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Genetic determinants and evolution of drug resistance in Mycobacterium tuberculosis in Cambodia, high tuberculosis burden country

Sokleaph Cheng

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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Déterminants génétiques et évolution de la résistance aux antibiotiques chez *Mycobacterium tuberculosis* au Cambodge, pays à lourde charge de tuberculose

Présentée par Sokleaph CHENG
Le 30 Décembre 2020

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Déterminants génétiques et évolution de la résistance aux antibiotiques chez *Mycobacterium tuberculosis* au Cambodge, pays à lourde charge de tuberculose

RESUME EN FRANÇAIS

La tuberculose (TB) est une menace de santé publique et fait partie des maladies infectieuses les plus tueuses dans le monde. Le Cambodge se classe au 15^{ème} rang mondial parmi les 30 pays ayant le taux d'incidence le plus élevé de TB. L'émergence croissante de la TB multirésistante (MDR) et ultrarésistantes (XDR) a été observée dans ce pays. Cependant, les mécanismes sous-jacents à ces émergences sont toujours mal connus. Cette thèse vise à comprendre l'émergence, la propagation et l'évolution de la résistance aux antibiotiques chez *M. tuberculosis* (M.tb) au Cambodge.

Deux collections de M.tb, (i) 404 isolats de patients présumés MDR-TB (2012-2017) et (ii) 19 isolats d'ours provenant d'un centre de sauvegarde et un isolat d'un gardien, ont été analysées par diverses techniques : spoligotypage, MIRU VNTR, séquençage des gènes de résistance et des tests de sensibilité aux anti-TB de première (FLD) et de second ligne (SLD).

Ce travail décrit, premièrement, la diversité génétique des isolats à l'étude et leur lien avec les résistances aux FLD. Les deux familles prédominantes, Beijing et EAI, représentent près de 90% de l'échantillon. L'analyse a montré une association statistique de la famille Beijing avec la MDR, la quadruple résistance et le groupement en clusters suggérant l'existence de transmissions récentes.

Deuxièmement, en se concentrant sur la résistance aux SLD, les données suggèrent que la proportion d'isolats XDR et pré-XDR reste faible mais est en augmentation par rapport aux précédents rapports. La famille Beijing était prédominante parmi ces isolats hautement résistants. Un isolat Beijing, nommé XDR+, était résistant à tous les anti-TB testés. Un cluster comprenant 2 isolats pré-XDR et XDR a été observé, suggérant une transmission potentiellement récente de ces souches.

Troisièmement, l'étude des déterminants génétiques et l'évolution de la résistance montrent une diversité de profils allant de la mono-résistance à la XDR, une grande diversité de mutations dans chaque famille et chaque cluster et l'existence de mutations compensatoires chez certains isolats résistants. Ces résultats suggèrent diverses trajectoires évolutives vers la résistance aux FLD et SLD. La famille Beijing est également associée à la MDR et XDR et à des mutations avec un faible coût biologique dans les gènes de résistance. Nos données suggèrent un effet cumulatif des mutations, un rôle de l'épistasie dans l'acquisition de la MDR et XDR et la propagation d'isolats hautement résistants et transmissibles dans la population.

Enfin, concernant la population de M.tb d'ours et l'isolat du gardien, mes travaux montrent une transmission entre l'homme et l'animal de M.tb sensibles et résistants avec la présence de deux clusters ; un cluster EAI et un cluster Beijing résistant à la streptomycine et l'isoniazide intégrant le cas humain. Les dates d'isolation et d'exposition suggèrent une transmission probable d'ours à l'homme, puis de l'homme à l'ours.

En conclusion, ce travail apporte des connaissances sur la diversité, la structure des populations de *M.tb* et la résistance aux FLD et SLD dans notre échantillon. Ces données prédisent une évolution de la résistance vers une situation plus problématique dans le futur. La famille Beijing est associée à la MDR et à la XDR ainsi qu'à des mutations associées à de faibles coûts biologiques, générant des niveaux de résistance élevés et une transmission dans la population humaine. Deuxièmement, la proportion d'isolats XDR et pré-XDR, bien que faible est en augmentation. Cela démontre la nécessité d'interventions rapides en termes de diagnostic et de traitement pour éviter la propagation des isolats Beijing pré-XDR et XDR dans la population et l'émergence de souches de plus en plus résistantes. Ce travail illustre également que la sensibilité d'une espèce d'ours en danger d'extinction, *Helarctos malayanus*, à la TB humaine pose des problèmes en termes de conservation de l'espèce mais également de santé publique.

Les mots clés : Tuberculose, multirésistance, résistance extrême aux antibiotiques, évolution, diversité génétique, transmission récente, Beijing, EAI, réservoir animal

Genetic determinants and evolution of drug resistance in *Mycobacterium tuberculosis* in Cambodia, high tuberculosis burden country

ABSTRACT

Tuberculosis (TB) is a public health threat and is among the world's most lethal infectious diseases. Globally, Cambodia ranks 15th among the 30 countries with the highest TB incidence rate. The increasing emergence of MDR-TB as well as pre-XDR and XDR-TB has been observed in this country. However, the mechanisms underlying this escalation of resistance are still poorly understood. In this context, this thesis aims to understand the emergence, spread and evolution of antibiotic resistance in *Mycobacterium tuberculosis* (M.tb) in Cambodia.

Two collections of M.tb are included in the study: (i) 404 isolates from MDR-TB presumptive patients between 2012 and 2017, (ii) 19 isolates from bears from a wildlife rescue center and one isolate from a staff member. The isolates were analyzed by two techniques: spoligotyping, 24-MIRU-VNTR, sequencing of drug resistance genes and drug susceptibility testing for first (FLD) and second line (SLD) drugs.

First, this work describes the genetic diversity of the clinical M.tb isolates and their link with FLD resistance. The two predominant families, Beijing and EAI, represent almost 90% of the sample. The analysis showed a significant association between the Beijing family and phenotypic drug resistance, in particular with MDR and quadruple resistance. The clustering suggests the existence of recent transmissions also associated with the Beijing family.

Second, while focusing on resistance to SLD, the data suggest that the proportion of XDR and pre-XDR isolates remains low but is on the rise compared to previous reports. The Beijing family was predominant among these highly resistant isolates. One Beijing isolate, named XDR+, was resistant to all anti-TB drugs tested (FLD and SLD). A cluster comprising 2 pre-XDR and XDR isolates was observed, suggesting recent transmission of these strains.

Third, the genetic determinants of resistance to FLD and SLD and the evolution of resistance were studied. The data demonstrated a diversity of drug resistant patterns from mono-resistance to XDR, an important diversity of mutation patterns in each M.tb family and each cluster and the existence of compensatory mutations in some resistant isolates. These results suggest various evolutionary trajectories towards resistance to FLD and SLD. The Beijing family was also associated with MDR and XDR and low fitness cost mutations in resistant genes. Our data suggest a cumulative effect of mutations, a role of epistasis in the acquisition of multiple resistance and the spread of highly resistant and transmissible isolates in the population.

Finally, concerning the M.tb in bear population and the staff isolate, my work has confirmed a human-animal transmission of M.tb sensitive and resistant with the presence of two clusters; EAI cluster and Beijing streptomycin and isoniazid resistant cluster including the human case. The timing and exposure indicated probable transmission from bear to human and back to bear.

In conclusion, this work provides knowledge on the diversity, population structure of M.tb and resistance to FLD and SLD in our sample collected from MDR-TB presumptive patients between 2012 and 2017. These data predict an evolution of resistance to a more problematic situation in the future. First, the Beijing family is associated with MDR and XDR as well as mutations associated with high level of resistance and low fitness cost and recent transmission. Second, the proportion of XDR and pre-XDR isolates remains low but appears to be increasing over time. This study strongly indicates the need for rapid interventions in terms of diagnostic and treatment to prevent the spread of the Beijing pre-XDR and XDR isolates in the population and the emergence of more resistant strains. This work also illustrates that the susceptibility of the endangered species, *Helarctos malayanus* sun bear, to human TB in particular to resistant TB poses problems in terms of conservation of the species but also of public health.

Keywords: Tuberculosis, multi-drug resistance, extensively drug resistance, evolution, genetic diversity, recent transmission, Beijing, EAI, animal reservoirs

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ACRONYMS AND ABBREVIATIONS

Abbreviations

AFB
BCG
Bdq
bp
CENAT
Cfz
Cm
COR
Cs
DIm
DNA
DOTS
DR
DS
DST
EAI
EMB
EP
Eto
FDC
FLD
FQ
Gfx
HBC
HC
HGDI
HIV
IGRA
INH
IPC
IPM-CLN
Km
LAM
LAMP
LED-FM
Lfx
LPA
LTBI

Full words

Acid-fast bacilli
Bacille Calmette-Guérin
Bedaquiline
Base pair
National Center for Tuberculosis and Leprosy Control
Clofazimine
Capreomycin
Crude odds ratios
Cycloserine
Delamanid
Deoxyribonucleic acid
Directly observed treatment-short course
drug-resistant
Drug susceptible
Drug-susceptibility testing
East African-Indian
Ethambutol
Efflux pumps
Ethionamide
Fixed-dose combination
First Line anti-TB drugs
Fluoroquinolones
Gatifloxacin
High burden countries
Health centers
Hunter-Gaston-Discriminatory Index
Human immunodeficiency virus
Interferon-gamma release assay
Isoniazid
Institut Pasteur du Cambodge
Imipenem-cilastatin
Kanamycin
Latin-American-Mediterranean
Loop-mediated isothermal amplification
Light-emitting diode fluorescence microscopy
Levofloxacin
Line-probe assays
Latent TB infection

Lzd	Linezolid
MDR	Multi-drug resistant
Mfx	Moxifloxacin
MIC	Minimal inhibitory concentrations
MIRU	Mycobacterial interspersed repetitive units
MPM	Meropenem
MSG	Millennium Development Goals
MTBC	<i>Mycobacterium tuberculosis</i> complex
NDRS	National Drug Resistant Survey
NGO	Non-governmental organization
NGS	Next Generation Sequencing
NRL	National TB Reference Laboratory
NTM	Nontuberculous mycobacteria
NTP	National tuberculosis control programme
OD	Operational districts
Ofx	Ofloxacin
OR	Odds ratio
PAS	P-aminosalicylic acid
PCR	Polymerase chain reaction
pDST	Phenotypic drug-susceptibility testing
PLHIV	People living with HIV
PMDT	Programmatic Management of Drug Resistant Tuberculosis
PTB	Pulmonary tuberculosis
Pto	Prothionamide
PZA	Pyrazinamide
QRDR	Quinolone resistance-determining region
RIF	Rifampicin
RNA	Ribonucleic acid
RR	Rifampicin resistance
RRDR	Rifampicin resistance-determining region
SDG	Sustainable Development Goals
SLD	Second-line drugs
SLID	Second-line injectable drugs
SNP	Single-nucleotide polymorphisms
STM	Streptomycin
STR	Shorter treatment regimen
TB	Tuberculosis
TDR	Totally drug resistant
TPT	Tuberculosis preventive treatment
Trd	Terizidone
TST	Tuberculin skin test
VNTR	Variable number tandem repeat

WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug resistant
ZN	Ziehl-Neelsen

Box 1: Definition of drug resistant categories

Drug-resistant (DR):	Resistance to any anti-TB drug.
Mono-resistant (Mono-R):	Resistance to one first-line anti-TB drug only.
Polydrug-resistant (Poly-R):	Resistance to more than one first-line anti-TB drug, other than both isoniazid (INH) and rifampicin (RIF).
Rifampicin-resistant (RR):	Resistance to RIF detected using Xpert [®] MTB/RIF, without further testing for INH resistance.
Multidrug-resistant (MDR):	Resistance to at least two of the most effective anti-TB drugs, INH and RIF.
Non-MDR:	Resistance to one or more FLDs but not to INH and RIF at the same time.
Second-line injectable drugs (SLIDs) resistant:	Resistance to at least one of three SLIDs (Amikacin, Kanamycin, and Capreomycin).
Extensively drug-resistant TB (XDR-TB):	MDR-TB resistance to any fluoroquinolones (FQs) and SLIDs simultaneously.
Pre-extensively drug-resistant TB (pre-XDR-TB):	MDR-TB resistance to either any FQ or SLIDs.

Chapter I: INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1. Introduction and objectives

Tuberculosis (TB) is one of the world's major public-health threats and ranks as the top infectious disease causing deaths worldwide. In 2019, 10 million people are estimated to have fallen ill with TB⁽¹⁾. In the same year, TB was responsible for 1.4 million deaths globally, including 208 000 deaths among human immunodeficiency virus (HIV)-positive individuals⁽¹⁾. Besides, antibiotic resistance is a growing threat and complicates the control of this disease with the worrying increase of multi-drug resistant TB (MDR-TB) and the emergence of extensively and totally drug resistant TB (XDR- and TDR-TB). This disease requires at least 6 months of antibiotic therapy in combination to prevent resistance and get healing. Despite drug strategies increasingly complex, we are facing the emergence of more and more resistant strains. The mechanisms and pathways that result in the emergence and subsequent fixation of MDR and XDR *Mycobacterium tuberculosis* (M.tb) in the population are essential to investigate in order to stop and prevent this escalation.

Despite the great achievement in TB control by successfully achieved Millennium Development Goal by the end of 2015, Cambodia with 16 million population is still one of the 30 countries with high burden of TB in the world⁽¹⁾. The incidence of TB is 287 cases per 100 000 population, and the mortality rate is 17 per 100 000 population⁽¹⁾. Although MDR-TB has not been a significant problem in the past, recent data show a 1.8% and 5.0% increase in MDR among new cases and retreatment TB cases, respectively^(2,3). It is therefore essential to study and understand the molecular mechanisms underlying this escalation of resistance in Cambodia where, up to now, little information is available. It is all the more important that its regional neighbors Vietnam, Thailand and Myanmar are among World Health Organization (WHO) designated highest burden countries for MDR-TB, indicating the need for innovative regional approaches to address the epidemic⁽²⁾. The acquisition of knowledge in Cambodia will help improve the diagnosis and treatment of both drug-susceptible and drug-resistant (DR) TB and prevent a further increase in the burden of MDR- and XDR-TB.

The knowledge of mechanisms and evolution of drug resistance are essential for the development of effective control strategies in terms of diagnostic and treatment but also for the implementation of new diagnostics or drug targets. In this framework, the overall objective of this thesis is to understand the emergence, spread and mechanisms of antibiotic resistance in M.tb in Cambodia by a genetic epidemiology approach. Two collection of M.tb isolates were included in this work; sampling 1 composed of clinical isolates from presumptive MDR-TB patients and sampling 2 contained isolates from sun bears and a staff member at one wildlife rescue center in Cambodia.

The specific objectives are as follows:

1. Inventory of the diversity of drug resistance in Cambodia from mono resistance to XDR patterns.
2. Characterization of the genetic determinants responsible for FLD and second line drug (SLD) resistance in M.tb.
3. Investigation of compensatory mechanisms linked to rifampicin (RIF) and isoniazid (INH) resistance in M.tb population.
4. Genetic characterization of M.tb isolates using standard methods of reference and study of the association between the genetic background and the FLD and SLD resistance patterns.
5. Study of the evolution of antibiotic resistance in M.tb in Cambodia

2. Literature review

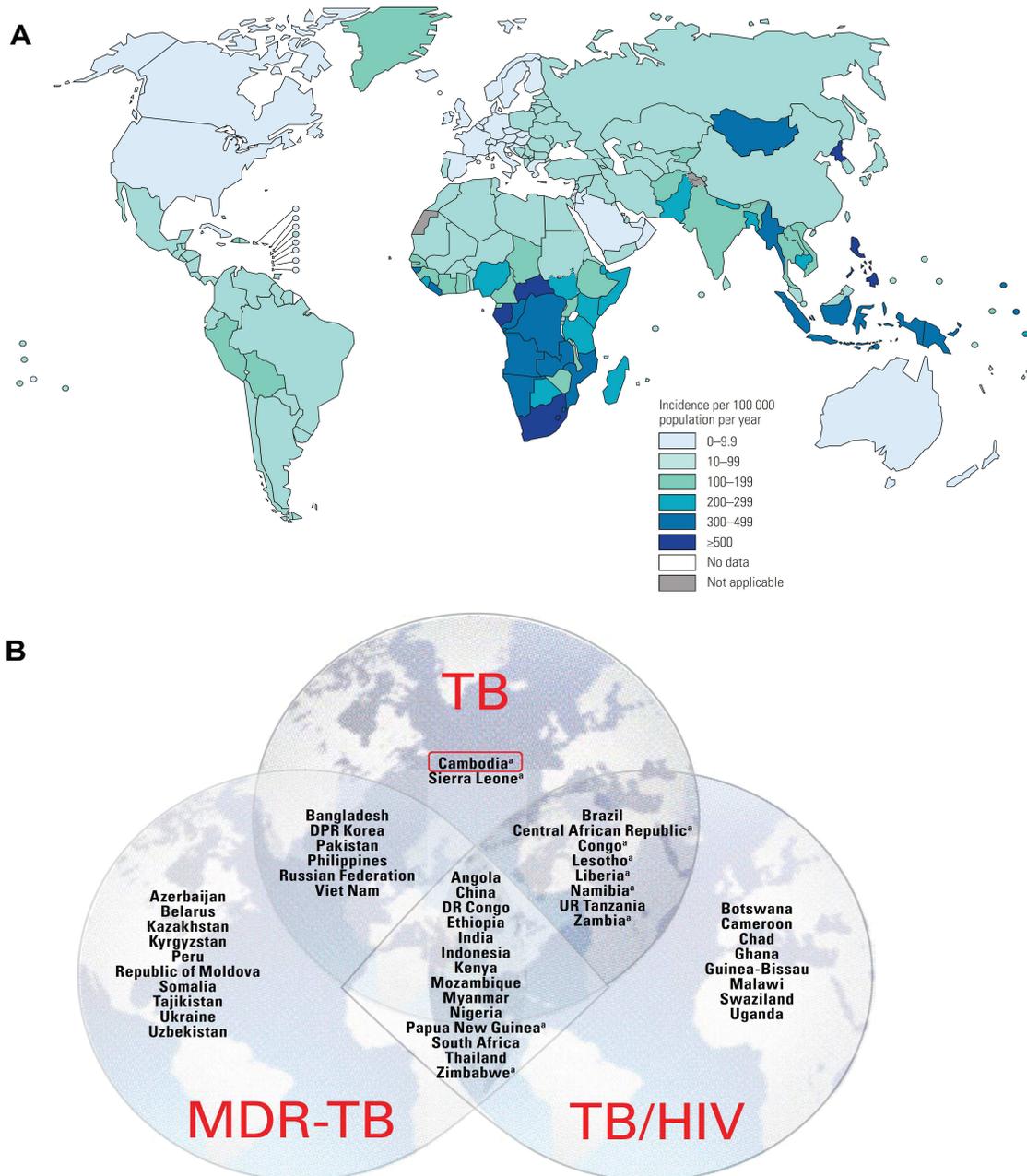
2.1. Tuberculosis epidemiological situation

2.1.1. Global incidence of tuberculosis

Tuberculosis is still today a major public health threat despite being a curable disease. It has been a global health problem since ancient times. The disease, historically called “White plague” or “Consumption”, would have emerged in humans about 70 000 years ago ⁽⁴⁾. Its causative agent, M.tb, has killed more people than any other microbial pathogens (above HIV/AIDS) ^(1, 5). Due to its long history, it is estimated that about one quarter of the world population is infected with M.tb - 1.7 billion people in total, and are thus at risk of developing active TB disease during their lifetime (1). In 2019, the latest figures from the WHO indicate an estimated 10.0 million people, equivalent to 132 cases per 100 000 population, developed TB disease: 5.6 million men, 3.2 million women and 1.2 million children ⁽¹⁾. 8.2% all those affected were people living with HIV. The five main attributable risk factors of developing TB disease in 2019 were undernutrition (2.2 million), HIV infection (0.76 million), alcohol abuse (0.72 million), smoking particularly among men (0.70 million), and diabetes (0.35 million), indicating the essential need of multisectoral action to address these factors ⁽¹⁾. As the leading cause of death from a single infectious agent since 2007, TB killed in total 1.4 million people, including 208 000 deaths among people living with HIV (PLHIV) in 2019. 85% of TB deaths occurred in the WHO African and South-East Asia regions; India alone account for 31% of TB death ⁽²⁾.

The TB epidemic varies greatly between countries and regions (Figure I-1A). Most of the TB cases occurred in the WHO South-East Asia Region (44%), followed by the WHO African Region (25%) and the WHO Western Pacific Region (18%) ⁽¹⁾. Smaller proportion of cases occurred in the WHO Eastern Mediterranean Region (8.2%), Region of the Americas (2.9%) and European Region (2.5%). Ninety percent of all cases were found in the WHO high burden countries (HBC) - for TB, MDR-TB and TB/HIV co-infection, 30 countries for each category - based on the absolute number of estimated

incident cases (20 countries) and incidence rates per capita (additional 10 countries) (Figure I-1B). Eight of the 30 TB HBC accounted for two third of all TB cases were India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%). Cambodia was among the HBC for TB, but not for MDR-TB nor TB/HIV, according to its high incidence rates per 10 000 population, ranked 17th of the 30 countries. However, among its 10 brotherhood countries in the Association of Southeast Asian Nations (ASEAN) community, five countries (Indonesia, Myanmar, Thailand, Philippines and Viet Nam) were also among the HBC; all for TB as well as MDR-TB and three of them (in bold) were also HBC for TB/HIV (Figure I-1B).



