclude outbreaks and the potential spread all the country scale. It is worth noting that the two molecular typing techniques (Spoligotyping and MIRU-VNTR) used in this study showed their usefulness for molecular epidemiology study. The regular use of these methods will permit to follow the trend of TB and drug resistant TB in Lao PDR and to evaluate the efficacy of the TB control over time.

➤ **Link between *M. tuberculosis* families and drug resistance and the drug resistance transmission in Lao PDR.**

The drug resistant isolates identified during our study belong to different *M. tuberculosis* families (EAI, Beijing, T, H and others). However, the EAI and Beijing were still the most prevalent in all three samplings. Nevertheless, conversely to the distribution in the whole *M. tuberculosis*, the proportion of Beijing family was higher in the drug resistant population compared to EAI and other families, and especially among highly drug resistant patterns (such as QDR and pre-XDR cases). Thus in addition to the epidemiogenic properties of the Beijing family demonstrated in the TBPS study, we also demonstrated that this species is specifically associated with drug resistance and highly drug resistance. This highlights a high risk of drug resistance increase due to the highly transmissible Beijing family in Lao PDR. This point is reinforced by the observation of genetic clusters of drug resistant isolates in the TBPS study (a cluster of three INH resistant isolates was found in the Northern Province with epidemiological data link). No cluster was observed in DRS and presumptive MDR-TB cases but for these two

samplings the isolate collection was not exhaustive. Since the samplings were only composed of cases detected in health centers or hospitals, it is thus probable that we missed many TB cases and that recent transmission was strongly underestimated. Nevertheless, it is interesting to note that during the DRS study, the prevalence of drug resistant isolates was 9 % among new cases and that some MIRU_VNTR profiles were very close (only one allele difference), both reflecting the spread of drug resistant strains. This finding illustrates the need to implement an effective TB control, especially in the Northern provinces to prevent the spread of drug resistant strain.

➤ **MDR-TB and pattern variety of drug resistant TB in Lao PDR**

During TBPS and DRS study, the rate of MDR-TB among new and previously treated cases were 0.9 % (n=2/222) and 0.6 % (n=5/820) respectively. During the DRS, the rate of MDR/RR-TB in Laos among new and previously treated cases (0.5%/1.2% and 2.3%/4.1% respectively) were lower compared to the global estimation 4.1% and 19 %, but also compared to the data of South East Asia region 2.8% and 13% and neighboring countries Cambodia, Thailand, Vietnam, Myanmar and China where the estimated rate ranged from 1.8% - 7.1% among new cases and 11% - 27% among retreated cases [1]. The low proportion of Beijing family could be an explanation for the low level of drug resistant TB in Laos. However, the data suggest a potential risk of Beijing increase and thus an undebatable risk of drug resistance increase.

While the NTCP mainly focuses on MDR/RR-TB, the mono resistant and poly-resistant TB (drug-resistant TB other than MDR-TB) are actually more common than MDR/RR-TB among our three samplings, TBPS, DRS and presumptive MDR-TB (5 %, 8 %, 12 % respectively). The data was in agreement with the global report. Indeed, the worldwide prevalence of MDR-TB in new cases is around 3% while the prevalence of mono- and poly-resistant strains is almost 17% [14]. Many of these cases contribute to the amplification of resistance and, eventually, can lead to MDR-TB if they are not properly managed [15]. In Lao PDR, the Xpert MTB/RIF has been

used as a frontline test for all presumptive TB and MDR-TB, leading to routinely undiagnosed mono-resistant and poly-resistant TB. This means that undiagnosed mono- and poly-resistant TB are likely often treated with standardized first line drug regimen (2RHEZ/4RH). Some of these patients may experience transient clinical and bacteriological improvement but are at risk for failure or relapse, often with higher resistance patterns. Appropriate treatment of mono- and poly-resistant TB can therefore prevent the development of MDR-TB.

Concerning INH resistance, the overall rate (among TBPS, DRS and presumptive MDR-TB) were 6 %, 5 % and 26 % respectively. Furthermore, the rate of mono INH resistance was 3 %, 3 % and 4 % respectively. As globally observed, the mono INH resistance is the most common form of mono resistance, with estimated prevalences ranging between 0 to 9.5% (0-12.8% among new cases and 0-30.8% among retreated cases) [14]. In our study, the distribution of mono INH resistant among presumptive MDR-TB was 57.9 %, 31.6 %, 10.5 % respectively among relapse, failure and return after loss follow up cases. The NTCP needs to consider these data since they can lead to TB treatment failure as previously observed [190]. Moreover when INH is used in combination with RIF during the 4 months of continuous phase for standard regimen, if INH resistance is undiagnosed, RIF is thus effectively used alone corresponding to mono drug therapy. There is thus a high risk of acquisition of RIF resistance as described by Hobby and Lenert 1979 [191].