^a Countries that are included in the list of 30 high TB burden countries on the basis of the severity of their TB burden in terms of incidence rates per capita.

Source: Adapted from WHO, 2020 (1).

(A) Estimated TB incidence rates per 100 000 population in the world;
(B) Countries with high-burden for TB, TB/HIV and MDR-TB defined by WHO for 2016-2020.

Figure I-1. Global estimated TB burden in 2019.

Although the disease burden caused by TB is falling globally and the Millennium Development Goals (MDGs) target to halt and reverse TB incidence has been achieved in 2015 on a worldwide basis, TB is still listed as a major health challenge in the Sustainable Development Goals (SDGs) as stated in Goal 3.3; one of which is to end the global TB epidemic by 2030 ⁽⁶⁾. The End TB Strategy was adopted by the WHO member states to end TB epidemic and progress towards targets set in the SDGs: a 90% reduction in TB deaths, an 80% reduction in the TB incidence rate and no costs faced by TB patients and their households by 2030, compared with levels in 2015. However, with the current rate of progress with only about 2% and 3% annual rate of decline in TB incidence rate and number of TB death, respectively, and the emerging new challenges including increase of MDR-TB, a large number of missing cases (only 7.1 million cases reported in 2019), and global migration, the SDG and End TB Strategy targets set for 2030 cannot be met without stepping up research and development in the field of vaccines, drugs and treatment regimes, and point-of-care testing ^(1,6). In addition, with the 2020 COVID-19 pandemic, causing enormous health, social and economic impacts, might have negative effect on the TB epidemic, people with TB and threaten to seriously undermine recent progress towards global TB targets by 2030 ⁽¹⁾.

2.1.2. Tuberculosis in children

Millions of children are exposed to TB each year, many of which become infected with M.tb. The risks of progression to TB disease after infection are high among children less than five years old, children with HIV infection, children with severe malnutrition and children that live in the same household as a person who has been recently diagnosed with smear positive TB. In 2019, WHO estimates that 1.19 million children under 15 years old suffer from TB worldwide, representing about 12% of annual TB cases. 30% and 48% of the cases were occurred in the WHO African and WHO South-East Asia regions, respectively, and approximately 230 000 children died, accounting for about 14% of total TB deaths ⁽¹⁾. The higher rate of TB deaths than TB burden among children suggests the poorer access to diagnosis and treatment.

Estimation of the burden of TB disease in children remains particularly challenging due to the inconsistent diagnostic criteria and investigations for childhood TB disease ⁽¹⁾. Childhood TB is often missed or overlooked due to non-specific symptoms and difficulties in diagnosis. Bacteriological diagnosing TB in children is a challenge due to the paucibacillary nature of the disease and the difficulty in obtaining expectorated sputum from children ⁽⁷⁾. In order to improve case notification, WHO recommended the use of GeneXpert MTB/RIF for TB diagnosis in children using easy-to-collected sample such as stool, expansion of access to chest radiography, and building capacity in the clinical diagnosis of TB presumptive children with negative bacteriological results or do not have access to bacteriological testing ^(1,8). Vulnerable children (e.g. those with pneumonia, malnutrition or HIV) need more attention.

2.1.3. Tuberculosis in people living with HIV

Tuberculosis is a major cause of death in persons infected with human immunodeficiency virus (HIV), especially in resource-limited settings where the ability to diagnose, treat, and control either disease may be limited⁽⁹⁾. When a patient has both HIV and TB each disease speeds up the progress of the other⁽¹⁰⁾. HIV, through immune suppression, is one of the most important factors contributing to the increased incidence of TB globally⁽¹¹⁾ due to either primary infection⁽¹²⁾, re-infection⁽¹³⁾ or progression from latent infection to active TB disease (reactivation)^(14, 15). The risk of progressing from latent to active TB is estimated to be between 15 and 21 times greater in people living with HIV than among those without HIV infection⁽¹⁾. Worldwide, an estimated 8.2% (815 000) incident TB cases in 2019 were infected with HIV and the majority of the case occurred in the African Region, the proportion exceeding 50% in some parts of southern Africa⁽¹⁾. The incidence of TB expressed per 100 person-years with HIV was 2.1%⁽¹⁾.

Among both HIV-infected adults and children, TB remains a leading cause of death and is the most common opportunistic infection, despite improved access to antiretroviral therapy (ART). In 2019, TB killed 208 000 people living with HIV (PLHIV) and 81.3% (169 000) of deaths were occurred in the WHO African region. Globally, the number of TB deaths among PLHIV has fallen by nearly 70% between 2000 and 2019 but still there were approximately 570 people with HIV die from TB each day including many who are receiving antiretroviral therapy⁽¹⁾. PLHIV also face the threat of DR-TB. If diagnosis is delayed there is increased risk of mortality from MDR and XDR-TB.

The clinical presentation and course of active TB in HIV infected persons are altered, particularly in those with advanced immunosuppression (CD4 counts <200 cells mm⁻³): active pulmonary TB can initially be asymptomatic; extrapulmonary TB is more common; the clinical course is accelerated; it is more difficult to diagnose; and mortality is particularly higher⁽¹⁶⁾. Early initiation of ART was shown to improve survival of people living with HIV, reduces the incidence of TB and delays the progression of HIV⁽¹⁷⁾. The WHO recommend starting HIV antiretroviral therapy (ART) between 2 and 8 weeks after starting TB treatment for HIV-infected individuals with CD4+ T-cell counts of less than 200 per mm³⁽¹⁷⁾. TB preventive treatment (TPT) substantially reduces the risk for progression of latent TB infection to active disease and death among patients with HIV infection and is also recommended by the WHO, but only 50% of those who are eligible to receive this therapy are actually treated, ranged from less than 1% in Thailand to 89% in Zimbabwe⁽¹⁾.

Diagnosis of active TB among HIV-infected persons still remains difficult because HIV patients have higher rates of sputum smear-negative disease. Moreover, the detection of TB in HIV infection is made more difficult by the higher rates of extrapulmonary disease and the need to distinguish TB from other opportunistic infections⁽¹⁸⁾. Besides, nontuberculous mycobacteria (NTM) are also common opportunistic infections in adult living with HIV⁽¹⁹⁾. In the 1980s, *Mycobacterium avium* complex (MAC) was identified as an important pathogen in patient with AIDS, highlighting the risk of these environmental organisms within the severely immunocompromised host⁽¹⁹⁾. WHO recommends that routine HIV testing should be offered to all patients with presumptive and diagnosed TB and

routine screening for TB symptoms is essential for all PLHIV. The use of rapid diagnostics such as Xpert MTB/RIF and lateral flow urine lipoarabinomannan (LF-LAM) assay are crucial for fast-tracking early diagnosis and treatment initiation ⁽⁷⁾. Diagnosis of TB in HIV-infected Children is even more challenged due to the low specificity of clinical and radiological features and the low sensitivity of tuberculin skin tests that are used in various scores and diagnostic approaches ⁽⁷⁾. Marcy *et al.* reported that Xpert performed on the combination of nasopharyngeal aspirate and stool sample is a promising diagnosis method for rapid confirmation of TB in HIV-infected children ⁽⁷⁾.

Prevalence of NTM infection in HIV-infected children: the PAANTHER Study

(Appendix 1. Article 1: Isolation of Nontuberculous Mycobacteria in Southeast Asian and African Human Immunodeficiency Virus-infected Children With Suspected Tuberculosis)

The disseminated NTM disease also occurs more frequently in HIV-infected patients once the CD4 cell count is < 50 cells/ μ L, indicating another challenge in diagnosis and management of mycobacteria infection in HIV-infected patients ⁽²⁰⁾. In collaboration with the Pediatric Asian African Network for Tuberculosis and HIV Research (PAANTHER) Study Group in a prospective cohort (ANRS 12229 PAANTHER 01 study, ClinicalTrials.gov identifier NCT01331811) followed in 4 countries with high and very high TB burden in Southeast Asia (Cambodia and Vietnam) and Africa (Burkina Faso and Cameroon) we assessed the rate of NTM isolation in HIV-infected children with a clinical suspicion of TB, as well as NTM species distribution and factors associated with NTM isolation (Appendix 1) ⁽²¹⁾. From April 2010 to May 2014, 427 HIV-infected children (median age, 7.3 years) were enrolled, of them, 59.2% were severely immunodeficient with suspected TB. NTM were isolated in 46 children (10.8%) and 45.7% of isolates were *Mycobacterium avium* complex (MAC). Compared with African origin, Children with Southeast Asian origin, age between 5 and 9 years, and severe immunodeficiency were independently associated with NTM isolation ⁽²¹⁾. These results suggest that the high rate of NTM among HIV children in a settings of high TB burden is making TB diagnosis even more challenging as NTM can also be detected by smear microscopy, and clinical and radiological features may mimic TB. Moreover, there is also a major challenge with diagnosis and management of NTM in HIV-infected children in high TB burden countries, as currently there is no criteria to help differentiate environmental contamination, or colonization, from disease. Further studies should be conducted in settings of high TB and NTM burden to assess clinical significance of NTM isolation in HIV-infected children and develop guidelines on NTM pulmonary disease diagnosis and treatment for this population ⁽²¹⁾.

2.1.4. Zoonotic tuberculosis caused by *Mycobacterium tuberculosis*

TB is a significant disease for both humans and animals. The *Mycobacterium tuberculosis* complex (MTBC), causative agent of TB, composed of several highly genetically related species that can affect humans and animals ⁽²²⁾. At least, nine lineages of the MTBC has been reported to infect a variety of

wild and domesticated animals ⁽²³⁾. These include *M. microti* (a pathogen of voles), *M. pinnipedii* (seals and sea lions), *M. orygis* (antelopes), *M. mungi* (mongooses), *M. suricattae* (meerkats), the “chimpanzee bacillus” (chimpanzees), the “dassie bacillus” (rock hyrax), *M. bovis* and *M. caprae* (domesticated cattle and goats and several wild animal species) ^(22, 24-30). The human-adapted MTBC lineages comprise *M. tuberculosis sensu stricto* (ie, MTBC lineages 1-4 and lineage 7) and *M. africanum* (i.e., MTBC lineages 5 and 6) ⁽²³⁾. Historically, zoonotic TB has been associated with *M. bovis* and remains an ongoing threat for humans despite the declined incidence and eradication in many countries. In 2019, WHO estimated a global incidence and mortality of 140 000 cases and 11 400 cases, respectively. More than 90% of the animal cases occurred in Africa, south-east Asia and Western pacific regions which account for 49.2%, 31.0% and 12.9%, respectively ⁽¹⁾. However, M.tb infections have been reported in a wide range of animal species including captive wildlife and pets ⁽³¹⁾. These animal species include free-ranging Asian and African elephants, cattle, chimpanzees, black rhinoceros, Rocky mountain goats, Lar gibbons, giraffes, tapirs, various non-domestic ungulates, seals, various non-domestic carnivores, dogs, cats, guinea pigs, rabbits, cattle and birds ⁽³²⁾. In addition, the animal-to-animal transmission has also been reported ⁽³¹⁾. The M.tb infection in animal has presented an impact of human diseases on animal ⁽³³⁾, in contrast, it is now emerging that animals could be a potential source of M.tb infection for humans. There are now several reports of transmission of M.tb from animals to humans ^(32, 34). Although the relative incidence is low, if no action taken, it could become an important occupational and public health concern.

2.1.5. Drug resistant TB: The global extent of the problem

The increase of antibiotic resistance in M.tb has posed a threat to the present global TB control measures as well as global Antimicrobial Resistance (AMR) challenge as more than 30% of AMR related deaths occurred among MDR-TB patients ⁽³⁵⁾. Globally, MDR-TB amounts to approximately 600,000 incident cases each year, challenging the prospect of ending TB by 2035 ⁽¹⁾.

There are three main categories of DR-TB: Rifampicin-resistant TB (RR-TB), multidrug-resistant TB (RR/MDR-TB) and extensively drug resistant TB (XDR-TB) (See definition in Box 1). Both MDR- and XDR-TB have become more prevalent in the last 25-30 years and pose formidable challenges in diagnosis and treatment. In 2019, there were approximately 465,000 people had RR-TB, of with 78% (362,000) developed MDR-TB, and 182,000 (39.1%) people died ⁽¹⁾. Three countries among WHO HBC list for MDR-TB accounted for 50% of the global RR-TB/MDR-TB cases: India (27%), China (14%) and the Russian Federation (8%) (Figure I-2). Globally, 3.3% of new and 18% of previously treated cases had RR/MDR-TB; in several countries of the former Soviet Union, the proportions of RR/MDR-TB were above 20% in new cases and above 50% in previously treated cases. An estimated 1.4 million cases (13.1% of new and 17.4% of previously treated cases) had INH resistance, of which, 1.1 million were susceptible to RIF ⁽¹⁾. The majority of people with INH-resistant TB were missed to diagnose, especially in settings where diagnostic algorithms prioritize the detection of RIF resistance, and do not receive

the recommended modified treatment regimen. In addition, an estimated 20.1% of people with MDR-TB harbor pre-XDR strains with additional resistance to any fluoroquinolone (FQ), including ofloxacin (Ofx), levofloxacin (Lfx) and moxifloxacin (Mfx) ⁽¹⁾. In 2019, a total of 12,350 cases of XDR-TB were reported, with 69.3% and 19.8% of cases being from the WHO European and South-East Asia region, respectively ⁽¹⁾.



Source: WHO, 2020 ⁽¹⁾.

Figure I-2. Estimated incidence of MDR/RR-TB for countries with at least 1000 incident cases in 2019.

Detection of MDR-TB remains a great challenge. MDR/RR-TB diagnosis requires detection of M.tb and its resistance to anti-TB drug using culture and drug susceptibility testing (DST) methods, rapid molecular tests or sequencing. Despite of the increasing use of the rapid molecular assay, Xpert® MTB/RIF assay for detection of M.tb and its resistance to RIF, only 206,030 MDR-/RR-TB cases, equivalent to 44.3% of the estimated cases, were detected and notified in 2019 and 177,099 cases (85.9% of notified cases and 38.8% of estimated cases) started MDR-TB treatment ⁽¹⁾. In addition, apart from RIF, coverage of other anti-TB drugs remains low. Undiagnosed, untreated or incorrectly treated patients with MDR-TB leads to further transmission of DR-TB. To curb this spread, there is urgent need to strengthen the laboratory capacities and improve the coverage of diagnosis, treatment and care for people with DR-TB ⁽³⁶⁾.

MDR-TB requires more complex treatment than drug-susceptible TB as it is caused by bacteria that do not respond to the two most powerful anti-TB drugs: INH and RIF. Both MDR and RR-TB need to treat for 9 to 20 months using SLD, which are typically less effective, more expensive, and more toxic compared to FLD ⁽³⁷⁾. By contrast, individuals with XDR-TB are unresponsive to even the most

powerful SLD. As reported by WHO, the treatment success rate were 57% for MDR-/RR-TB and 39% for XDR-TB ^(1,36). In addition, more extreme drug resistance patterns to all available FLD and SLD drugs have already been reported in several countries around the world. The terms are not yet recognized by the WHO ⁽³⁸⁻⁴⁰⁾ but Totally drug-resistant TB (TDR) ⁽³⁹⁾ also known as extremely drug-resistant (XXDR) by Migliori *et al.* ⁽⁴¹⁾, have been reported and were untreatable with currently available drugs. This suggests the need to develop urgently new drugs and correctly apply the existing policies and strategies of TB and MDR-TB control programme ⁽³⁹⁻⁴¹⁾.

2.2. Natural course of tuberculosis infection and disease development

Although the TB disease was known for many centuries to be a major cause of illness called “White plague” or “Consumption” and death, its causative agent remained unknown until Robert Koch who discovered the germs, later known as Koch’s tubercle bacillus or M.tb, in stained sputum from patients with the disease in 1882 ⁽⁴²⁾. The microorganism is a slow-growing acid-fast rod-shaped bacilli, with a replication time of approximately 20 hours, shielded by a unique and robust wax-rich cell wall. These special characteristics allow the bacilli to survive in host phagocytes and forms the basis for the chronic nature of infection and disease. They complicate the microbiological diagnosis and lead to long-term drug treatment ⁽⁴³⁾.

M.tb is an obligate pathogen normally acquired through respiratory tract ⁽⁴⁴⁾. The lung represents both the main port of entry and an important site of disease manifestation (>90%), although TB can manifest itself at any tissue site and form extrapulmonary TB ⁽⁴³⁾. The disease is spread by aerosolization of droplet nuclei containing M.tb particles expelled by individuals with active pulmonary or laryngeal M.tb. The infection is established in approximately one-third of individuals exposed to the tubercle bacillus ^(45, 46). Once the particles, usually 1-5 µm in diameter, are inhaled and phagocytosed by alveolar macrophages, a vigorous host cellular immune response involving type I interferon responses, chemokines, and cytokines occur and induce bacterial containment in small granulomatous lesions of the lung ^(47, 48). A dynamic balance between bacterial persistence and host defense develops and its outcomes are highly variable, ranging from rapid clearing by the innate immune response to lifelong latent infection ⁽⁴³⁾. Most patients, particularly immunocompetent individuals, with latent TB infection (LTBI) (>90%) never develop clinical diseases as their prominent immune responses contribute substantially to bacterial containment and limit infection to the primary site of invasion, the lung parenchyma and the local draining lymph nodes (“Ghon complex”) ^(49, 50). The remaining 5-10% of patients with latent TB infections develop TB disease later in life, typically pulmonary TB, due either to reactivation of existing infection within months or decades after a long latency period or secondary reinfection ^(43, 45). If remains untreated, the active disease is fatal in 50% of patient. In immunologically incompetent individuals, such as newborns, the aged and immunosuppressed persons, the primary infection habitually progresses into disease as a continuous process within months or so after infection, rather than the normal years to decades ⁽⁴³⁾.

Once the disease progresses, cavitory lesions develop and continual bacillary replicated in the caseous detritus. When cavitation reaches the alveoli, the patient becomes infectious, and then, the infection circle continues (an infectious individual might infect 3-10 people per year) ^(43, 51).

Pathology of TB disease in humans varies considerably, ranging from asymptomatic infection to a life-threatening disease ⁽⁵²⁾. Patients having LTBI is asymptomatic and non-transmissible, but when the disease progresses, the clinical manifestation is variable in regard to severity, duration, therapeutic response, and tissue tropism ⁽⁴⁹⁾. The progression and nature of disease are influenced by a number of factors including bacteria genetic variation and intrinsic host susceptibility or comorbid conditions that weaken the host immune system, such as HIV co-infection, poorly controlled diabetes mellitus, renal failure, chemotherapy and malnutrition ^(53, 54). Typically in immune-incompetent individuals, the infection can disseminate via lymphatics or the blood stream to lymph nodes and diverse other organs that can produce a spectrum of clinical manifestations ranging from disseminated TB, meningitis, miliary TB and extra pulmonary granuloma ⁽⁵⁵⁾.

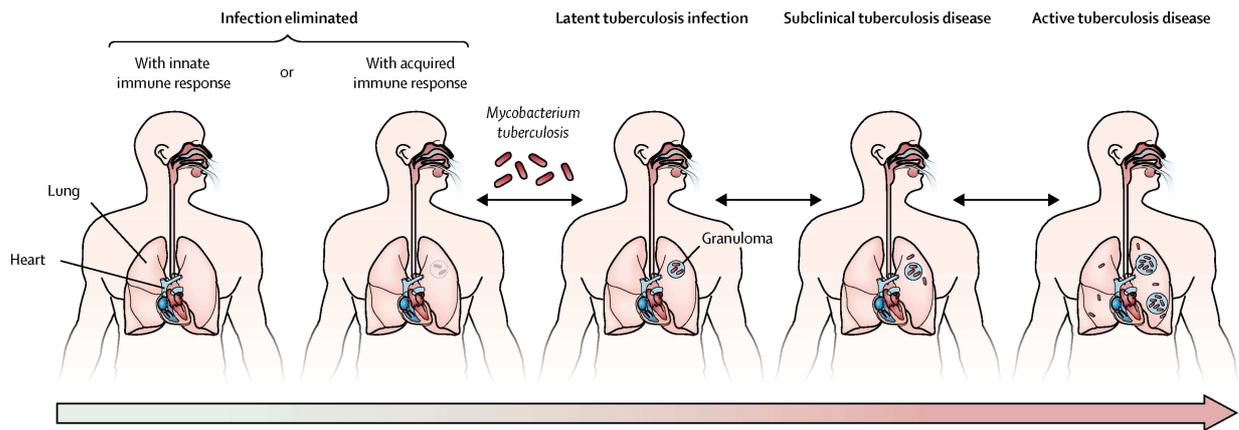
2.3. Diagnosis of active TB and LTBI: WHO-recommended diagnostic techniques

Timely access to accurate TB diagnostics and drug susceptibility tests enables TB programme to identify TB cases, select appropriate treatment and interrupt transmission. Hence, the WHO's End TB Strategy prioritizes the early diagnosis of TB and universal DST, highlighting the critical role of laboratories for rapidly and accurately detecting TB and drug resistance. Although multiple advances have been made in TB diagnosis, no reliable, simple, point-of care test exists to definitively diagnose the disease and access to these innovations, remaining a challenge for the majority of people living with the disease ⁽⁵⁶⁾. In 2017, 2.8 million TB cases were either not diagnosed, or not notified to the national tuberculosis programme (NTP) and only 56% of 5.5 million pulmonary TB (PTB) patients were bacteriologically confirmed with a WHO-recommended test including GeneXpert[®] MTB/RIF. The rest was likely managed on the basis of clinical suspicion or non-specific tests (e.g. chest x-rays) ⁽³⁶⁾.

M.tb infection is acquired by inhalation of infectious aerosol particles released from close contacts. It has variable outcomes in different hosts (**Figure I-3**). Only about 12% of the infections lead to symptomatic or active TB disease ⁽⁵⁷⁾. The diagnosis of active TB aims to detect M.tb bacilli in specimens from the respiratory tract (pulmonary TB) or from other body sites (extrapulmonary TB).

A majority of infected individuals, through innate or adaptive immune function, has completely cleared the invading bacilli or inhibited the growth of M.tb, resulting in the bacteria becoming dormant; this condition is often referred to as LTBI. Persons with LTBI do not present symptoms and do not transmit the disease to others but may develop it in the near or remote future, a process called TB reactivation. About 5 - 10% of LTBI cases are at risk to progressing from infection to active TB ^(36, 58, 59). Since one fourth of the world population have latent infection, the development of new diagnostics and screening tools for LTBI has become necessary in order to control the disease. Currently, Interferon-gamma release assays (IGRAs) are used to diagnose LTBI, although the

tuberculin skin test (TST) still remains the most cost-effective test. Both method work by measuring memory T-cell response to M.tb antigens ⁽⁵⁸⁾.



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Source: Furin et al., 2019 ⁽⁵⁶⁾.

Figure I 3. Spectrum of TB infection and disease and expected outcome of diagnosis tests

2.3.1. Sputum smear microscopy

Sputum smear microscopy, a technique developed more than 100 years ago, plays an important role in the early laboratory diagnosis of TB as it is often the only available diagnostic method in resource-limited countries ⁽⁶⁰⁾. This technique is based on the high lipid containing cell walls of mycobacteria which makes them resistant to decolorization by acid-alcohol after the primary staining. Because of this characteristic, all members of *Mycobacterium* spp., not only M.tb, are referred to as acid-fast bacilli (AFB). To determine that a clinical specimen contains AFB, a very thin layer of the sample is placed on a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye. Currently, two types of acid-fast staining method are used:

- Carbol-fuchsin staining (Ziehl-Neelsen or ZN method and its modification, Kinyoun cold staining, performed without heating the dye) examined with conventional light microscopy;
- Fluorochrome staining (auramine or auramine-rhodamine) and fluorochrome staining (Auramine) examined with fluorescence microscopy or Light-emitting diode fluorescence microscopy (LED-FM).

Smear microscopy is simple, rapid, inexpensive and efficient in detecting the most infectious cases of pulmonary TB. It is also necessary for treatment follow up of patients with susceptible TB.

A major limitation of smear microscopy is its low sensitivity (variable between 20-80%) and the high number of AFB required for positive result (5000 bacilli per ml of sample). The sensitivity is significantly reduced in patients with extrapulmonary TB, children and in TB/HIV co-infected patient ⁽⁶¹⁾. The performance of smear microscopy is also influenced by numerous factors including the disease prevalence, the type and quality of specimens, the number of mycobacteria in the sample and the quality of the smear preparation, staining and reading process. Furthermore, microscopy for AFB cannot distinguish MTBC from nontuberculous mycobacteria, viable from nonviable organisms and drug-susceptible strains from DR strains ⁽⁶²⁾. Fluorescence microscopy is more sensitive (10%) than conventional ZN microscopy, and smear examination takes less time ⁽⁶³⁾. Since 2009, WHO recommended to use LED fluorescence microscopy as an alternative to conventional ZN light microscopy ⁽⁶¹⁾.

2.3.2. Culture and identification

Culture and identification of M.tb provide a definitive diagnosis of TB. The primary advantage of culture is their higher sensitivity, allowing significantly increasing in the number of cases identified by 30-50% when compared with microscopy ⁽⁶³⁾. However, culture is more complex and expensive than microscopy; it requires specific laboratory equipment, technicians with specialized skills, appropriate pre-treatment and processing of samples and adapted biosafety conditions ⁽⁶³⁾. Because mycobacteria grow slowly and need a long incubation time (4-8 weeks on solid culture), several automated systems have been commercially developed for rapid detection of mycobacteria in liquid medium (BD BACTEC MGIT 960 system and bioMérieux MB/BacT). Liquid culture increases the case yield by approximately 10% over solid media, and automated systems reduce the diagnostic delay to days rather than weeks. However, liquid systems are more prone to contamination, and the manipulation of large volumes of infectious material mandates appropriate additional biosafety measures ⁽⁶⁴⁾.

2.3.3. Drug-susceptibility testing

DST provides a definitive diagnosis of DR-TB as it determines whether a strain is susceptible (resistant or susceptible) to particular anti-TB agents. Deux techniques including phenotypic and genotypic methods can be tested directly with a concentrated specimen or indirectly with a pure culture grown from the original specimen ⁽⁶³⁾. Phenotypic DST (pDST), a culture-based method, currently remains the reference standard for DST. Compared to genotypic methods, pDST takes longer to get the result. However, liquid culture systems for DST reduce the result turnaround time to approximately 10 days (28-42 days needed for conventional solid media) which can contribute significantly to improving patient management ⁽⁶⁴⁾.

The accuracy of pDST varies according to the anti-TB agent being tested. For first-line anti-TB agents, pDST is most accurate in detecting susceptibility to RIF and INH but the results are less reliable and reproducible for streptomycin (STM), ethambutol (EMB) and pyrazinamide (PZA). However, recent studies shown that liquid DST fails to detect some clinically relevant “borderline RIF resistant strains” with *rpoB* mutations^(8, 65). For SLD, pDST is reliable and reproducible for only second-line injectable agents (SLID), including kanamycin (Km), amikacin (Am) and capreomycin (Cm), and FQ (Ofx, Lfx, Mfx, gatifloxacin). DST for other SLD, such as ethionamide, prothionamide, cycloserine, terizidone, p-aminosalicylic acid, clofazimine, amoxicillin/clavulanic acid, clarithromycin and linezolid, is not recommended⁽⁶³⁾.

2.3.4. Molecular testing

Genotypic methods have considerable advantages on the programmatic management of DR TB in particular with regard to their speed, the standardization of testing, their potentially high throughput and the reduced requirements for biosafety⁽⁶³⁾. Robust and accurate molecular technologies would have the greatest impact on improving diagnosis if they were made available at microscopy centers, where most people with signs and symptoms of TB seek a diagnosis. Although candidate products are described in this landscape report, to date, no molecular test has been sufficiently assessed to demonstrate the potential to do this⁽⁶⁶⁾.

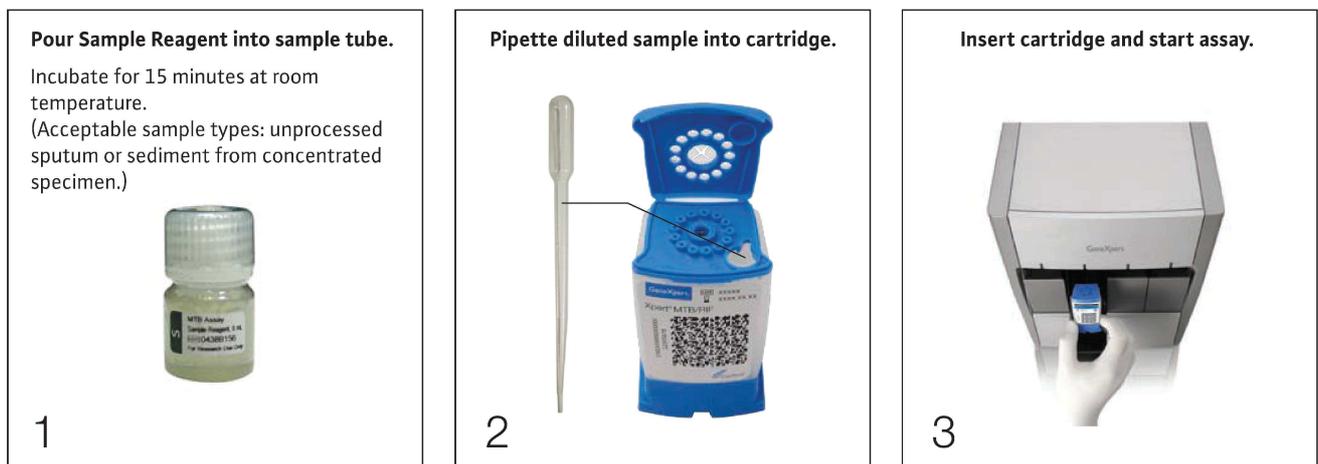
2.3.4.1. LINE-PROBE ASSAYS

Endorsed by WHO, molecular Line-probe assays (LPA) for first and second line drug enable rapid detection of resistance to RIF and INH and for FQ and SLID for second line drugs in less than 48 hours^(67, 68). The technique involves DNA extraction from *M.tb* isolates or directly from clinical specimens, polymerase chain reaction (PCR) to amplify drug resistant genes and reverse-hybridization of the labeled PCR products with specific oligonucleotide probes immobilized on a strip. As a high-throughput molecular tool for use at central and regional levels, LPA have been adopted by many countries for rapid FLD DST (to RIF and INH) on smear-positive specimens or cultures. However, LPA does not eliminate the need for pDST as its sensitivity to detect resistance to INH is lower (approximately 85%) and it is unable to identify resistance to specific SLID⁽⁶³⁾.

2.3.4.2. XPERT MTB/RIF ASSAY

The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is an automated semi-quantitative nested real-time PCR for the rapid detection of MTBC DNA and RIF resistance simultaneously, directly from sputum, processed sputum sediment and selected extrapulmonary specimens within 2h^(63, 69)

(Figure I-4). The assay is very simple to run under a biosafety precautions similar to those for smear microscopy and the training is minimal, which allows the technology to be used at relatively low levels in a laboratory network. In addition, The sensitivity (88%) and specificity (99%) of the Xpert MTB/RIF assay for detecting TB is similar to that of liquid culture ⁽⁶³⁾. For smear-negative culture-positive TB, the pooled sensitivity of Xpert MTB/RIF were 68%. As a tool for detecting RIF resistance, Xpert MTB/RIF has a sensitivity and specificity of 95% and 98%, respectively, when compared with phenotypic reference standards ⁽⁷⁰⁾.



Source: Adapted from Cepheid Xpert MTB/RIF brochure, April 2009 (71).

Figure I-4. Xpert MTB/RIF assay

Following initial WHO recommendations issued in December 2010, Xpert MTB/RIF has been quickly adopted by countries as an effective tool for the rapid detection of TB and RIF resistance in MDR-TB presumptive and HIV/TB co-infected patient at lower levels of the health system ⁽⁷²⁾. A policy update in 2013 expanded its recommended use, including for the diagnosis of TB in children, on selected specimens for the diagnosis of extrapulmonary TB, and for all individuals suspected of having PTB (conditional recommendations) ⁽⁷⁰⁾. The assay is currently the WHO-recommended rapid diagnostic most frequently used worldwide. With increasing use of Xpert MTB/RIF, a growing number of RR-TB cases (without further testing for INH resistance) which also requires treatment with SLD, are being detected and notified ^(36, 37).

Although Xpert MTB/RIF represents a major milestone for global TB and MDR-TB diagnosis and care, it has some disadvantages including the short shelf life of the cartridges, and the need for a stable uninterruptable electrical supply, annual calibration, and ambient operating temperature below 30°C of the instrument. In addition, the use of Xpert MTB/RIF does not eliminate the need for conventional microscopy, culture and DST, which are required to monitor the progress of treatment and to detect resistance to anti-TB agents other than RIF ⁽⁶³⁾. To address some of these challenges, the Xpert Omni, a new machine smaller, lighter and less expensive than the current Xpert machine were developed for point of care (POC) testing and a next generation cartridge called the GeneXpert Ultra was launched ^(73, 74). The Xpert MTB/RIF Ultra test performed better than the MTB/RIF assay in TB diagnosis of children, HIV-infected patients and patients with extra PTB, however, there is a need for more research to be conducted to improve the specificity of the new test ⁽⁷⁴⁾.

2.3.4.3. TB-LAMP

A commercial molecular assay Loopamp MTBC Detection Kit was developed by Eiken Chemical Company Ltd. (Tokyo, Japan) for the detection of MTBC in less than 1h using loop-mediated isothermal amplification technology (LAMP) ⁽⁷⁵⁾. The LAMP is manual assay consisting of three steps, sample preparation, amplification at unique temperature, and visual detection of fluorescence light from the reaction tube using UV light (Figure I-5). Additionally, the technique is simple and robust and can be used at peripheral health centres, where microscopy is performed. The pooled sensitivity of TB-LAMP (78%) was shown to be higher than that of smear microscopy (63%) but lower than that of Xpert MTB/RIF (89%) ⁽⁷⁶⁾. In 2016, WHO issued a conditional recommendation on the use of TB-LAMP as a replacement test for smear microscopy or a follow-on test to smear microscopy to diagnose pulmonary TB in adults with signs and symptoms consistent with pulmonary TB. However, TB-LAMP should not replace Xpert MTB/RIF that detects TB and resistance to RIF but can be its plausible alternative where the Xpert assay cannot be implemented. Further operational research is needed to gather more evidence on the implementation of TB-LAMP ⁽⁷⁶⁾.

2.3.5. Testing for latent TB infection

As one fourth of the world population have LTBI and 5-10% of them will develop active TB disease over their lifetime, the development of new diagnostic and screening tools and management standards for LTBI has become necessary in order to control the disease ⁽³⁶⁾. Diagnosis of LTBI is important as those found positive may be initiated on prophylactic treatment, thus preventing development of active TB and indirectly preventing transmission. Currently, WHO recommended to use either a tuberculin skin test (TST) or interferon-gamma release assay (IGRA) to test LTBI ⁽⁷⁷⁾. Both methods work by measuring the response of T cells to TB antigens.

2.3.5.1. TUBERCULIN SKIN TEST

The TST is one of the oldest diagnostic tests developed in the 19th century but which is still being widely used as it is not expensive, and does not require any special laboratory infrastructure or supplies ⁽⁷⁸⁾. The standard recommended test is the Mantoux test, which is administered by intradermal injection of a 0.1 mL of liquid containing tuberculin units of purified protein derivative (PPD) and the test is read 48-72 h after the injection ⁽⁷⁹⁾. Although widely used, the test has several limitations; it requires two patient visits, an injection into the skin, and adequately trained staff. Moreover, it has poor specificity in BCG-vaccinated populations, cross-reactivity with non-tuberculous mycobacteria and poor sensitivity in immunocompromised persons ⁽⁶³⁾.

2.3.5.2. INTERFERON-GAMMA RELEASE ASSAY

The IGRA measures the release of Interferon- γ (IFN- γ) from T lymphocytes following stimulation of the cells with M.tb-specific antigens (ESAT-6, CFP-10, TB 7.7) using an enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunospot (ELISPOT). Two commercially available IGRA (QuantiFERON[®]-TB Gold In-Tube and T-SPOT[®].TB) were recommended by the WHO ⁽⁷⁷⁾. IGRA require a single patient visit, results are available in 24-48 hours, and prior BCG vaccination does not cause false positive results. However, these assays are not routinely used in Resource-Limited Settings because they are expensive, require special laboratory infrastructure and supplies and advanced technical expertise ⁽⁶³⁾.

2.3.6. Non-commercial culture and drug-susceptibility testing

In 2011, the WHO recommended non-commercial culture and DST methods for screening patients at risk for MDR TB including (a) microscopic observation of drug susceptibility (MODS): a micro colony direct method in liquid culture, based on inoculation of specimens into drug-free

and drug-containing media, followed by microscopic examination of early growth, (b) colorimetric redox indicator (CRA): indirect methods based on the reduction of a colored indicator added to liquid culture medium on a microtiter plate after exposure of MTB strains to anti-TB drugs in vitro and (c) nitrate reductase assay (NRI) methods: a direct or indirect method based on the ability of M.tb to reduce nitrate, which is detected by a color reaction ^(63,80). MODS and NRI are recommended as direct or indirect tests to screen patients suspected of having MDR-TB while CRI methods are recommended as indirect tests on M.tb isolates from patients suspected of having MDR-TB.

Non-commercial methods of culture and DST are less expensive than commercial systems but are prone to errors due to a lack of standardization and to local variations in the methods. Similar to commercial systems, the non-commercial culture and DST systems and methods are suitable for use only by central or regional reference laboratories. In contrast, non-commercial methods have not been validated for use with second line agents ⁽⁶³⁾.

2.3.7. Lateral flow lipoarabinomannan assay

A lateral flow Lipoarabinomannan (LF-LAM) is based on the detection of the mycobacterial lipoarabinomannan (LAM) antigen in urine. The LAM antigen is a lipopolysaccharide present in mycobacterial cell walls, which is released from metabolically active or degenerating bacterial cells and appears to be present only in people with active TB disease ⁽³⁶⁾. Urinary LAM assays are not suitable for use as general screening tests for TB due to suboptimal sensitivity. However, compared with traditional diagnostic methods, they have demonstrated improved sensitivity for the diagnosis of TB among individuals co-infected with HIV, especially among patients with low CD4 counts. The advantages of LAM include the use of urine which is easily and rapidly obtained even from very ill patients compared to sputum, it is a potential point-of-care tests which can also be performed by trained nurses. In 2015, the urine LAM strip-test (Alere Determine™ TB LAM Ag test, Alere Inc, USA) is currently recommended by WHO in HIV-positive adults with CD4 counts less than or equal to 100 cells/UL and with signs and symptoms of TB ⁽⁸¹⁾.

2.4. Tuberculosis treatment and prevention

The treatment of TB aims to cure the disease process, prevent relapses and the development of drug resistance and rapidly reduce transmission ^(82,83). Effective TB treatment needs to overcome the organism's ability to persist in diverse microenvironments under extreme conditions, including immunological attack, prolonged antibiotic exposure, and nutrient and oxygen depletion, such as in pulmonary cavities, empyema pus, or solid caseous material, where penetration of antibiotics is difficult or the pH is sufficiently low to inhibit the activity of most antibiotics ^(84,85). Therefore, the current TB treatment requires multiple antibiotics taken for many months ⁽⁸²⁾. Standardized treatment

regimens and fixed-dose combination medications has been implemented in order to simplify good clinical care in resource-limited settings ⁽⁸²⁾. In order to ensure treatment success and preventing the emergence of acquired drug resistance, Directly observed therapy (DOT), in which the TB treatment is directly monitored by a trained third party acceptable to the patient and the health system, has been adopted worldwide ^(86, 87). DOT is one of the core components of the WHO recommended DOTS strategy (Directly Observed Treatment Short course) which has been recognized as a highly efficient and cost-effective strategy for TB control and a core component of TB programme ^(86, 87).

2.4.1. First-Line Anti-tuberculous Treatment for drug-susceptible tuberculosis

Clinical trials in the twentieth century established current first-line drug regimens ^(88, 89) which includes four drugs including RIF, INH, PZA and EMB in the intensive phase of two months and two drugs (RIF and INH) in the continuation phase of 4 months, named as “short-course” regimen (**Table I-1**). RIF and INH are the most potent drugs for susceptible TB and are taken throughout the course of first-line treatment. INH is highly bactericidal against replicating tubercle bacilli, while RIF has bactericidal action and a potent sterilizing effect against tubercle bacilli in both cellular and extracellular locations ⁽⁸³⁾. PZA synergistically reinforces the sterilizing activity of RIF and, when added to the first two months of treatment during the intensive phase, shortens the period of treatment to six months ^(82, 88). EMB is added to the regimen for two months to prevent or delay the emergence of resistant strains and is continued for the full duration of therapy for new patients in populations with high prevalence of INH resistance ⁽⁸³⁾. The treatment success rates is at least 85% for drug-susceptible TB cases ⁽¹⁾. However, the treatment effectiveness has been eroded by the evolution and transmission of DR TB in many countries, indicating that new regimens will need to be increasingly incorporated into TB treatment ⁽⁸²⁾.

Table I-1. Tuberculosis treatment regimens currently recommended by the WHO

Type of case and phase	Length of regimen (months)	Drugs used
New patients with pulmonary TB		
Intensive phase	2	RIF-INH-PZA-EMB
Continuation phase	4	RIF-INH (low risk of INH resistance) or RIF-INH-EMB (high risk ^a of INH resistance)
Treatment of drug resistant TB		
Shorter MDR/RR-TB regimen		
Intensive phase	4-6	Am-Mfx-Pto/Eto-Cfz-PZA-INH _{high-dose} -EMB
Continuation phase	5	Mfx-Cfz- PZA-EMB
Longer standardized MDR/RR-TB regimens ^b		
Intensive phase	6	At least four effective medicines likely to be effective: all three Group A agents including Bedaquiline and at least one Group B agent (see Table I-2).
Continuation phase	12-14	At least 3 agents for the remaining duration of treatment after bedaquiline is stopped.
Shorter, all-oral MDR/RR-TB regimens (under operational research conditions.)		
Intensive phase	4-6	Bdq-Lfx/Mfx-Eto- Cfz-PZA- INH _{high-dose} -EMB
Continuation phase	5	Lfx/Mfx-Cfz-PZA-EMB
Patients with confirmed RIF-susceptible and INH-resistant TB (Hr-TB)		
-	6	RIF-EMB-PZA-Lfx

^a Using local epidemiological data.