Besides, the mono resistance to OFX and CAP were identified among new cases from DRS sampling. Since OFX is recently used in TB regimen for MDR-TB treatment (late 2013) and CAP was never used in TB regimen before. The question of acquisition of these resistances arises. The OFX resistance might be due to the extensive use of this antibiotic for the treatment of respiratory diseases other than TB. Nevertheless, the reason for CAP resistance is still unclear. Whatever the etiology of drug resistance acquisition, the detection of this mono-resistance underlines a real risk of pre-XDR and XDR emergence in the country. The best management of these OFX and CAP resistance cases is to strictly follow up of patients to

prevent the failure of standard treatment regimen which could lead to pre-XDR and XDR emergence in the future.

PZA is one of the core drugs used in the standard first regimen and the second line treatment. Nevertheless, since the conventional DST for PZA is not yet standardized [168, 189], the PZA resistance was neither assessed in the first DSR nor in routinely diagnosis. The sequencing that we carried out in this work showed several mutations in *pncA* gene (nine in total). The majority of them were reported associated with PZA resistance. One third of these mutations were identified among highly drug resistant patterns (QDR, pre-XDR). This is alarming since critical treatment outcomes were previously observed consequently to PZA resistance [192, 193].

Additional resistances to MDR-TB were identified in our study (the sampling of presumptive MDR-TB cases), leading to QDR and pre-XDR TB forms (n=9/17 QDR and 3/17 pre-XDR). The patients infected by FQ resistant are non-eligible for the short course nine-month regimen for MDR-TB. As resistance to both FLDs and SLDs was identified in Lao PDR in our study, the most important questions are how to control and prevent the transmission of such deadly drug resistant strains and what combination of drugs has to be used for an effective treatment. These findings underline an urgent need to screen the resistance to all drugs used for TB and MDR-TB treatment in Laos. All presumptive TB and MDR-TB cases need to have access to the full drug resistance testing, since it is critical for the prescription of appropriate treatment. It is first to increase the cure rate among patients but also to limit the spread of TB and drug resistant TB.

➤ **Genetic determinants associated with anti-TB drug resistance and molecular method use for rapid diagnosis of drug resistant TB**

The sequencing of the genes involved in drug resistance showed a diversity of mutations associated with drug resistance. However, the most common mutations were mainly observed as previously described: mutations in *rpoB* gene at codons 526 and 531 [152, 171, 194–196];

mutations in *katG* gene at codon 315 [104, 196, 197] and mutations in *rpsL* at codon 43 [198, 199]. These prevailing mutations are strongly selected in the population [200, 201]. The pattern is different for *pncA* gene since we observed a diversity of mutations without preeminent ones as described in many studies (four unique mutations among presumptive MDR-TB cases and 5 unique mutations among DRS). The majority of them were previously correlated with PZA resistance.

The main mutations found in this work include mutations related to different levels of drug resistance (eg: Ser315Thr in *katG*, (-) 15CT in *inhA*-promoter), the mutation outside the target region (Ile572Phe in *rpoB*) and mutations disputed for DST results (eg: His26Ser in *rpoB*, Val360Met in *embB*). The combination of mutations on different regions generally referred to high level of drug resistance and cross-resistance to other drug (e.g. -15(CT)/Ile21Val in *inhA* promoter and *inhA* coding gene). Regarding second line drugs, even though the number of FQ resistance was very small (n=7), we observed four different mutations in *gyrA* gene. The mutations in targeted genes were not found among all CAP resistant isolates, in some RIF, INH, STR and OFX resistant isolates. This underlines the existence of other mechanisms that need to be determined. This study gives the first information on drug resistance associated mutations in *M. tuberculosis* and it is necessary to continue the molecular characterization to follow the evolution of drug resistance in Lao PDR.

By assessing the performance of molecular methods for the detection of drug resistance, the current GeneXpert MTB/RIF used in the country showed higher sensitivity and specificity for detection of RIF resistance than MTBDR*plus*, however its sensitivity was lower than DNA sequencing. The data demonstrated that the use of a limited number of targets for the detection of drug resistance is not enough to get the full drug resistant patterns and determine the appropriate treatments. Higher the number of genes/regions will be explored, the more we will be close to the true drug resistance. In this context, NRL needs to either implement new

methods such as MICs, targeted gene sequencing or WGS or to find a new diagnostic tool to complete the diagnosis of drug resistance for all the drugs used.