^b The duration may be modified according to the patient's response to therapy. A total treatment duration of 18–20 months or a treatment duration of 15–17 months after culture conversion is suggested for most patients.

Source: WHO, 2019 ⁽³⁷⁾.

2.4.2. Recent WHO recommended treatment regimens for MDR/RR TB

The treatment of DR TB is evolving and changing rapidly due to the limited number of available drugs and the uncertainties about the relative effectiveness of regimens used, especially those to treat MDR/RR-TB. The conventional MDR-TB treatment regimen (subsequently referred to as longer therapy) lasts up to 24 months and requires the use of “reserve” or second line TB drugs by including at least five medicines considered to be effective based on patient history or DR patterns ⁽⁹⁰⁾. These drugs are less effective, far more toxic than first-line agents and more difficult to administer, as most regimens contain injectable agents such as amikacin. Treating MDR-TB using conventional regimens is also resource intensive, costing from US\$2,500 to US\$10,000 per patient compared with US\$100–

US\$1,000 for drug-susceptible TB cases ⁽⁹¹⁾. Nevertheless, it has been possible to achieve treatment success rate for MDR-TB of only 56% worldwide ⁽²⁾.

In May, 2016, WHO made a conditional recommendation on use of the shorter MDR-TB regimen (9-12 months), also known as Bangladesh regimen. The regimen consists of an intensive phase of four months (extended to six months in case of delayed sputum smear conversion) containing high-dose Gfx or Mfx, Km, prothionamide (Pto), clofazimine (Cfz), high-dose INH, PZA and EMB followed by a continuation phase of five months containing Gfx or Mfx, Cfz, EMB and PZA ⁽⁹⁰⁾. Shorter regimen increases adherence and retention in care, lower the cost to less than US\$1,000 per patient and reduces number of adverse events which lead improvement in treatment success rate, with more than 80% of patients cured ^(90, 92, 93).

In 2019, WHO announced changes to treatment guidelines for MDR-TB in which includes policy recommendations on treatment regimens for INH-resistant TB and longer treatment and shorter regimen for MDR/RR-TB ⁽³⁷⁾. In patients with confirmed RIF-susceptible and INH-resistant TB, a full 6-month course of RIF, EMB, PZA and Lfx is recommended ⁽³⁷⁾. The updated guideline continued recommendation of using shorter regimen whenever possible in MDR/RR-TB patients who have not been previously treated for more than 1 month with SLD used in the shorter MDR-TB regimen or in whom resistance to FQ and SLID agents has been excluded ⁽³⁷⁾. The drugs used to compose the longer MDR/RR-TB regimens were reclassified into three groups A, B and C (**Table I-2**). Group A medicines includes three drugs [Lfx/Mfx, bedaquiline (Bdq) and linezolid (Lzd)] to be prioritised and used, if possible, in all regimens. Group B includes two drugs [Cfz and cycloserine (Cs)/terizidone (Trd)] to be possibly added to all regimens. Group C includes “other” agents [EMB, delamanid (Dlm), PZA, imipenem-cilastatin (IPM-CLN), meropenem (MPM, administered with clavulanic acid), Am (STM), ethionamide (Eto)/Pto and p-aminosalicylic acid (PAS)] to be used as a substitute to complete a regimen of at least four drugs when agents from groups A and B cannot be used ⁽³⁷⁾. Km and Cm were excluded from all regimens, given an increased risk of treatment failure and relapse when used in longer MDR-TB regimens.

Table I 2. Grouping of medicines recommended for use in longer MDR-TB treatment regimen

Groups & steps	Medicine	
Group A: Include all three medicines	Levofloxacin or	Lfx
	Moxifloxacin	Mfx
	Bedaquiline	Bdq
	Linezolid	Lzd
Group B: Adding of one or both medicines	Clofazimine	Cfz
	Cycloserine or	Cs
	Terizidone	Trd
Group C: Adding to complete the regimen and when medicines from Groups A and B cannot be used	Ethambutol	EMB
	Delamanid	Dlm
	Pyrazinamide	PZA
	Imipenem–Cilastatin or	lpm–Cln
	Meropenem	Mpm
	Amikacin	Am
	Or Streptomycin	STM
	Ethionamide or	Eto
Prothionamide	Pto	
	P-Aminosalicylic Acid	PAS

Source: Adapted from WHO, 2019⁽³⁷⁾.

In summary, WHO recommended an injection-free therapy (groups A and B drugs) in the MDR-TB treatment by replacing injectables with Bdq, which can cause patients pain and distress and serious adverse events that lead to interruption of treatment (**Table I-2**)⁽³⁷⁾. Group C agents (oral and parenteral) should be administered when groups A and B drugs cannot be used. The all-oral regimen containing Bdq may be used in patients with XDR-TB who had no previous exposure to Bdq and Lzd more than two weeks⁽⁹⁴⁾. However, the effectiveness and safety of all-oral regimen need to be explored under operational research conditions.

2.4.3. Tuberculosis prevention

TB is a preventable disease. There are a number of options available to prevent TB and reduce the burden of TB in the community. These include (i) stopping the transmission of TB from one adult to another through early detection and rapid initiation of effective treatment of TB cases and airborne infection control measures in clinical and community settings, (ii) preventing people with latent TB from developing active and infectious TB disease with tuberculosis preventive treatment (TPT), (iii) preventing humans from getting bovine TB through pasteurization of milk and (iv) interrupt the transmission of TB among adults with vaccination for TB⁽⁸²⁾.

2.4.3.1. BACILLE CALMETTE-GUÉRIN VACCINE

Bacille Calmette-Guérin (BCG) is the only available vaccine against TB for the past 100 years. It is the first live-attenuated bacterial vaccine derived from *M. bovis* and one of the most widely used vaccines yet controversial in the world ⁽⁸²⁾. Since its introduction in 1921, more than 3 billion people, mostly in countries with an intermediate or high incidence of TB where it is part of the national childhood immunization programme, have been vaccinated with BCG ⁽⁹⁵⁾. BCG has several advantages: it can be given at birth or at any time thereafter; a single dose can produce long-lasting immunity; it is relatively stable and inexpensive ^(60, 82).

The efficacy of BCG in preventing pulmonary TB varies between populations, ranging from 0% in South India to 80% in the United Kingdom ⁽⁹⁶⁻⁹⁸⁾. Considerable evidence indicates consistently high estimates of efficacy (>70%) for infant BCG vaccination against tuberculous meningitis and disseminated miliary TB ^(99, 100). There is evidence that BCG provides partial protection against leprosy (20-80% efficacy) and Buruli ulcer (~50% efficacy) in African region ^(82, 101). This fact, along with consistent protection against severe systemic forms of TB, has supported the use of vaccination in countries or settings with a high incidence of TB and/or high leprosy burden as well as where Buruli ulcer occurs ⁽¹⁰²⁾. Providing insufficient and inconsistent protection against pulmonary TB in adults, there is a need for development of a new, more efficient vaccines, and alternative strategies to replace or complement BCG.

In the past two decades, there has been a renewed effort to develop vaccines against TB that would provide greater protection than BCG. About 40 vaccine candidates are at various stages of preclinical testing, and 15 vaccine candidates are currently in clinical trials ⁽⁸²⁾. Among the candidates in the pipeline, M72/AS01E candidate developed by GSK was found to be significantly protective against active pulmonary TB disease in adults infected with *M.tb* in a Phase 2b trial conducted in Kenya, South Africa and Zambia ⁽¹⁰³⁾. The point estimate of vaccine efficacy was 54% (90% CI, 14% to 75%; $p = 0.04$) ⁽¹⁰³⁾. This promising result raise hope for TB prevention.

2.4.3.2. TUBERCULOSIS PREVENTIVE TREATMENT

About one-fourth of the world's population is estimated to be infected with *M.tb* and about 5-10% of those infected develop active TB disease in their lifetime ⁽¹⁾. The risk for active TB disease after infection depends on several factors, the most important being the person's immunological status ⁽¹⁰⁴⁾. Therefore, the aim of TPT is to prevent the progressing from TB infection to disease among people at highest risk including PLHIV, individual in contacts with TB patients and those with immunodeficiency conditions ⁽¹⁰⁴⁾. TPT is strongly recommended once TB disease is ruled out by an appropriate clinical evaluation and risk assessment or TB infection is confirmed by TST or IGRA test ⁽¹⁰⁴⁾.

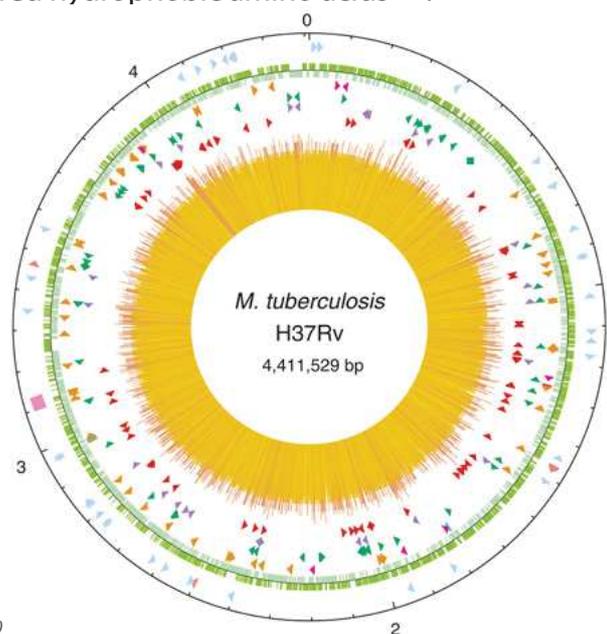
There are two options currently recommended by the WHO for the treatment of LTBI across all disease burden settings and target populations including the PLHIV: (i) 6 or 9 months of daily INH, or

(ii) rifamycin based shorter preventive treatment including a 3-month regimen of weekly rifapentine plus INH, a 3-month regimen of daily INH plus RIF, a 1-month regimen of daily rifapentine plus INH or 4 months of daily RIF alone. The choice will depend on availability of appropriate formulations and considerations for age, safety, drug-drug interactions, adherence and the assumption that the infecting strain is susceptible to these medicines ⁽¹⁰⁴⁾. Among contacts exposed to MDR-TB patients, the use of Lfx with or without EMB/Eto daily for six months is recommended however the susceptibility to a FQ need to be established ⁽¹⁰⁴⁾.

2.5. Molecular genetics of *Mycobacterium tuberculosis*

2.5.1. *Mycobacterium tuberculosis* genome

Mycobacterium tuberculosis contains a single circular chromosome approximately 4 mega base pairs in length and which in general appears to have similar structure and organization to the genomes of other mycobacterial relatives. The complete genome sequence of the best-characterized strain of M.tb H37Rv was first available in 1998 by Cole *et al.* which allowed us to better understand the biology of this slow-growing pathogen, the nature of its complex cell wall, certain genes related to its virulence and persistence, and the apparent stability of its genome (**Figure I-6**) ⁽¹⁰⁵⁾. Cole *et al.* revealed that the genome of M.tb H37Rv comprises 4,411,529 base pairs, contains around 4,000 genes, and has a very high guanine plus cytosine (G+C content; 65.5 %) throughout the genome ⁽¹⁰⁵⁾. The characteristically G + C-rich genome supported the hypothesis that horizontal gene transfer events are probably absent ⁽¹⁰⁶⁾. Several regions showing very high G + C content (>80%) appear to be unique in mycobacteria and correspond to sequences belonging to the family of PE or PPE proteins. There are few genes with particularly low G+C content (<50%) coding for transmembrane proteins which required hydrophobic amino acids ⁽⁶⁰⁾.



Source: Cole *et al.* 1998 ⁽¹⁰⁵⁾.

Figure I-6. Circular map of the chromosome of *M. tuberculosis* H37Rv.

This initial genome annotation in *M.tb* H37Rv strain were re-annotated in 2002 by Camus *et al.* ⁽¹⁰⁷⁾. This re-annotation allowed the incorporation of 82 additional protein-coding genes and identification of four sequencing errors, making the current sequence size change from 4,411,529 to 4,411,532 bp ⁽¹⁰⁷⁾.

Fifty genes were coding for functional RNAs with a single ribosomal RNA operon (*rrn*) ⁽¹⁰⁸⁾. Unlike most eubacteria that have one or more *rrn* operons near to *oriC* to exploit the gene-dosage effect during replication ⁽¹⁰⁹⁾, the unique *rrn* operons of *M. tb* locates about 1,500 kilobases (kb) from the putative *oriC* ⁽¹⁰⁵⁾. This arrangement may contribute to the slow growth phenotype of *M.tb* ⁽¹¹⁰⁾.

Regarding protein coding genes, 3,924 open reading frames were identified in 1998 and additional 82 in 2002, accounting for 91.3% of the coding capacity of the genome ^(105, 107). A few of coding genes appear to have in-frame stop codons or frameshift mutations and may either use frameshifting during translation or correspond to pseudogenes ⁽¹⁰⁵⁾. Although ATG (61%) is the most common translational start, the alternative initiation codon GTG is used in 35 % in H37Rv compared to 14 % or 9 % in *Bacillus subtilis* or *Escherichia coli* respectively. This contributes to the high G+C bias in the codon usage of mycobacteria ⁽⁶⁰⁾. With respect to the direction of replication, ~59% are transcribed with the same polarity as replication, compared with 75% in *B. subtilis* and with ~ 100% in other bacteria. Such a bias could also be part of the slow growing phenotype and infrequent replication cycles of the tubercle bacillus ⁽¹⁰⁵⁾. About 84% of encoded proteins have precise or predictable functions, while the remaining 16% resembled no known proteins and may account for specific mycobacterial functions ⁽¹⁰⁵⁾. It was also found that the fraction of the proteome that arose by gene duplication is similar to that seen in *E. coli* or *B. subtilis* (~51%), but the degree of conservation of duplicated genes is higher in *M.tb* ^(111, 112). The lack of divergence following gene duplication is consistent with the hypothesis that *M.tb* is of a recent evolutionary descent or a recent bottleneck ^(106, 113).

The genome is rich in repetitive DNA, particularly insertion sequences, and in new multigene families and duplicated housekeeping genes. Sixteen copies of insertion sequence *IS6110*, six copies of the more stable element *IS1081* and another 32 different insertion sequences that belong mainly to the *IS3* and *IS256* families reside within the genome of H37Rv ^(114, 115). Most of the insertion sequences appear to have inserted in intergenic or non-coding regions, often near tRNA genes and clustered in insertional hot-spot region preventing the genes from being inactivated ⁽¹⁰⁵⁾.

Two prophages *phiRv1* and *phiRv2* were detected in the genome sequence; both are similar in length (~10 kb) and also similarly organized ⁽¹⁰⁵⁾. Some of their gene products are similar to those encoded by certain bacteriophages from *Streptomyces* and saprophytic mycobacteria ⁽¹⁰⁵⁾. The first prophage *phiRv1* exhibits variability of its position between strains ⁽¹¹⁶⁾, while the second prophage *PhiRv2* were be much more stable with less variability among strains ⁽¹¹⁷⁾.

The genome sequence reveals that *M.tb* has the potential to synthesize all the essential amino acids, vitamins and enzyme co-factors and switch from one metabolic route to another including aerobic (e.g. oxidative phosphorylation) and anaerobic respiration (e.g. nitrate reduction). The

ability is useful for its survival in the changing environments, ranging from high oxygen tension in the alveolus to microaerophilic/anaerobic environment within the tuberculous granuloma, within the human host. Another distinct characteristic of M.tb is the presence of genes involving in the biosynthesis and degradation of almost all kinds of lipids from simple fatty acids such as palmitate and tuberculostearate to complex molecules such as mycolic acids⁽¹⁰⁵⁾. In total, ~250 distinct enzymes were involved in fatty acid metabolism, compared to only 50 in the genome of *E. coli*⁽¹¹⁸⁾. Given the complexity of the environmental and metabolic choices, M.tb codifies a substantial transcriptional regulators including 13 putative sigma factors and more than 100 regulatory proteins⁽¹⁰⁵⁾.

The PE (Pro-Glu) and PPE (Pro-Pro-Glu) multigene families, which account for almost 10% of the coding capacity of the genome, are among the most interesting protein gene families found in M.tb. These proteins are believed to play an important role in survival and multiplication of mycobacteria in different environments⁽¹¹⁹⁾. There are about 100 members of the PE family and the most important protein is the highly repetitive PGRS class that contains 61 members. Proteins in this class have been found to be exclusive to the M.tb complex⁽¹¹⁹⁾ and resemble the Epstein-Barr virus nuclear antigens (EBNA), which are known to inhibit antigen-processing pathway through the major histocompatibility complex (MHC) class I molecules⁽¹⁰⁵⁾.

2.5.2. Drug resistance mechanisms

The mechanisms of drug resistance in M.tb can be subdivided in two categories, intrinsic or acquired drug resistances. The understanding of these resistance mechanism will allow the development of tests capable of rapidly detecting resistance strains and ultimately for new drug with novel mechanisms of action including agents used as adjunctive therapy to compensate for resistance mechanism promoted by the bacteria^(120, 121).

2.5.2.1. INTRINSIC DRUG RESISTANCE

During evolution, M.tb developed intrinsic resistant mechanisms toward many antibiotics, making the disease difficult to treat⁽¹²¹⁾. This involves cell envelope, efflux systems and other mechanisms (drug degradation and modification, target modification)⁽¹²²⁻¹²⁴⁾.

The mycobacterial cell wall is extremely thick and multilayered with innermost layer of peptidoglycan covered by a layer of arabinogalactan; both layer are hydrophilic thus hindering the penetration of hydrophobic molecules⁽¹²⁵⁾. In addition, the bilayer complex is covalently bonded to mycolic acids that form a hydrophobic barrier restricting the entry of hydrophilic molecules⁽¹²⁶⁾. This low permeable cell wall functions as an effective barrier for penetration of diverse classes of drug. For example, the penetration of β -lactams through the mycobacterial cell wall is 100-fold slower than going through the *E. coli* cell wall⁽¹²⁷⁾.

M.tb possesses active efflux systems which are transmembrane proteins usually play roles in transporting nutrients, wastes, toxins or signaling molecules across the cell wall. These active efflux systems also provide resistance by expelling the drug molecules that enter the cell through efflux pumps (EP) ⁽¹²⁸⁾. There are at least 18 transporters in mycobacteria including 14 members of the major facilitator family and numerous ABC transporters found to confer low-level antibiotic resistance ^(105, 129). *M.tb* exposure to INH induces the overexpression of *MmpL7* and *mmR* efflux pumps genes ^(130, 131). Furthermore, several EP are involved in resistance to several drugs ; EP Tap mediates low-level resistance to tetracycline (TC) and aminoglycosides, whereas EP encoded by the *Rv0194* gene is associated with resistance to beta-lactams, STM, tetracycline, chloramphenicol and vancomycin ⁽¹³²⁾. Some of these transporters are expressed under the control of antibiotic-responsive transcription regulators. Lsr2 protein, a nucleoid-associated transcription regulator of expression of *IniBAC* that confers multi-drug tolerance in *M. bovis* BCG through an associated pump-like activity, is inducible by INH or EMB ⁽¹³³⁾. Besides drug resistance, recent studies suggested that drug efflux has also been associated with pathogenicity, virulence, biofilm formation and quorum sensing ⁽¹³⁴⁾.

Other potential intrinsic resistance mechanisms include:

- Target alteration: *M.tb* and related mycobacteria reduce binding of macrolides and lincosamides to their ribosomes RNA by methylating the 23S ribosomal RNA.
- Target mimicry: Studies found that *MfpA* which closely resembles the 3D structure of a DNA double helix, sequesters FQ in the mycobacterial cytoplasm by mimicking the DNA structure, thus freeing DNA from the drug's action ⁽¹³⁵⁾.
- Drug modification: Eis (enhanced intracellular survival) protein that functions in protecting mycobacteria against the host immunity acetylates multiple amine groups of aminoglycosides (kanamycin) using acetyl coenzyme A as an acetyl donor thereby inactivating the antibiotics ⁽¹³⁶⁾.
- Drug degradation: *M.tb*, produce BlaC, the most important β -lactamase belongs to the Ambler class A β -lactamases, leading to resistance to a broad range of β -lactams antibiotics including carbapenems ⁽¹³⁷⁾.
- Phenotypic tolerance: During the latent infection, *M.tb* is believed to enter a dormant-like state, called "persisters", which replicate extremely slowly or completely stop growth, leading to increased antibiotic tolerance due to low metabolic rates ⁽¹³⁸⁾.

The intrinsic resistance mechanisms provide *M.tb* with a high background of resistance to the existing antibiotics and make the development of new drugs more difficult ⁽¹²⁸⁾. However, drugs inhibiting these intrinsic systems would enable those antibiotics to gain its potential action against *M.tb* ⁽⁶⁰⁾.

2.5.2.2. ACQUIRED DRUG RESISTANCE

Acquired antibiotic resistance in other bacterial species may occur through horizontal gene transfer mediated by phages, plasmids or transposon elements ⁽¹²⁸⁾ but in *M.tb*, antibiotic resistance is mainly acquired through mutations in chromosomal genes ⁽¹³⁹⁾. The majority of them are single-nucleotide polymorphisms (SNP), insertions or deletions (indels), and to a certain extent, deletions in genes that encode drug targets or drug-activating enzymes within the bacilli ^(140, 141). These chromosomal alterations lead to modification of the drug-targets (RIF, EMB, FQ, STM, Km, Am and Cm) structure, failure to activate a prodrugs (INH, PZA, Eto), inactivation of the drugs (Km) or poor drug permeability and increased drug efflux (Cfz and Bdq) (see **Table I-3**) ^(121, 142, 143).

Table I-3. Modes of action of anti-TB drugs and common genes involved in drug resistance in *Mycobacterium tuberculosis*

Drug	Gene	Gene Product	Mutation frequency (%)	Compensatory mechanisms
Isoniazid: inhibition of cell wall mycolic acid synthesis				
	<i>katG</i>	Catalase-peroxidase	70	
	<i>inhA</i> and its promoter	Enoyl-ACP reductase	~10	<i>oxyR'</i> and <i>ahpC</i>
Rifampicin: inhibition of RNA synthesis				
	<i>rpoB</i>	β -subunit of RNA polymerase	95	<i>rpoA</i> and <i>rpoC</i>
Ethambutol: inhibition of cell wall arabinogalactan biosynthesis				
	<i>embB</i>	Arabinosyl transferase	~70	unknown
Pyrazinamide: reduction of membrane energy; inhibition of trans-translation; inhibition of pantothenate and coenzyme A synthesis				
	<i>pncA</i>	Pyrazinamidase	~99	unknown
Streptomycin: inhibition of protein synthesis				
	<i>rpsL</i>	16S rRNA	~6	unknown
	<i>rrs</i>	S12 ribosomal protein	<10	
	<i>gidB</i>	7-Methylguanosine methyltransferase	-	
Fluoroquinolones: inhibition of DNA synthesis				
	<i>gyrA</i>	DNA gyrase subunit A	~90	<i>gyrA</i> (T80A and A90G)
	<i>gyrB</i>	DNA gyrase subunit B	<5	putative <i>gyrB</i>
Amikacin, Kanamycin and Capreomycin: inhibition of protein synthesis				
	<i>rrs</i>	S12 ribosomal protein	60–70	<i>rrs</i> (C1409A and G1491T)
	<i>eis</i>	Aminoglycoside acetyltransferase	~80 (low-level kanamycin)	
	<i>tlyA</i>	rRNA methyltransferase	~3 (capreomycin)	
Ethionamide: inhibition of cell wall mycolic acid synthesis				
	<i>ethA</i>	Flavin monooxygenase	~96%	unknown
	<i>inhA</i> and its promoter	Enoyl-ACP reductase		

Drug	Gene	Gene Product	Mutation frequency (%)	Compensatory mechanisms
Para-aminosalicylic acid: inhibition of folic acid and thymine nucleotide metabolism				
	<i>thyA</i>	Thymidylate synthase	~40	unknown
	<i>folC</i>	Dihydrofolate synthase	-	
	<i>ribD</i>	enzyme in riboflavin biosynthesis	~90	
Bedaquiline: inhibition of mycobacterial ATP synthase				
	<i>rv0678</i>	Transcriptional regulator (drug efflux)	-	unknown
	<i>atpE</i>	ATP synthase, subunit F0	-	
Clofazimine: inhibits mycobacterial growth and binds preferentially to mycobacterial DNA. It may also bind to bacterial potassium transporters, thereby inhibiting their function.				
	<i>rv0678</i>	Transcriptional regulator (drug efflux)	~80% with cross-resistance to bedaquiline	unknown
	<i>rv1979c</i>	(Possible permease involved in amino acid transport)	~20%	
	<i>rv2535c</i>	(PepQ putative aminopeptidase)	-	
Delamanid/pretonamid: specific and selective inhibition of mycolic acid biosynthesis, essential for cell wall formation				
	<i>fgd1</i>	Glucose-6-phosphate dehydrogenase	-	unknown
	<i>fbjC</i>	Protein FbjC	-	
	<i>fbjA</i>	Protein FbjA	-	
	<i>fbjB</i>	Protein FbjB	-	
	<i>ddn</i>	Deazaflavin (F420)-dependent nitroreductase	-	
Linezolid: inhibition of protein synthesis				
	<i>rplC</i>	50S ribosomal protein L3	~90	unknown
	<i>rrl</i>	23S RNA	1.9–11	

Source: Adapted from Dookie et al, 2018 & Zhang and Yew, 2015 ^(143, 144)

In general, mutations occur spontaneously during DNA replication at a rate of 10^{-4} - 10^{-5} per base pair (bp) per round of replication ⁽¹⁴⁵⁾. However, correction by proofreading exonucleases and mismatch repair enzymes reduces the mutation rate to $\sim 10^{-10}$ per bp ⁽¹⁴⁶⁾. At this error rate, DR mutants of M.tb are expected to be rare and are predicted to arise as a consequence of drug selective pressure. Multiple factors could influence the rate of selection of DR mutants in the host, including the relative fitness of individual mutants, patient compliance with prescribed drug regimens, pharmacokinetic variability amongst patients, spatial heterogeneity in drug distribution and the size of the infecting bacterial population ⁽¹³⁹⁾.

For each anti-TB drug, mutations in one or several genes have been described, and each mutation relates to different levels of drug resistance. Mutation frequency can also be variable ⁽¹⁴⁷⁾.

▪ Isoniazid

Isoniazid (INH), the hydrazide of isonicotinic acid, was synthesized in 1912 but first used as antituberculosis agent in 1951⁽¹⁴⁸⁾. It is a prodrug requiring an activation by the catalase-peroxidase (KatG) enzyme encoded by the *katG* gene. Once activated, INH inhibits the biosynthesis of cell wall mycolic acids via multiple cellular targets including the NADH-dependent enoyl-acyl carrier protein reductase, encoded by the *inhA* gene^(144, 149, 150). This drug has bactericidal activity, with minimal inhibitory concentrations (MIC) between 0.02 and 0.2 mg/L, against only growing M.tb but not non-growing bacilli (persisters). This tolerance mechanism to INH in persisters bacilli was caused by mycobacterial DNA-binding protein 1 (MDP1), a histone-like protein, which downregulates *katG* transcription⁽¹⁵¹⁾. Although the molecular mechanisms of INH resistance in MTBC have not been fully elucidated, the resistance to INH is mostly mediated by mutations in *katG*, *inhA* gene or within the promoter region of the *inhA* gene causing overexpression of InhA (Enoyl acyl carrier protein reductase), an enzyme involved in mycolic acid synthesis (152).

Mutations in *katG*, *inhA* and *inhA* promoter accounted for ~84% of global phenotypic INH resistance⁽¹⁵³⁾. The mutations in *katG* disrupt INH activation to varying degrees. The *KatG* S315 mutation is the most common mutation in INH-resistant strains, accounting for 50-95% of INH-resistant clinical isolates. This specific mutation usually does not completely eliminate catalase activity, and the strains acquiring such mutation may still maintain fitness and virulence. This may explain its frequent occurrence among clinical isolates⁽¹⁴⁴⁾. Recent whole genome sequencing (WGS) analysis showed that INH resistance precedes RIF resistance and often associated with the *katG* S315T mutation⁽¹⁵⁴⁾. Therefore, this marker is ideal for pre-MDR screening⁽¹⁵⁴⁾. In contrast to *katG* mutations which is associated with high-level INH resistance, mutations in *inhA* or its promoter region are usually associated with low-level resistance (MIC 0.2-1 µg/ml) and are less frequent than *katG* mutations^(152, 155, 156). Regarding the mutations of the *inhA* promoter region, the most common was found at position -15, resulting in the upregulation of *inhA*. This mechanism not only causes INH resistance, it also confers cross-resistance to a structural analogue, Eto⁽¹⁴⁴⁾. Mutations in the active region of the *inhA* gene, resulting in a decreased affinity of the INH - NAD product, are less frequent^(156, 157). A recent study reported that mutations occurring in the *inhA* regulatory region and coding region resulted in high-level resistance to INH and cross-resistance to Eto⁽¹⁵⁷⁾.

Mutations in several other genes have also been reported to be associated with INH resistance, but occur less frequently, and their association with INH resistance is less clear. Those genes include *furA-katG* intergenic region that affect *KatG* expression, *kasA* which also encodes an enzyme involved in mycolic acid synthesis, *furA*, a ferric acid upregulator, *ndh* coding for NADH dehydrogenase, *nat* coding for arylamine N-acetyltransferase which inactivates INH, *mshA*, the glycosyltransferase involved in mycothiol synthesis, *efpA* coding membrane efflux protein EfpA, *fadE24* coding acyl-CoA dehydrogenase FadE24, *iniA* coding INH inducible gene protein IniA, *iniB* coding INH inducible gene protein IniB, *iniC* coding INH inducible gene protein IniC, *Rv1772*, *Rv1592c*, *Rv0340*, and *srmR* genes^(144, 158). Mutations in *oxyR-ahpC* intergenic region which lead to increasing expression of the

encoded alkyl hydroperoxidase enzyme responsible for resistance to reactive oxygen and nitrogen derivatives are believed to compensate for the reduction or loss of activity of *katG* activity and not confer INH resistance ⁽¹⁵⁸⁻¹⁶¹⁾.

▪ **Rifampicin**

Rifampicin (RIF), introduced in 1972 in the treatment of TB along with INH, is one of the most effective anti-TB drugs. Due to its efficient antimicrobial action against both actively metabolizing and slow-metabolizing bacilli, it is considered, together with INH, to be the key component of the short-course treatment regimen of DS-TB ^(159, 162-164). In *M.tb*, RIF binds to the β subunit of the ribonucleic acid (RNA) polymerase, resulting in the inhibition of elongation of messenger RNA (mRNA) ⁽¹⁶⁵⁾.

RIF monoresistance is rare as RIF resistance occurs in conjunction with resistance to other drugs, most commonly INH, making RIF targets a surrogate marker of the MDR phenotype ⁽¹⁶⁶⁾. Resistance to RIF in MTBC is most commonly associated with mutations in a well-defined, 81- base pair region (codons 507-533) of the *rpoB* gene coding for the RNA polymerase β subunit ⁽¹⁶⁷⁾. Mutations in this region, which is also known as the rifampicin resistance-determining region (RRDR), generally impede binding of RIF to the beta subunit of DNA-dependent RNA polymerase in MTBC and result in RIF resistance ⁽¹⁶⁸⁾. Approximately, 95% of RIF resistance occurs in RRDR ^(144, 169) and codons 526 and 531 harbor the most common mutations associated with RIF resistance ^(162, 163, 165, 170). Mutations in *rpoB* have been associated with cross-resistance to all rifamycin antibiotics.

Mutations outside the RRDR region have also been reported in RIF-resistant isolates ⁽¹⁷¹⁾. A lack of alteration in the *rpoB* gene has also been found in RIF-resistant isolates, suggesting other mechanisms of RIF resistance ⁽¹⁷¹⁾. Meanwhile mutations in the *rpoA* and *rpoC* genes, which encode the α and β' subunits of the RNA polymerase were reported as compensatory mechanisms in isolates with mutation in the *rpoB* gene. These mutations are associated with increased fitness and transmissibility of resistant strains ⁽¹⁷²⁾. Recently, the phenomenon of RIF -dependent/-enhanced strains which grow poorly in normal culture media that lacks RIF has been reported in 39% of the MDR-TB strains by Zhang *et al.* However, the mechanisms under which such strains emerge is yet to be elucidated, it is believed that they are selected by repeated treatment with the drug ^(173, 174).

▪ **Pyrazinamide**

Pyrazinamide (PZA) is a nicotinamide analogue that has been used for more than 70 years as a FLD to treat TB and has shorten the total treatment duration to 6 months. It is also been added in the DR-TB treatment regimen ⁽¹⁷⁵⁾. In contrast to common antibiotics that kill or inhibit growing bacteria, PZA is bactericidal to semi-dormant bacilli (non-growing persister bacteria) located in acidic environments (TB lesions) ⁽¹⁶⁴⁾. The need for acidic pH for the culture become the reason why the DST for PZA is technically challenging and unreliable. Currently, Bactec MGIT 960 liquid culture method is the only WHO-recommended methodology for PZA susceptibility testing, though even this testing is associated with a high rate of false-positive resistance results ^(176, 177). Despite

its common use, the precise mode of action of PZA and how mycobacteria resist this drug have remained poorly understood ⁽¹²⁸⁾. PZA is a prodrug that needs to be enzymatically activated into its active form, pyrazinoic acid (POA) by the pyrazinamidase/nicotinamidase (PZase) enzyme, encoded by the *pncA* gene ^(178, 179). Once activated, PZA has multiple effects on M.tb, including interference with membrane energy production ⁽¹⁸⁰⁾, and inhibition of 30S ribosomal protein S1 (RpsA), which inhibits trans-translation by preventing the interaction of RpsA with tmRNA ⁽¹⁸¹⁾, and PanD, which is involved in pantothenate and co-enzyme A synthesis ^(144, 182, 183).

The mutation in *pncA* accounts for most of the resistance cases reported in M.tb (72–99%, average 85%) ⁽¹⁷⁹⁾. *pncA* mutations are highly diverse and scattered along the gene and its promoter, with 600 unique mutations in 400 positions ⁽¹⁷⁵⁾. A wide variety of *pncA* mutations were associated with high MICs in MGIT, including *pncA* a-11g, t-7c, A3E, L4S, I6T, D8G, Q10P, D12G, C14R, G17D, Y34D, H51Q, P54L, H57D, H57Y, F58L, P62Q, W68C, T76P, F94L, G97D, R123P, V128G, G132A, G132S, T135P, H137P, V139G, T142K, V155G, L172P, M175T, M175V, combined frameshifts and premature stop codons ⁽¹⁸⁴⁾. The mutations were found to have low associated MICs included E37V, S65A, D110G, A170V and V180I ⁽¹⁸⁵⁾. Mutations with no association with PZA resistance include c-125del, I31T, L35R, T47A, I6L, K48T, and T114M ⁽¹⁸⁵⁾. Unfortunately, PZA-resistant strains with mutations in the *pncA* gene do not display any loss of fitness or virulence ⁽¹⁷⁹⁾.

A small proportion of PZA resistant isolates showed no mutations in the *pncA* gene, suggesting another mechanism of resistance exists ⁽¹⁸⁶⁾. Those isolates are reported to have mutations in the *rpsA* (ribosomal protein I) gene which are usually associated with low-level PZA resistance (MIC 200–300 µg/ml). Loss of the codon encoding residue Alanine 438, located near the C-terminus of RpsA, was shown to be essential for pyrazinoic acid binding ^(181, 187). Overexpression of *rpsA* has also been implicated in increased resistance to PZA ⁽¹⁸¹⁾. However, there was no clear demonstration of the significance of *rpsA* in PZA resistance among M.tb clinical isolates ^(188, 189). More recently, mutations in *panD* gene such as H21R and I49V double mutations decrease the binding affinity for PZA found to be involved in PZA resistance ⁽¹⁹⁰⁾.

▪ Ethambutol

Ethambutol (EMB), first introduced as an anti-TB drug in 1966, has activity against multiplying bacilli by disrupting the biosynthesis of cell wall arabinogalactan ⁽¹⁹¹⁾. In M.tb, EMB targeted an enzyme involved in the synthesis of arabinogalactan, arabinosyl transferase, encoded by *embB* ⁽¹⁹²⁾. Resistance to EMB is mediated via mutations in *embCAB* operon (particularly in *embB* and occasionally *embC*), encoding arabinosyl transferase enzyme ⁽¹⁹²⁾. *embB* mutations in the 576 bp EMB resistance-determining region (ERDR) are thought to decrease the hydrophobicity index of the region leading to sub-optimal interaction of the drug with the binding site ⁽¹⁹³⁾. Alteration in *embB* codon 306 (Met306Ile and Met306Val) is the most frequent mutation in clinical isolates resistant to EMB, accounting for about 68% of resistant strain ^(192, 194). Beside 306 codon, some mutations were found in *embB* codon 406, codon 497 and between codons 296 and 426, while

more occurred in the *embC–embA* intergenic region of the *embCAB* locus⁽¹⁹⁵⁾. It was further reported that the codon 306 mutations were rather responsible for predisposition to develop resistance to other drugs such as EMB, INH and RIF and is not necessarily involved in EMB resistance^(193, 196). Furthermore, the *embB306* mutation results in varying degrees of EMB resistance depending on the type of mutations⁽¹⁹⁷⁾. However, approximately 30% of EMB resistant isolates (MIC 10 mg/ml) do not have *embB* mutations⁽¹⁹⁸⁾, suggesting a different mechanism of resistance^(162, 163). Mutations in *ubiA*, encoding the decaprenyl-phosphate 5-phosphoribosyltransferase (DPPR) synthase involved in cell-wall synthesis, have recently been reported to cause higher-level EMB resistance in conjunction with *embB* mutations. This *ubiA* mutation is also lineage specific, and is predominant in the African isolates^(197, 199).

▪ **Fluoroquinolones**

The fluoroquinolone (FQ) is potent bactericidal drugs which have excellent activity against MTBC⁽²⁰⁰⁾. The later generation FQ (Lfx and Mfx) are among the most important anti-TB drugs used in the second-line treatment regimen for drug resistant TB or intolerant of current first-line therapy^(37, 201). FQ kill bacterial cells by targeting DNA gyrase, thereby inhibiting DNA replication, transcription and repair^(105, 202). DNA gyrase encoded by *gyrA* and *gyrB* genes is an ATP-dependent enzyme that introduces negative supercoils into the double stranded DNA^(105, 202). The primary resistance mechanism to the FQ in MTBC is conferred through mutations occurring in a conserved region known as the quinolone resistance-determining region (QRDR) in the *gyrA* (codons 74 to 113) and *gyrB* genes⁽¹⁰⁵⁾. *gyrB* QRDR mutations are presented using two different numbering systems, codons 500 to 538 based on M.tb 1998 numbering⁽¹⁰⁵⁾; or codons 461 to 499 based on M.tb 2002 updated numbering⁽²⁰³⁾. The majority of FQ resistance (~64%) has been associated with mutations occurring in the *gyrA* QRDR, most frequently at codons 88, 90, 91 and 94^(185, 201). In contrast, only 3% has mutations in *gyrB* QRDR that typically occur with lower sensitivity and specificity^(185, 201, 204). It has been reported that double mutations in the *gyrA* or concomitant *gyrA* and *gyrB* mutations result in high MIC⁽²⁰⁴⁾. Some mutations have been found to lie outside the QRDR, which negatively impact the performance of diagnostic tests such as the MTBDRs/ assay that allows to identify resistance in the QRDR⁽³⁶⁾. This places an emphasis on the reliability of phenotypic DST used in conjunction with these rapid molecular tests. Efflux mechanisms and DNA mimicry have also been reported to mediate FQ resistance⁽²⁰⁵⁾. However, the latter two mechanisms have been described only in laboratory strains, not clinical M.tb isolates^(204, 206-208). Although there is cross-resistance within the FQ group, Mfx MICs are usually lower than those of other FQ, making it as potentially effective drug to treat Ofx-resistant TB⁽²⁰⁴⁾.

▪ **Second-line injectable agents**

The SLID included cyclic polypeptides (Cm) and aminoglycoside (Am and Km). They continue to be used despite reported adverse side effects, including hearing loss, and invasive intramuscular

administration. Currently classified as group C drugs, only Am and its alternative, STM, are the injectable agents recommended for use in the treatment of MDR-TB, while kanamycin and capreomycin are no longer recommended due to its poorer outcomes ⁽³⁷⁾.

▪ **Streptomycin**

The use of aminoglycosides against TB began with the discovery of STM in the 1940s. Streptomycin (STM), originally isolated from the soil microorganism *Streptomyces griseus*, was the first antibiotic used to treat TB in 1942 ⁽²⁰⁹⁾. Unfortunately, as a result of being administered as monotherapy, its resistance was rapidly emerged ⁽²¹⁰⁾. It is active against actively growing bacilli through inhibition of protein synthesis by binding to the 30S subunit (ribosomal protein S12 and 16S rRNA) of bacterial ribosome, causing misreading of the mRNA message ^(211, 212). The main mechanism of resistance to STM is believed to be mediated via mutations in the S12 protein encoded by the *rpsL* gene and 16S rRNA encoded by the *rrs* gene, accounting for ~60%–70% of STM resistance ⁽²¹³⁾. A substitution in codon 43 from lysine to arginine in *rpsL* conferring high-level resistance to STM has been the most commonly reported. In *rrs*, the most common substitutions occur around nucleotides 530 and 915 ⁽²¹⁴⁾. Other mechanisms have also been reported such as mutations in the *gidB* gene, encoding a 7-methylguanosine methyltransferase specific for methylation of the G527 in loop of the 16S rRNA ^(215, 216) and mutations in the promoter region of *whiB7*, conferring cross-resistance to STM and Km due to increased expression of the tap efflux gene and *eis* controlled by *whiB7* ⁽²¹⁷⁾. The *gidB* gene mutation was account for about 20–30% of STM-resistant strains with low-level resistance (MIC, 32 µg/ml) ⁽²¹⁵⁾.

▪ **Amikacin, Kanamycin and Capreomycin**

Although, belonged to different classes of antibiotics, all three drugs have the same mechanism of action by inhibiting the protein synthesis ^(162, 163). Aminoglycosides are chemically diverse and bind differently to the A-site on the 16S rRNA ⁽²¹⁸⁾. Different from STM chemical structure, Km and Am are among aminoglycosides containing 2-deoxystreptamine ring and interacting with highly conserved nucleotide bases of the helix 44 of 16S rRNA through hydrogen bonding. This binding leads to incorrect pairing of mRNA and aminoacyl-tRNA or interference with translocation, and results in mistranslation of proteins in *M.tb* ⁽²¹⁹⁾. Cm is bacteriostatic agents with a similar structure and inhibits protein translation through its binding to the ribosomal interface between helix 44 of the small 30S subunit and Helix 69 of the large 50S subunit ^(220, 221).