4.2. Conclusion

This study provides the first genetic insights into the *M. tuberculosis* population in Lao PDR. The presence of the main families detected in neighboring countries was determined, mainly EAI and Beijing families. The 11% of recent transmission rate and mini outbreak of drug resistant isolates represent a challenge in Lao PDR. EAI family is predominant in Laos, more prevalent in rural area and in older people but associated with a lower rate of TB transmission and known to be mildly virulent. Conversely, Beijing family, known to be highly transmissible and drug resistant, represents a low frequency in the global population of the TBPS sampling. Nevertheless, this family was more detected among hospital based sampling collected in the framework of DRS and in the presumptive MDR-TB samplings. It is worth noting that Beijing was especially observed in highly drug resistant patterns such as in QDR and pre-XDR isolates (7/10 QDR and 3/3 pre-XDR isolates respectively). Moreover, this family was significantly observed in younger age group (<35 yrs), and also involved in recent transmission, suggesting a risk of rapid spread in the country from north to south. This situation could change the TB epidemiology in the near future in Lao PDR. This underlines the need to reinforce the efforts to maintain an efficient TB control and surveillance system in order to prevent the emergence of highly transmissible and drug-resistant strains in Lao PDR, as observed in neighboring countries.

Even though the MDR-TB prevalence in Lao PDR is still low compared to neighboring countries, the overall rate and variety of patterns of drug resistance have to attract the attention of NTCP. If mono- and poly-drug resistances are undiagnosed and inappropriately treated, this could lead to critical treatment outcome and amplify the risk of MDR, pre-XDR and XDR emergence in the future. Moreover, our data showed that the MDR-TB cases had additional

resistances to other drugs, leading to QDR and pre-XDR-TB. The NTCP should take into account these information since these patients are non-eligible for the shorter 9 month regimen and present a risk to develop XDR that could be then transmitted in the population.

Every single drug resistance needs to be identified, even mono or poly-resistances, in order to monitor on the best way the treatment course and the treatment outcome. The full drug resistance testing is critical for the prescription of appropriate treatment. The consequence it is not just to increase the cure rate among patients but also to limit the spread of TB and drug resistance TB in the community.

The DNA sequencing provided crucial information on mutations associated with FLD and SLDs drug resistance. The result revealed various mutations reflecting the diversity of drug resistant mechanisms in *M. tuberculosis* in Lao PDR. These findings showed that the surveillance of drug resistance is essential in order to follow the evolution of drug resistance in the country and to prescribe the most appropriate treatment for the patient care. Nevertheless, the available molecular tests target only single or few loci limits the ability for accuracy diagnosis. In order to detect more MDR-TB cases, it is necessary to intensify case finding able to screen MDR-TB among both new and previously treated cases. Furthermore, to complete the knowledge on drug resistance to FLDs and SLDs, molecular methods, especially DNA sequencing should be considered to be applied for all TB cases. Indeed, there is a current need for the development of rapid molecular tests that detect mutations associated with drug resistance in strains of *M. tuberculosis* with a feasible implementation in the limited resources countries.

4.3. Perspectives

This first molecular epidemiology study reflects the extent threat of TB. The data demonstrated the need to emphasize TB control in general, but also in specific areas (high risk provinces such as Northern provinces invaded by Beijing transmission and drug resistance

emergence). The data obtained will be the baseline of the families/subfamilies/genotypes of *M. tuberculosis* population and of the mutations associated with drug resistance in Lao PDR. These data could be used to explore the evolution of TB and drug resistant TB in the country by comparison with further analysis. This follow up will permit to evaluate the impact of control improvement and new strategy development set up by the NTCP. The data on mutations associated with drug resistance and the diversity of mutations will be used to complete the database of drug resistant mutations in the region (Laos, Vietnam and Cambodia). These data will be used to develop a diagnostic tool based on DNA chip technology to improve the drug resistance detection in the region. Patients, with mono- and poly-resistant strains identified during our study, who underwent for standard regimen of treatment need to be followed up and assessed for treatment outcome in order to evaluate the efficiency of standard regimen in these patients and the risk of MDR or pre-XDR emergence. This will help NTCP to make appropriate decision concerning the regimen of each patient. The strain with discordant result between phenotypic DST and sequencing, as well as uncommon mutations will be further studied by WGS in order to determine the mechanisms of resistance. These data regarding molecular epidemiology and drug resistance (mono/poly/MDR/pre-XDR) will be reported to NTCP and MoH. These kind of data are an essential source of information to guide the national making decision towards better strategy for TB control, to adapt the algorithm for diagnosis of TB and drug resistant TB, and to implement the best treatment strategies which combine the effective anti-TB drugs and the appropriate treatment according to the individual drug resistant patterns. The final goal is to limit the increase of drug resistant TB in Lao PDR. For this, the next step is to implement a molecular diagnostic test based on targeted gene sequencing to get rapidly the full drug resistant patterns and to permit a rapid clinical making decision for the determination of the appropriate treatment regimen for each patient.

ANNEX

Table S 3.1 Complete data (clinical, epidemiological, demographic and genetic data) for the 74 isolates included in Molecular analysis of drug resistance in *Mycobacterium tuberculosis* population collected during the first national anti-tuberculosis Drug Resistance Survey in Lao PDR (2016-2017) (xlsx)

The data was stored in google drive, please follow the link below:

<https://drive.google.com/drive/folders/1-lzHwzjLL5v69ER6QaELGbPt-zSXqxEB>

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