Mutations in the 16S rRNA (*rrs*) confers cross-resistance to several aminoglycosides ^(222, 223). The substitution A1401G of the *rrs* gene is the most common molecular mechanism of resistance to all three drugs. This mutation is associated with ~70%–80% of capreomycin and amikacin resistance and 60% of kanamycin resistance, globally ^(224, 225). An increased fitness is also reported in clinical isolates bearing the *rrs* A1401G mutation and is thought to occur due to the presence of compensatory mutations ⁽²²⁶⁾. Cm resistance with mutations in the *tlyA* gene, encoding rRNA

methyltransferase required for 2'-O-methylation of ribose in rRNA ^(215, 223) has been observed in isolates with or without *rrs* mutations ⁽²²²⁾. More recently, mutations in the promoter region of the *eis* gene, such as polymorphisms at positions –10 and –35, have been reported to account for 80% of the clinical isolates with low-level resistance to kanamycin ^(210, 227). These polymorphisms overexpress the enzyme aminoglycoside acetyltransferase (*eis*) which induce acetylation of Km, modifies the drug structure and alters Km binding to its target ⁽²²⁷⁾.

▪ **Para-aminosalicylic acid**

Para-aminosalicylic acid (PAS) was one of the first antibiotics used in the treatment of TB together with INH and STM and is now used in second-line treatment regimens DRTB. PAS acts as a bacteriostatic agent with an MIC of 0.5–2 µg/ml for *M.tb* ⁽¹⁴⁴⁾ by interfering with folic acid biosynthesis ⁽²²⁸⁾ and inhibition of iron uptake ⁽²²⁹⁾. The mechanism of PAS resistance is not well understood. Mutations in the *thyA* gene encoding thymidylate synthase, which reduce the utilization of tetrahydrofolate, accounts for 40% of PAS resistance ^(228, 230). However, the T202A *thyA* mutation was found to be a phylogenetic marker associated with the Latin American strain families. Mutations in *folC* gene encoding dihydrofolate synthase ⁽²³¹⁾ and mutations in *ribD* gene encoding an enzyme of the folate pathway has been associated with resistance to PAS ⁽²³²⁾. Further studies are needed to elucidate PAS resistance mechanism in clinical isolates ⁽²³³⁾.

▪ **Ethionamide**

As a derivative of isonicotinic acid and a structural analogue of INH, Ethionamide (Eto), is a prodrug that requires activation by the mono-oxygenase enzyme encoded by the *ethA* gene. Eto is a bactericidal agent only against *M.tb* at the MIC of 0.5–2 µg/ml ^(234, 235). Once activated, ethionamide inhibits mycolic acid synthesis pathway during cell wall biosynthesis by inhibiting the same target as INH, i.e., enoyl-acyl carrier protein reductase (ENR) enzyme encoded by *inhA* gene ⁽¹⁵²⁾. Resistance to ethionamide is mediated by mutations in the *etaA/ethA*, *inhA* genes and *ethR*, a transcriptional repressor of *ethA* gene ⁽²²¹⁾. Mutations in the *inhA* gene confer resistance to both INH and ethionamide ^(234, 236). High-level resistance to Eto (MIC of ≥ 25 mg/L) had various mutations in one or more of the ethionamide resistance genes, while low-level resistance to Eto (MIC of 2.5–10 mg/L) was associated mainly with mutations in the *ethA* gene ⁽²³⁷⁾. A recent study has demonstrated the role of the *mshA* gene, encoding an enzyme essential to mycothiol biosynthesis, in spontaneous INH- and ethionamide-resistant mutants ⁽²³⁸⁾. EthA also activates thioamide drugs such as thioacetazone, thiocarlide, and thiobenzamide, which explains the cross-resistance between these agents when mutations in EthA occurred ⁽²³⁹⁾.

▪ **Bedaquiline**

Bedaquiline (TMC207) is a new class of TB drug, the diarylquinolines. It is highly active against both growing and non-growing *M.tb* population (MIC 0.03 mg/L) by targeting mycobacterial *F₁F₀*

proton adenosine triphosphate (ATP) synthase, inhibiting bacterial respiration^(240, 241). The synergy combination between Bedaquiline and PZA has been demonstrated the highest sterilizing effects in the mouse model⁽²⁴⁰⁾. Resistance to bedaquiline is caused by mutations in the *atpE* gene encoding the mycobacterial F₁F₀ proton ATP synthase, a key enzyme in ATP synthesis and membrane potential generation^(240, 241). *atpE* D28N and A63V mutations were described in two clinical isolates of M.tb associated with an MIC of 0.12 and 1.00 mg/L, respectively⁽²⁴²⁾. Mutations in *rv0678* leading to upregulation of efflux pump MmpL5 and in *pepQ* gene were recently found to cause low-level bedaquiline resistance and cross-resistance to clofazimine⁽²⁴³⁻²⁴⁵⁾.

▪ Delamanid

Delamanid (formerly known as OPC-67683) inhibits the synthesis of mycobacterial cell wall through methoxy-mycolic and keto-mycolic acid pathways⁽²⁴⁶⁾. Delamanid is a prodrug that requires activation by deazaflavin-dependent nitro-reductase, which is encoded by *ddn* gene. Delamanid has been reported to be highly active against M.tb with an MIC range of 0.006–0.24 mg/L⁽²⁴⁷⁾. Resistance to Delamanid has been shown to be associated with mutations in the genes involving in prodrug activation (*ddn* and *fgd1*), or in genes associated with the F420 biosynthetic pathway (*fbiA*, *fbiB* and *fbiC*)⁽²⁴⁸⁾. Mutation D49Y in the *fbiA* gene and a frameshift mutation in codon 49 of the *fgd1* gene have been recently reported to link with increasing phenotypic delamanid resistance by Bloemberg *et al*⁽²⁴⁹⁾.

▪ Linezolid

Linezolid (Lzd), a member of oxazolidinones, has long been used for the treatment of resistant Gram-positive bacterial infections such as methicillin-resistant *Staphylococcus aureus* (MRSA)⁽²⁵⁰⁾. It also has significant activity against M.tb, with an MIC of 0.125–0.5 µg/ml⁽²⁵¹⁾. Due to its toxicity issues including peripheral neuropathy, optical neuropathy, hepatic dysfunction, etc.,⁽²⁵²⁾ the use of Lzd is only recommended in patients with MDR or XDR-TB or with very few or no treatment options remaining⁽²⁵³⁾. The newer Lzd derivatives with less toxicity are currently being tested in phase I and II clinical trials⁽²⁵⁴⁾. Lzd acts as an inhibitor of the bacterial protein synthesis in M.tb by binding to the V domain of the 23S rRNA peptidyl transferase and blocking the binding of tRNA to the peptidyl-transferase center (PTC) on the 50S bacterial ribosomal^(255, 256).

Resistance to linezolid has been associated with mutations in the 23S rRNA (*rrl*) gene⁽¹⁴⁴⁾ with mutations at G2061T and G2572T conferring high-level resistance to Lzd (MIC 16–32 mg/L) in *in vitro* selected mutants⁽²⁵⁷⁾. In contrast, low-level resistance mutants (MIC 4–8 µg/ml) have no mutations in the *rrl* gene⁽²⁵⁷⁾. Mutations in the *rpIC* gene, such as C154R and T460C, encoding the 50S ribosomal L3 protein, which had been previously reported to cause resistance to Lzd in *S. aureus* has also been reported for M.tb, mostly observed in isolates with low-level resistance to Lzd^(257, 258). Recent study in China suggests that the resistance to linezolid were observed in 11% of MDR-TB strains but only 30% of them acquired mutations in 23S rRNA gene or the *rpIC* gene⁽²⁵⁹⁾, suggesting that the mechanisms of resistance to linezolid still need to be uncovered⁽²³²⁾.

▪ Clofazimine

Clofazimine (Cfz), conventionally used for the treatment of leprosy caused by *M. leprae* ⁽²⁶⁰⁾, is also active against *M.tb* at MIC 0.5–2 µg/ml ⁽²⁶¹⁾. The drug was added in the MDR-TB Bangladesh regimen (9-12 months standardized regimen comprising high-dose Gfx, alongside Cfz, EMB and PZA throughout, supplemented by Km, Pto and high dose INH during the intensive phase of a minimum of 4 months) and showed a promising result with a high relapse-free cure rate of 88% ^(93,262). Therefore, Cfz is now included in the new standardized short-course regimen recommended by WHO for the treatment of DR-TB ⁽⁹⁰⁾. The precise mechanism of action of Cfz are poorly understood. However, it is probably a prodrug that may involve in the production of reactive oxygen species ⁽²⁶³⁾, the inhibition of energy production through inhibiting NDH-2 (NADH dehydrogenase) and membrane disruption ⁽²⁶⁰⁾. The resistant mechanism to Cfz has not been clearly understood either. Recent studies showed that resistance to clofazimine has been linked to cross-resistance with bedaquiline through mutations in a transcriptional regulator Rv0678, leading to upregulation of efflux pump MmpL5 ^(243, 244). Two new genes, *pepQ* and *rv1979c*, were found to be associated with Cfz resistance ⁽²⁶⁴⁾. The mutation in the *rv1979c* gene do not show any cross-resistance to bedaquiline ⁽²⁶⁵⁾.

2.5.3. Compensatory evolution

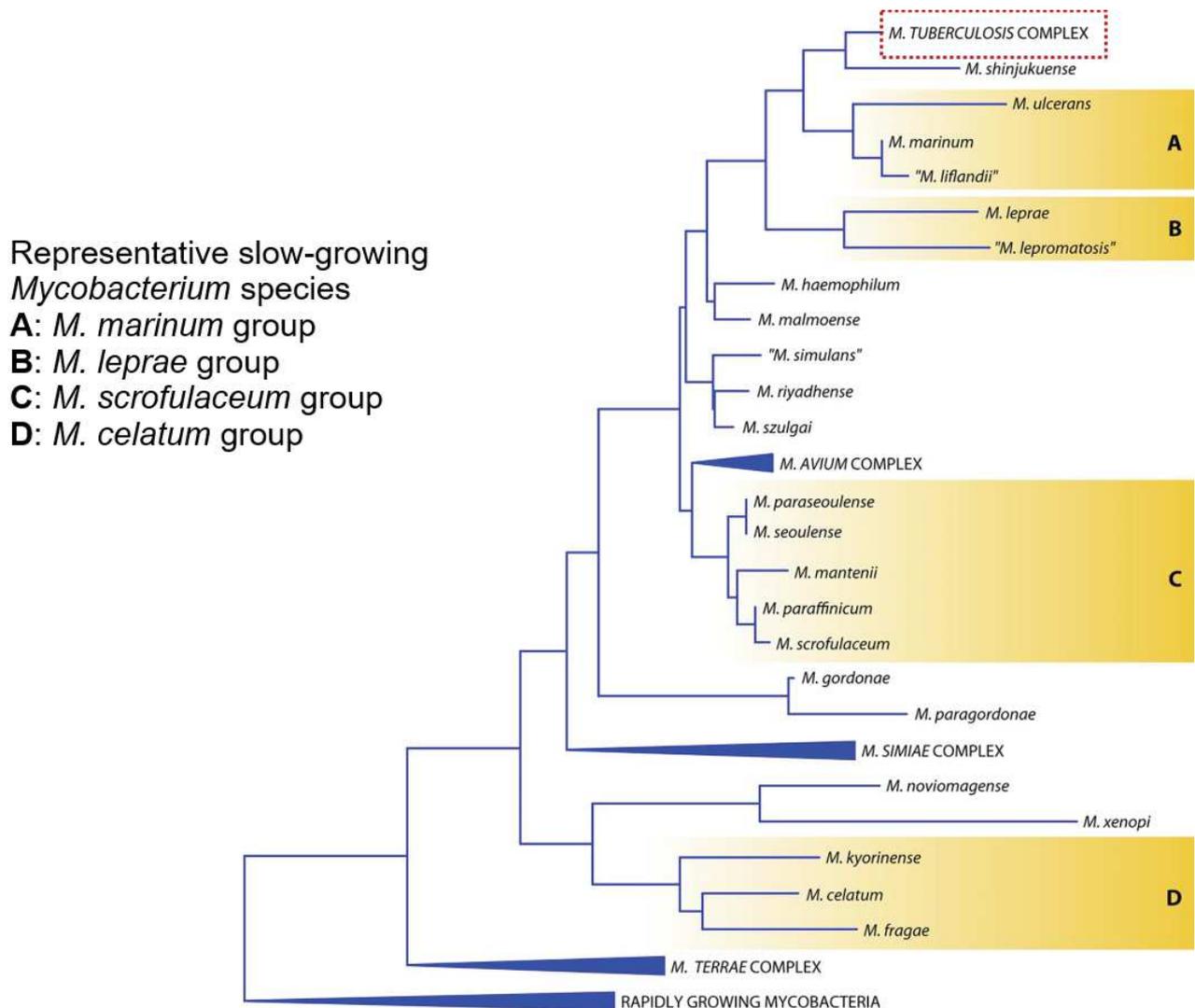
Resistance mutations bear a fitness cost to the bacteria, however in *M.tb*, studies have demonstrated compensatory mechanisms for the impaired fitness through the co-occurrence of secondary mutations. Most of these mutations occurred in genes encoding the same protein or genes involved in similar metabolic pathways (**Table I-3**) ⁽¹⁷²⁾. However, the data on compensatory mutation are still scarce and mainly focused on INH and RIF resistant isolates ⁽²⁶⁶⁾. Mutations in the regulatory region of the *ahpC* (alkyl hydroperoxidase reductase) gene, leading to overexpression of this gene are believed to be compensatory for the loss of *katG* activity in INH-resistant *M.tb* isolates ⁽²⁶⁷⁾. More recently, mutations in RNA polymerases *rpoA*, *rpoC* and secondary mutation in *rpoB* were compensatory for the loss of fitness mediated by mutations in the *rpoB* gene in RIF-resistant isolates ⁽²⁶⁸⁻²⁷⁰⁾. These compensatory mutations associated with increased fitness and transmissibility of drug resistant strains may have implications for the emerging epidemic of MDR-TB ⁽¹⁷²⁾. Little is known for compensatory mechanisms toward other drugs.

2.5.4. Phylogeny and genetic diversity of *Mycobacterium tuberculosis*

2.5.4.1. ORIGIN OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

The *Mycobacterium* genus currently encompasses almost 200 species, most of which are environmental organisms ⁽²⁷¹⁾. Mycobacteria traditionally has been divided into fast-growers and slow-growers and classified in to three major groups; *Mycobacterium tuberculosis* Complex (MTBC) – causative agent of TB, *Mycobacterium leprae* – causative agent of leprosy and NTM, also

known as environmental mycobacteria ^(272, 273). The members of MTBC include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. microti*, *M. orygis*, *M. mungi* and *M. canettii* ⁽²⁷⁴⁾. Robust phylogenetic reconstructions of the evolutionary pathway of mycobacteria by comparing genetic sequences of conserved genes suggested that rapidly growing species descended directly from ancestral mycobacteria, while slowly growing mycobacteria belong to a single evolutionary branch originated from the rapidly growers **(Figure I-7)** ^(274, 275). Therefore, the MTBC, a group of closely-related slow-growing mycobacteria, were believed to adapt from environmental organism to an obligate pathogen which acquire the ability to infect, cause disease and transmit between individuals ⁽²⁷⁶⁾.



Source: Adapted from Tortoli, 2014 (275).

Figure I-7. Phylogenetic tree of the *Mycobacterium* genus based on the 16S rRNA gene.

2.5.4.2. EVOLUTIONARY HISTORY AND CLONAL EXPANSION OF MTBC

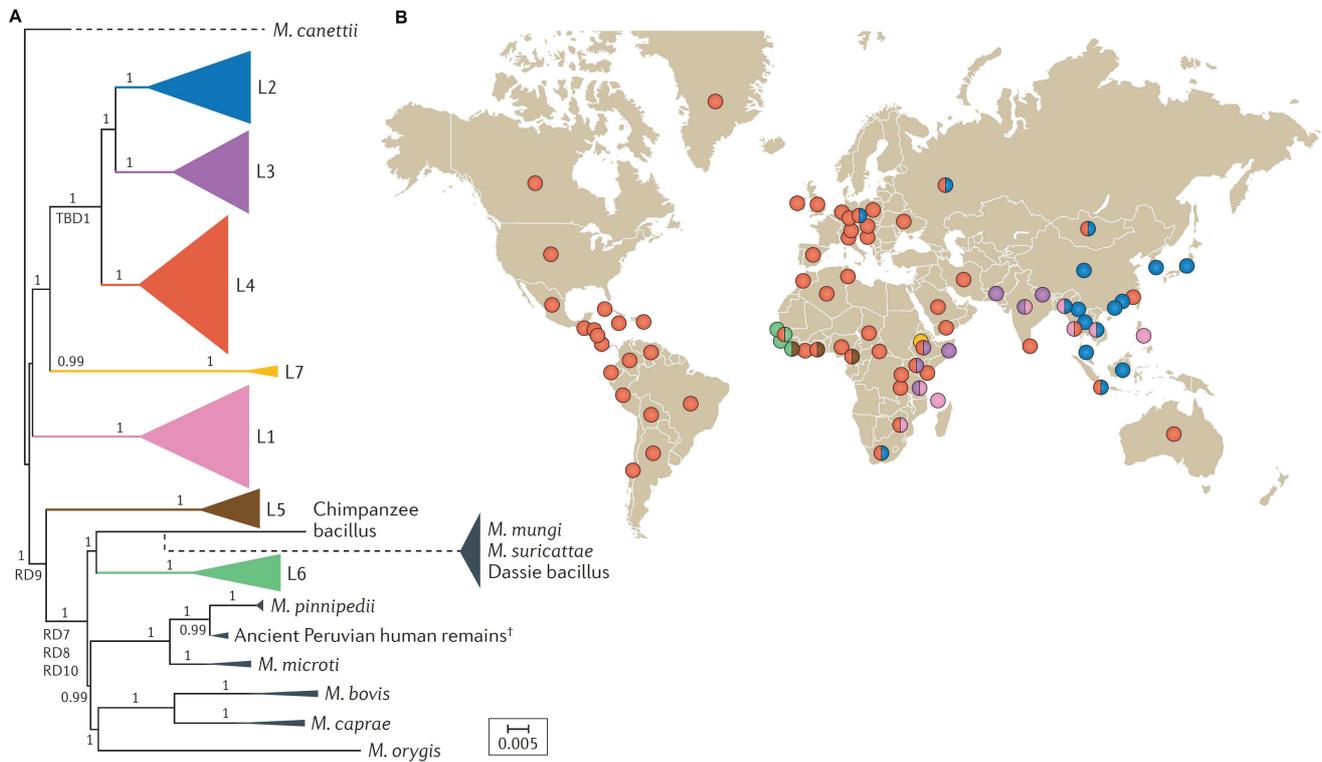
A recent advances in whole genome sequencing allows a better understanding of the origin of the MTBC and its molecular evolution and population genetic characteristics ⁽²⁷⁷⁾. The member of MTBC are sharing 99.9% DNA sequence identity. Although the members of the MTBC display different phenotypic characteristics and primary host ranges, their unusually high degree of conservation in their housekeeping genes indicates that the total population resulted from clonal expansion

from a recent evolutionary bottleneck occurred between 15 000 and 35 000 years ago ^(113, 272, 277, 278). Most evidence to date supports that the *Mycobacterium canettii*, a smooth tubercle bacillus (STB), is the closest relative of the common ancestor of the MTBC ^(272,277). Africa is the most likely region of origin of MTBC since it is the only continent with the largest diversity of human-adapted MTBC, including ancient L5, L6 and L7 which are restricted to the African continent, and it is where *M. canetti*, the probable common ancestor of the MTBC, were exclusively reported ⁽²⁷⁷⁾. Accompanied migrations of modern humans out of Africa and expanded as a consequence of the human population density increase during the Neolithic period, MTBC has evolved together with humans and spread worldwide ⁽⁴⁾.

2.5.4.3. LINEAGE CLASSIFICATION AND DISTRIBUTION OF THE MAIN HUMAN-ADAPTED MTBC LINEAGES

Human TB is mainly caused by *M. tuberculosis sensu stricto* and *M. africanum*, which can further divided into seven phylogenetic lineages (L): Lineage 1 (Indo-Oceanic, mainly East African-Indian family), Lineage 2 (East-Asian, mainly Beijing family), Lineage 3 (East-African-Indian, mainly CAS family), Lineage 4 (Euro-American) and Lineage 7 (Ethiopian lineage) belong to *M. tuberculosis sensu stricto*, whereas Lineage 5 and Lineage 6 belong to *M. africanum* West Africa 1 and 2, respectively **(Figure I-8)** ⁽²⁷⁹⁾. Beside, animal-adapted strains branch next to L6 *M. africanum* and affect various mammalian animal species ⁽²⁸⁰⁾. Based on the presence or absence of a genomic deletion known as TbD1, MTBC can be classified into “ancient” and “modern” strains. The TbD1-deleted strains, also known as modern strains, are comprised of three lineages (L2, L3 and L4), whereas others lineages (L1, L5, L6 and L7) with the presence of TbD1 are referred as ancient strains.

Among the human-adapted MTBC lineages, some occur globally and others are strongly associated with specific geographical regions ⁽²⁸¹⁾. Africa harbors the largest diversity of human-adapted MTBC and it is the only continent that all seven human-adapted MTBC lineages present. In addition, L5, L6 and L7, which are restricted to the African continent; L5 and L6 are restricted to Western African countries ⁽²⁸²⁾ and L7 is limited to Ethiopia ⁽²⁸³⁾. L1 (Indo-Oceanic) ⁽²⁸³⁾ occurs around the Indian Ocean, extending from Eastern Africa to Melanesia ⁽²⁸⁴⁾. Similar to L1, L3 has its distribution around the Indian Ocean with a stronger presence in Northern Africa, and a broader distribution across Southern Asia, but it does not extend into Southeastern Asia ⁽²⁸⁴⁾. L2 and L4 are generalist lineages which are found globally ⁽²⁸⁵⁾. L2 including the Beijing family has expanded and emerged worldwide with a predominance in East- and South East-Asia ^(284, 285). The recent emergence of L2–Beijing sublineage has been linked to increased transmission, virulence and drug resistance ^(280,286). L4 (Euro-American mainly LAM, T, X, H and S families) is well dispersed with a broad distribution in Europe and America but also in Africa and the Middle-East ⁽²⁸⁴⁾. The global population structure of MTBC strains is influenced by significant demographic changes and large-scale movements in human populations, therefore the increases in human population, urbanization and global travel could lead to homogenization of the global population structure of MTBC ⁽²⁸⁷⁾.



Source: Adapted from Gagneux, 2018 (277).

(A) Genome-based phylogeny of the *Mycobacterium tuberculosis* complex (MTBC) rooted with *Mycobacterium canettii*;
 (B) Global distribution of the seven main human-adapted MTBC lineages.

Figure I-8. Figure Global phylogeography of the human-adapted MTBC.

2.6. Current methods for molecular typing of *M. tuberculosis*

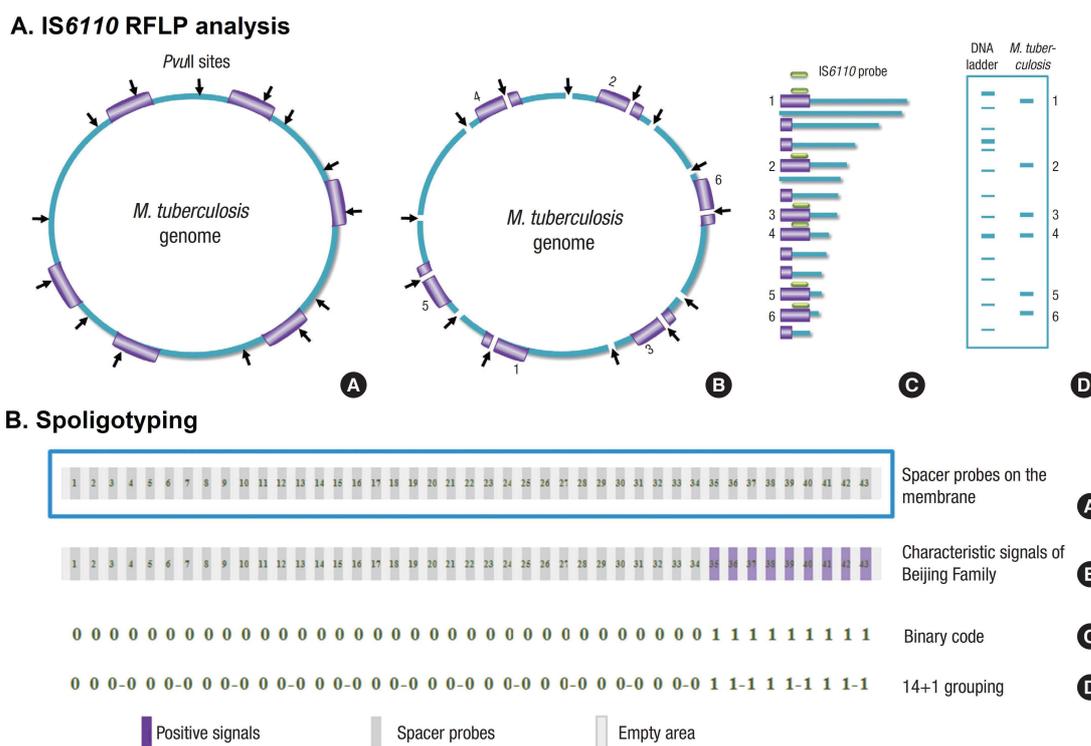
Molecular typing, first introduced since the early 1990s, has revolutionized epidemiological studies of infectious diseases, including TB ⁽²⁸⁸⁾. In combination with conventional epidemiological approaches, so-called molecular epidemiology is widely used to understand the key issues in the epidemiology of TB, including (i) disclosing sources of infection, (ii) estimating the extent of recent transmission, (iii) identifying risk factors for TB transmission in a community, (iv) distinguishing between exogenous reinfection and endogenous relapse, (v) identifying laboratory cross-contamination, (vi) tracking the geographic distribution and clonal expansion of specific strains, and (vii) determining the genetic basis behind specific phenotypic characteristics (virulence, organ tropism, transmissibility, or resistance to antimicrobial drugs) ⁽²⁸⁹⁾. Molecular typing is based on the exploitation of the presence of various polymorphisms of the genetic markers in the genome of the bacteria, such as CRISPRs, unique SNPs, insertion sequences, Regions of Difference (RD) or tandem repeats etc., in order to differentiate various strains and to study the evolutionary relationships between them ⁽²⁹⁰⁾. Ideally, those molecular methods should be reproducible, stable, easy to perform and interpret, cost-effective and time-effective and have high discriminatory power and epidemiologic concordance ⁽²⁹⁰⁾.

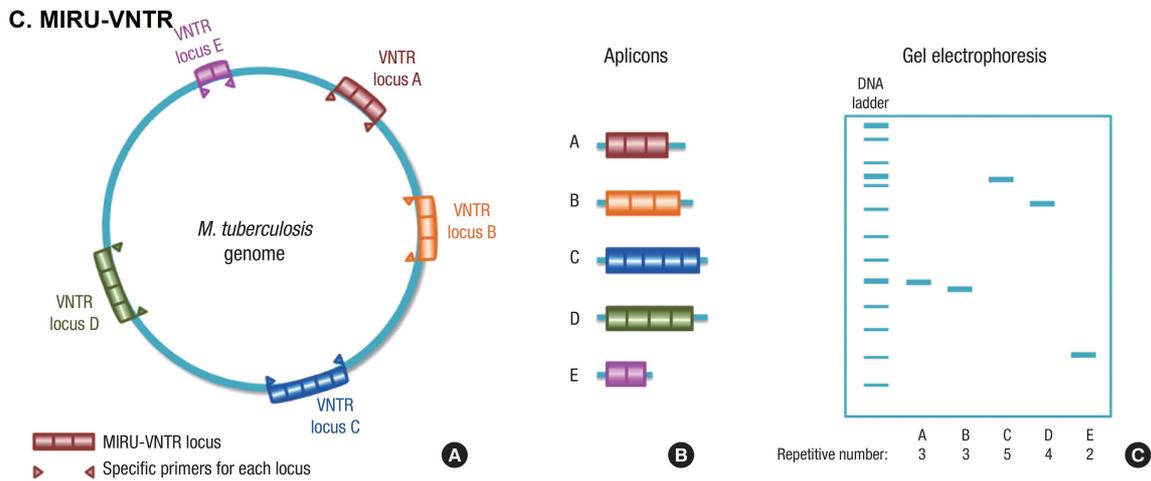
Over the last years a diverse range of molecular tools have been developed to characterize and type MTBC, however, only some of these methods have been widely applied. The gold standard for molecular genotyping of *M.tb* is insertion sequence IS6110 restriction fragment length polymorphism

(RFLP) analysis ^(288, 291). However, similar discriminatory power and greater convenience has been demonstrated with other methods such as spacer oligotyping (Spoligotyping) ⁽²⁹²⁾; mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) ⁽²⁹³⁾ and repetitive-sequence-based PCR (rep-PCR) ⁽²⁹⁴⁾. With a rapid improvements in whole genome sequencing (WGS) and the next-generation sequencing (NGS) technologies, comparison of nearly complete genome sequences (whole genome sequencing) of *M.tb* strains is progressively becoming the method of choice, especially in complex outbreak situations ⁽²⁹⁵⁻²⁹⁷⁾.

2.6.1. IS6110-RFLP Analysis

IS6110-RFLP analysis, also known as IS6110 DNA fingerprinting, has been the standard approach to genotyping of *M.tb* for more than a decade ⁽²⁹¹⁾. Insertion sequence IS6110, mobile elements with 1,355 bp long belonged to the IS3 family, is found only within the MTBC. In most other members of the complex, except for *M. bovis* that normally contains only one copy, the sequence IS6110 is present in multiple copies ranging from 0 to 25 ⁽²⁹⁸⁾. IS6110-RFLP technique is based on the high degree of IS6110 polymorphism in terms of copy number and positions in the genomes of clinical isolates ⁽²⁹⁹⁻³⁰¹⁾. A schematic diagram of the RFLP method is shown in **Figure I-9A** ⁽²⁸⁸⁾. In brief, the methodology includes (**Figure I-9A-A**) genomic DNA digestion with the *PvuII* restriction enzyme ; (**Figure I-9A-B**) cleavage of IS6110 sequence into fragments of different size ; (**Figure I-9A-C**) DNA fragments separation by gel electrophoresis are transferred onto a membrane through Southern blot hybridization which is carried out with a peroxidase-labeled probe complementary to part of the 3'-end of the IS6110 sequence and (**Figure I-9A-D**) visualized fragment represents a single copy of IS6110 surrounded by different lengths of flanking DNA sequences of different lengths ⁽²⁹¹⁾.





Source: Adapted from *Ei et al, 2016* ⁽²⁸⁸⁾

Figure I-9. Principle of the three commonly used genotyping methods of MTBC.

The *IS6110* DNA method is highly discriminatory and reproducible, and its pattern are stable over time ⁽²⁸⁸⁾. In addition, the use of computer-based analysis software (e.g., Bionumerics), the creation and sharing of databases ⁽³⁰²⁾, and the international standardization of the method ⁽²⁹¹⁾ allow its worldwide application ⁽²⁸⁸⁾. However, *IS6110*-RFLP method has several important limitations including (i) low discriminatory power in isolates presenting five or fewer *IS6110* copies frequently isolated from Asian, South East Asian and some African countries ⁽³⁰³⁻³⁰⁵⁾; (ii) labor intensive and requires large amounts of DNA purified from well-grown cultures for restriction enzyme digestion and (iii) requires sophisticated and expensive computer software as well as experienced personnel of high technical expertise ^(289,306). Due to the limitations of this referent method, different alternative techniques based on PCR such as MIRU-VNTR or spoligotyping have been evaluated ⁽³⁰⁶⁾.

2.6.2. Spoligotyping

Spoligotyping, PCR-based method, is one of the most frequently used genotyping methods to study the phylogeography of MTBC ⁽³⁰⁶⁾. This hybridization assay is based on the reverse blot detection of variability of 43 spacer sequences in the direct repeat (DR) region that is present in all MTBC isolates ⁽²⁹²⁾. The DR region comprises a series of well-conserved 36 bp direct repeats sequence (DRs) separated by unique, nonrepetitive spacer sequences of 34–41 bp ⁽³⁰⁷⁾. In the spoligotyping method (**Figure I-9B**), the entire DR locus is amplified by PCR using primers (one of the primers is biotinylated) that are complementary to the sequence of the short DRs and hybridized to a membrane with 43 covalently bound synthetic oligonucleotides (**Figure I-9B-A**). The hybridization signals are detected by chemiluminescence through biotin labeling of the PCR products and a streptavidin-peroxidase conjugate system and then visualized by autoradiography ⁽³⁰⁶⁾. Each of the spacers produces either a dark band (indicating the presence of the spacer) or no band (indicating the absence of the spacer) and a complete 43-spacers set represents the polymorphic spacers of each isolates (e.g. characteristic signals of Beijing family with spacers 35–43) (**Figure I-9B-B**). The pattern is converted to a 43-digit

binary code system that has 1 and 0 (1: the band is present and 0: the band is absent) (Figure I-9B-C). Then the 43-digit binary code is converted to a 15-digit octal numbers (Figure I-9B-D). The code-like patterns represent each strain can be analyzed using international spoligotyping database ^(292, 308-310).

Spoligotyping is a simple, cost-effective, high-throughput method and can differentiate *M.tb* strains with low *IS6110* copy numbers (≤ 5 bands in RFLP patterns) ⁽³¹¹⁾. For high-throughput analysis, the Luminex platform and multiplexed primer extension-based spoligotyping assay using automated matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can be used as an alternative to a conventional spoligotyping by eliminating the membrane step ⁽³⁰⁶⁾. Another important advantage of spoligotyping is its high sensitivity, estimated at 10 fg of chromosomal DNA equivalent to DNA from 2-3 bacterial cells, allowing the method to be applied directly in clinical samples ⁽³¹²⁾. However, compared with *IS6110* DNA fingerprinting and 24-locus MIRU-VNTR typing, spoligotyping has a lower discriminatory power due to its targets for only a single genetic locus, covering less than 0.1% of the MTBC genome ⁽³⁰⁶⁾. By adding some of the newly discovered spacers, the 68-spacer format was shown to slightly improved the resolution of the method for the *M. africanum* subspecies and for the East African-Indian (EAI) clade of *M.tb* ^(313, 314). In molecular epidemiological studies, spoligotyping, when used alone, is not sufficient for epidemiological linking studies. It could be performed as a first-line screening test, to be followed by another high-resolution method ^(315, 316).

2.6.3. MIRU-VNTR typing

Mycobacterium tuberculosis was among the first bacterial species in which tandem repeat loci similar to minisatellite loci in eukaryotic genomes were identified. Their repeat numbers are highly variable in many loci and therefore are called "variable number tandem repeat" (VNTR) loci ^(317, 318). However, as new VNTR-type loci have been discovered, they have been referred to under different names, such as major polymorphic tandem repeat (MPTR), exact tandem repeat (ETR) ⁽³¹⁹⁾ and mycobacterial interspersed repetitive units (MIRU) ⁽²⁹³⁾. MIRU were originally described by Supply *et al.* ⁽²⁹³⁾ as 46–101 bp repetitive sequences scattered at 41 loci throughout the mycobacterial chromosome, mainly in intergenic regions ⁽³²⁰⁾. The MIRU-VNTR markers are stable and evolve more slowly than *IS6110* which is suitable for long-term epidemiologic analysis ⁽³²¹⁾. In 2001, Supply *et al.* select 12 hypervariable loci for genotyping of *M.tb* isolates ^(320, 321). With the 12-loci, the discriminatory power of MIRU-VNTR typing is slightly less than that of *IS6110*-RFLP for analysis of *M.tb* isolates with high copy numbers of *IS6110* ⁽³²¹⁻³²³⁾ and for discriminating the Beijing family strains ^(324, 325). In 2006, Supply *et al.* ⁽³²⁶⁾ has optimized and standardized the combination of MIRU-VNTR loci for strain genotyping; the 15-loci (including 6 previously investigated) were selected as the new standard for epidemiologic discrimination of *M.tb* and the 24-loci (including 12 previously investigated) as a high-resolution tool equal to that of *IS6110*-RFLP for phylogenetic studies ^(327, 328). Additional hypervariable loci such as VNTRs 3232, 3820, and 4120 has been recommended to be added to standardized loci set for second-line typing of Beijing strains ⁽³²⁹⁾.

A schematic diagram of the MIRU-VNTR genotyping method is shown in **Figure I-9C**. The principle of the MIRU-VNTR typing system is PCR analysis of selected variable tandem repeat loci (**Figure I-9C-A**) with specific primers complementary to the flanking regions by gel electrophoresis or, fluorescence-based sequencer (**Figure I-9C-B**). The estimated size (bp) of the amplicon reflects the numbers of tandem repeat and is converted into numerical code (**Figure I-9C-C**)⁽³³⁰⁾. The final multidigit numerical code (so-called MIRU-VNTR code), corresponding to the repeat number at each locus was used for analysis and compared across laboratories worldwide^(323, 326). This PCR-based MIRU-VNTR typing is rapid, easy-to-perform and highly specific and highly reproducible. It can generate portable digit-based data which allow the investigators can easily compare the genotypic data of different independent studies with a large number of strains^(320, 323, 330, 331). At the same time, a free web-based tool, named *MIRU-VNTRplus*, was set up for the analysis and construction of M.tb MIRU-VNTR genotypes database⁽³²³⁾ which allows the worldwide epidemiologic surveillance of tuberculosis^(319, 330). Another attractive potential of MIRU-VNTR is its use for phylogenetic analysis, although more comprehensive marker sets, such as large sequence polymorphisms and SNP are required. With the advantages, VNTR surpassed IS6110 RFLP and became the first line genotyping method for molecular epidemiological studies⁽³³²⁾. Despite its great advantages, for certain strains, some specific loci could not be typed using MIRU-VNTR typing due to unexpectedly large numbers of repeat units or deletion of some loci⁽³²⁹⁾.

2.6.4. SNPs typing

The analysis of genetic polymorphisms at the nucleotide level, SNP, are amenable to targeting multiple genetic markers that allow to determine the occurrence of mutations causing drug resistance and to differentiate clinical isolates as well as to study the phylogenetic relatedness of clinical strains⁽⁴⁹⁾. Two types of SNP, synonymous SNP (sSNP) and nonsynonymous SNP (nsSNP), provide useful genetic information^(49, 333). In general, sSNP are considered functionally neutral and do not alter the amino acid profile. When occurring in structural or housekeeping genes, the sSNP can provide the basis to study genetic drift and evolutionary relationships among mycobacterial strains as well as the different epidemiology in a given population⁽⁴⁹⁾. In contrast, nsSNP cause changes in amino acids under internal or external selection pressure. When occurring in drug resistance-determining genetic loci, it can result in phenotypic drug resistance^(49, 334). The detection of nsSNP in drug resistant genes can help us to understand the spread, the mechanisms of drug resistance and the nature of the drug resistant isolates within the populations⁽⁴⁹⁾.

The SNP might be detected by restriction endonuclease analysis (REA)⁽³³⁵⁾ or by a variety of PCRs^(336, 337). Modern technology provides several efficient methods to analyze several SNP sites at a time. These include using molecular beacons⁽³³⁸⁾, melting temperatures obtained by real-time PCR curve analysis⁽³³⁹⁾, SNaPshot analysis (Thermo Fisher Scientific) with a capillary electrophoresis instrument⁽³⁴⁰⁾, iPLEX Gold technology⁽³⁴¹⁾, microarray technology⁽³⁴²⁾, Multiplexing by a multiplex

ligation-dependent probe amplification assay (MLPA)⁽³⁴³⁾, Magpix reader (Luminex, Austin, TX, USA)^(344, 345). SNP is considered as one of the most reliable markers for MTBC lineage classification^(333, 346, 347), however, the use of SNP typing was limited by the need for a large set of genes to obtain reach satisfactory discriminatory power⁽³⁴⁸⁾.

2.6.5. Whole-genome sequencing

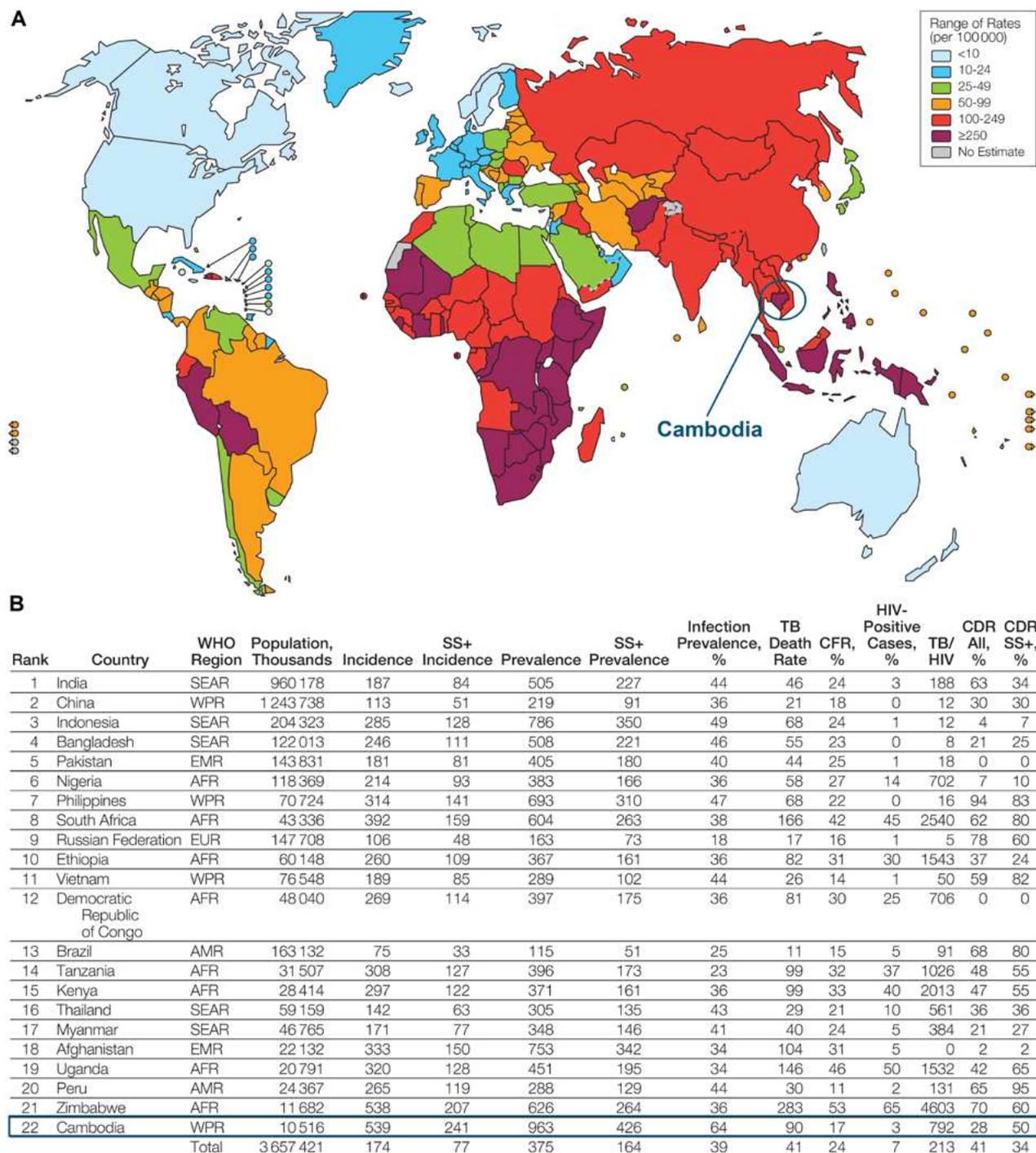
The most recent breakthrough in the field of molecular epidemiology is the availability of relatively affordable, high-throughput WGS technology which allow us to examine outbreaks of the strains genetically indistinguishable by previously described methods^(296, 349, 350). WGS-based genotyping using Next Generation Sequencing (NGS) technology offers a comprehensive genetic information including genomic markers, drug resistance profile, virulence determinants, and genome evolution^(351, 352). It can fully detect recent transmission chains and distinguish between genetically closely related strains which could not be distinguished using other genotyping methods which only rely on polymorphic genetic markers that represent less than 1% of the genome⁽³⁵³⁾. The conceptual NGS workflow includes DNA extraction, library preparation, sequencing and data analysis. Several widely used NGS technologies which allow the production of massively parallel DNA reads with simple and stepwise preparation of the sample prior to DNA sequencing include Roche/(454) FLX, Illumina/Solexa Genome Analyzer, Applied Biosystems SOLiD™ System, Helicos Heliscope™, Ion Personal Genome Machine (Ion PGM), Pacific Biosciences Single Molecule Real Time (SMRT) and nanopore sequencing instruments⁽³⁵⁴⁾.

With increasingly affordable, the WGS, which has a higher discriminatory power and a single rapid analysis simultaneously of drug resistance prediction and epidemiologic genotyping, may become a new standard for routine typing of M.tb in the near future⁽³⁵⁵⁾. Recently, WHO also suggested using WGS could improve tuberculosis (TB) management and control through the rapid and accurate detection of all clinically relevant mutations⁽¹⁸⁵⁾. However, some important limitations are needed to be addressed, such as the lack of standardization of WGS analysis pipelines, the need for specialized software for data analysis, the need of databases for sharing WGS data at a global level, and a better understanding of the molecular clocks of SNP and LSP. A number of studies were carried out to improve the standardization of WGS genotyping such as such as CLC Genomics Workbench (www.clcbio.com/products/clc-main-workbench/), and UniproUGENE (<http://ugene.unipro.ru/>)⁽³⁵²⁾, and the creation of Web-assessable databases for global TB surveillance⁽³⁵⁶⁾. More evidence remains necessary to illustrate the potential impact of WGS on patient care or design of public health strategies⁽³⁵⁷⁾.

2.7. Tuberculosis situation in Cambodia

2.7.1. Status of the TB epidemic

Tuberculosis is a serious problem of public health in Cambodia. Decades of civil war left Cambodia's health system in tatters and its prominent burden of TB. The prevalence of all form of TB was estimated at 1670 per 100.000 in 1990 ⁽³⁵⁸⁾. After the success of introduction and expansion of directly observed treatment-short course (DOTS) in 1994, the TB prevalence rate was dropped



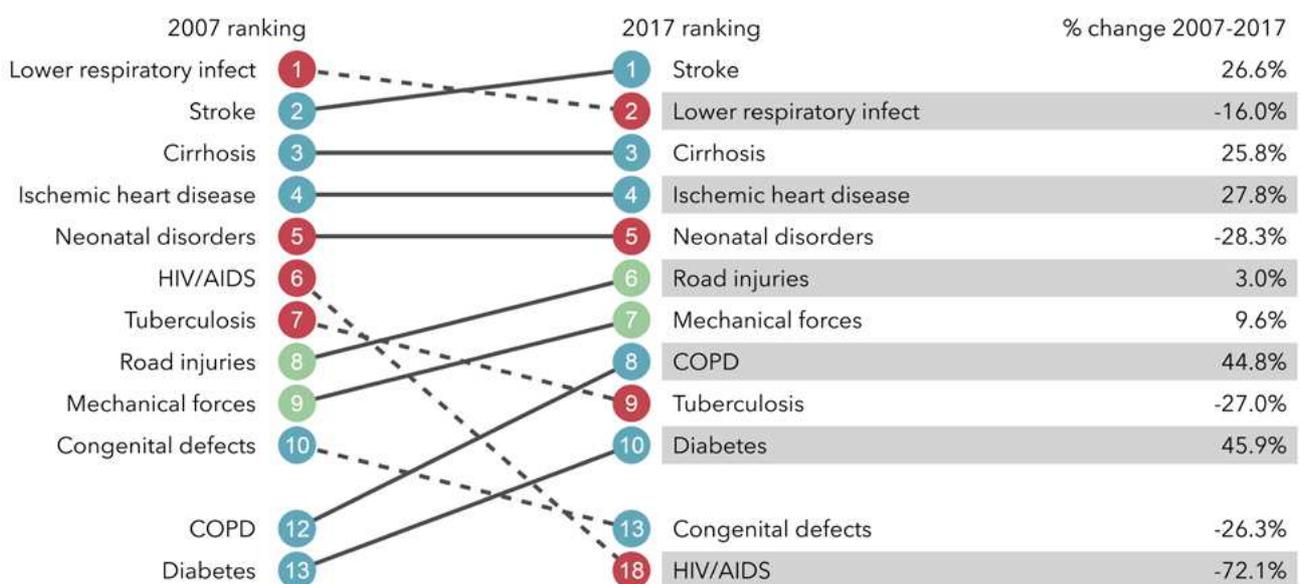
Source: Adapted from Dye C. et al, 1999 ⁽³⁵⁹⁾.

A) Estimated incidence rates of tuberculosis by Country;
 B) Estimated TB burden in the 22 highest-incidence countries.

Figure I-10. Estimated burden of all forms of tuberculosis in 1997.

to 963 per 100,000 population in 1997, a decline of more than 40% (**Figure I-10**)^(359, 360). However, Cambodia still had the highest TB prevalence rate at that time and the WHO expert estimated that approximately 64 % of Cambodian population (equivalent to 6,738,000 people) especially those living in unhygienic and overcrowded conditions, were infected with M.tb⁽³⁵⁹⁾. TB incidence rate and TB death rate were estimated at 539 per 100,000 population and 90 per 100,000 population⁽³⁵⁹⁾.

Despite a struggling medical infrastructure left in ruins after Khmer Rouge rule and civil war with only fewer than 50 doctors remained, Cambodia has made a remarkable progress in TB control and has reached its Millennium Development Goal by halving the 1990 level of TB mortality rate and TB prevalence rate in 2014⁽³⁶¹⁾. The mortality rate was dropped by 63% from 157 per 100,000 populations to 58 per 100,000 populations and the TB prevalent rate were dropped by 50% from 1670 per 100,000 population to 668 per 100,000 population⁽³⁵⁸⁾. Since 2004 after the expansion of DOTS strategy to all health centers (HC) nationwide and the introduction of WHO recommended 6 months regimen, the mortality rate and TB prevalent rate were decreased respectively 5% and 6% every year. The TB incidence rate was decreased as well, with a declined rate of 3.5% per year compared to an average global declined rate of 1.5% per year⁽³⁵⁸⁾. However, given the historically high disease burden, Cambodia continues to stand among the top 30 countries with high burden (HBC) of TB in the WHO new TB HBC list (2016–2020), compared to 22 HBC in the previous one^(2, 361). According the latest WHO Global TB report, in 2019, Cambodia has an estimated TB incidence rate of 287 cases per 100,000 population, accounting for around 47,000 new incident TB cases⁽¹⁾. As a result of a decline in the TB mortality rate with a current estimated at 17 deaths per 100,000 population (equivalent to 2,900 deaths among HIV negative TB patients), TB was shifted from the seventh to the ninth leading cause of death in Cambodia (**Figure I-11**)^(2, 362). Unfortunately, there are still about 8 people die of TB each day.



Source: Institute for Health Metrics and Evaluation, 2018⁽³⁶²⁾.

Figure I-11. Top 10 cause of death in Cambodia in 2007 and 2017.

Of 47,000 estimated incident TB cases, only 30,017 cases (63.9%) were detected and notified to the National TB Control Program (NTP) in 2019 ⁽¹⁾. Hence, approximately one third of TB cases are undetected in Cambodia. Those missing cases could potentially cause the continuous transmission of TB in the community and contribute to the challenge to end TB in the country ⁽³⁶³⁾. In order to interrupt TB transmission chains, narrowing the gap in diagnosis by targeting those missing population is essentially to reduce TB burden in Cambodia ⁽³⁶³⁾. Of all notified TB cases, 64% had pulmonary TB and half of them (54%) were bacteriologically confirmed, 52% were men and 42% were women. Children under 15 years were accounted for 6% (~1800 cases) of all notified cases, dropped from 19% equivalent to 5342 cases reported in 2018 ^(2, 364).

2.7.1.1. TB/HIV EPIDEMIOLOGICAL SITUATION

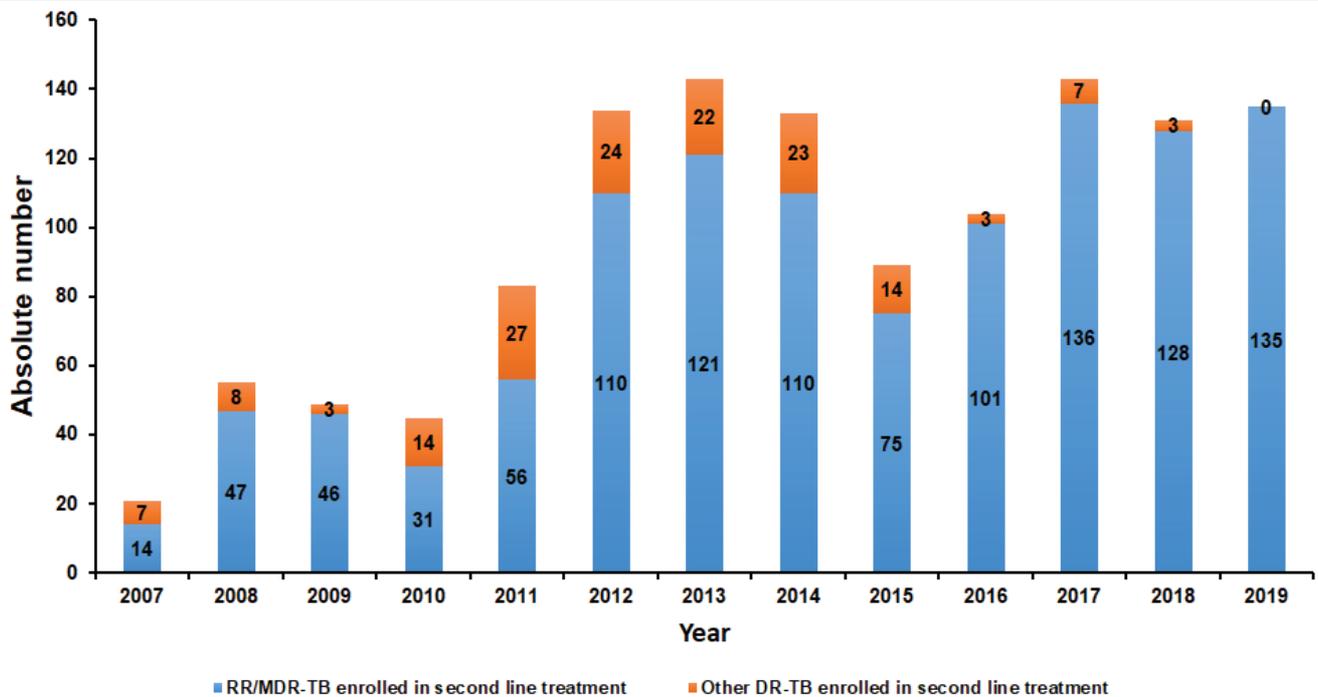
The HIV epidemic has caused explosive increases in TB incidence in Cambodia ⁽³⁶⁵⁾. After facing the fastest growing HIV epidemics in the mid-1990s, the epidemic become generalized in the country within the last two decades ⁽³⁶⁶⁾. The HIV prevalence among the adult population aged 15-49 years reached its pick at 3.3% in 1997-1998 ⁽³⁶⁷⁾ and now declines at 0.5% in 2019 ⁽³⁶⁸⁾. The overlap of the TB and HIV epidemics have synergistic health impacts in terms of disease development and progression for Cambodian over the last decade ⁽³⁶⁹⁾. The HIV prevalence among TB patients has increased from 2.5% in 1995 ⁽³⁷⁰⁾ to a peak of 11.8% in 2003 ⁽³⁷¹⁾ and declined to approximately 2.7% in 2019 ^(364, 372) along with the decline of HIV prevalence in general population. Thus, Cambodia is no longer country with high burden of TB/HIV based on the WHO new TB HBC list (2016–2020) ⁽³⁷³⁾.

2.7.1.2. DRUG RESISTANT TUBERCULOSIS

Cambodia is not among WHO HBC of MDR-TB, however the number of DR-TB is increasing overtime. In the 2000-2001 National Drug Resistant Survey (NDRS), the proportion of MDR-TB were found in 0.0% and 3.1% among new cases and retreatment cases, respectively ⁽³⁾. In 2006, during the second NDRS, the proportion of MDR-TB were found in 1.4% among new cases and 10.5% among retreatment cases ⁽³⁷⁴⁾. The proportion of MDR-TB was found even higher among new cases (3.7%) and previously treated cases (28.9%) in TB/HIV co-infected patients in one study conducted between 2007 and 2009 by Walls *et al.* ⁽³⁷⁵⁾. The author also suggested that the prevalence of drug resistant TB in Cambodia might be higher than previously reported ⁽³⁷⁵⁾. Ten years after the second survey, one XDR-TB case was reported for the first time ⁽³⁷⁶⁾.

In 2019, WHO estimated 1000 MDR/RR-TB cases among notified PTB cases (1.8% among new cases and 8.2% among retreatment cases) in the country but only 135 (13.5%) cases were identified in 2019 ^(1, 364) (**Figure I-12**). This could translate to about 85% of MDR-TB patients are not correctly diagnosed and treated. Those missing cases could play a major role in the transmission of MDR strain

in the community and the inappropriate treatment regimen could lead to the emergence of more and more resistant strain which could become untreatable with the current available anti-TB drugs. Although available data suggest that MDR-TB has not been a significant problem in the past, the missing MDR-TB cases could be major challenges in fighting the disease and pose a serious threat to the national and global health security. A more systematic routine screening would be needed to reach those missing cases.



Source: Adapted from CENAT, 2020⁽³⁷⁷⁾.

Figure I-12. Drug resistant TB cases notified between 2007 and 2018.

Under the PMDT, the resistance to SLD were performed among Xpert RIF-resistant patients using LPA test at IPC. Between 2018 and 2019 (January to July), there is an increasing in FQ-resistant from 6% (8/128) to 15% (12/80). All 20 patients with FQ-resistance had *gyrA* mutations. SLID resistance was stable at 4% (4/128 in 2018 and 3/80 in 2019). Overall, among RIF-resistance, pre-XDR were observed in 9.4% and 18.8% in 2018 and 2019, respectively⁽³⁷⁸⁾. No XDR patients were observed since the first report in 2016.

2.7.2. Tuberculosis control in Cambodia

2.7.2.1. ORGANISATION OF TB SERVICE NETWORK

Cambodia has since the early 1990's put intensive effort onto TB control after the recognition of the heavy burden of TB in the country and the lapse of TB control activities during the Khmer Rouge genocide (1975-1979) and the subsequent decade of United Nations sanctions⁽³⁶⁰⁾. The National Tuberculosis Control Program (NTP), which had struggled to establish itself in the early

1980s, relaunched its activities in 1993 with the support from WHO and international donors ⁽³⁶⁰⁾. The NTP has been operating under the responsibility of the National Center for Tuberculosis and Leprosy Control (CENAT) and within the overall national health system.

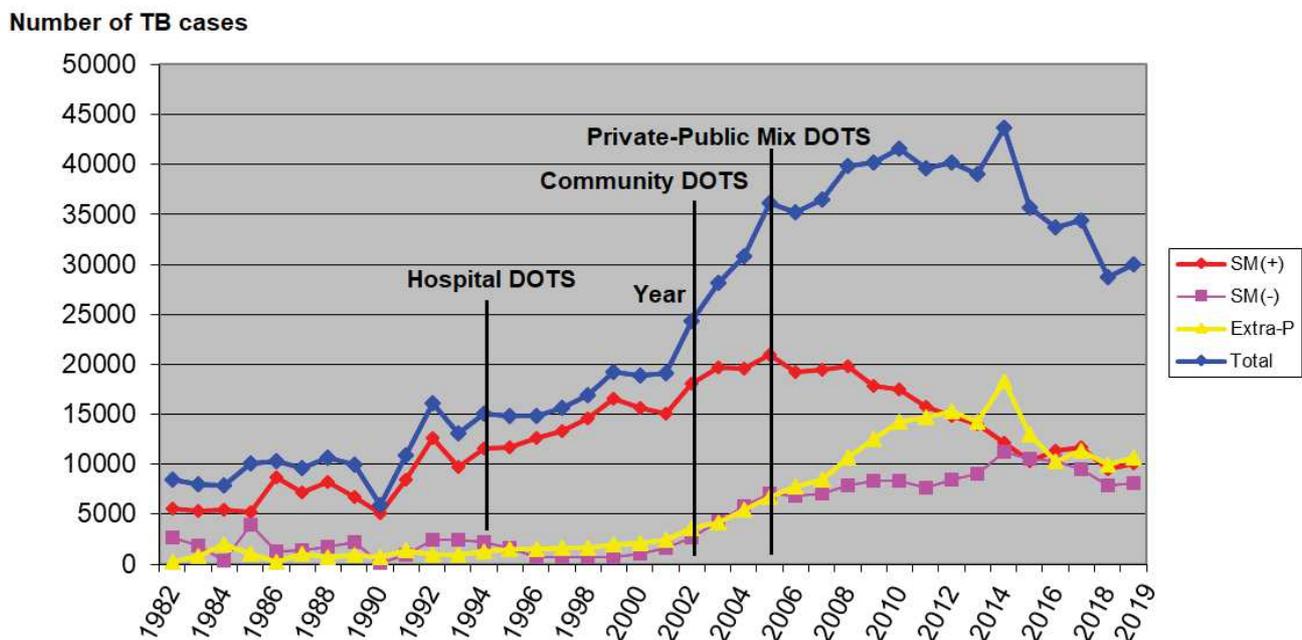
Integrated in the national health system, the TB control network is in place in all level of public health system including CENAT at the central level, 25 Municipal/provincial Health Departments (M/PHD), 94 operational districts (OD) and 1255 health facilities (102 referral hospital and 1153 HC) ⁽³⁷⁹⁾. At the central level, the CENAT is coordinating TB control activities in the country including planning, supervision, training, monitoring and research. Located in the capital city of Phnom Penh, CENAT has the NTP unit of Ministry of Health, the National TB reference Laboratory (NRL), a TB Clinic and a Chest Hospital with 130 beds ⁽³⁸⁰⁾. At the provincial level, Provincial Health Department (PHD) is responsible for planning, coordination and supervision of TB control in each province, including supporting and supervision TB activities in OD. Each OD has its referral hospital and one Health center for every 10,000 population. Each referral hospital has a TB unit with beds and TB laboratory. The OD TB supervisor is responsible for supervising the TB activities at Health Centers (HC) and reporting OD level activities to the PHD level. At the HC, primary delivery service level, usually 2 staffs were trained on DOTS including smear preparation and assigned as TB staffs. Most of the HC does not have the laboratory (about 10% of HC had TB laboratory) and the crossed trained TB staff refer TB suspects for diagnosis and facilitate DOTS of TB patients. In addition to this, a large network of community volunteers have been engaged in the Community Directly Observed treatment-short course (C-DOTS) programme to promote TB related information, identify and refer TB suspects, and serve as DOT watchers for patients in their communities ⁽³⁸¹⁾.

2.7.2.2. DIRECTLY OBSERVED TREATMENT-SHORT COURSE (DOTS)

Modern TB control began in Cambodia with a constrained introduction of DOTS, the WHO strategy for TB control, in 1994 ^(360, 382). It was administered through hospital-based TB units, including 120 TB units inside general public hospitals at provincial and district level, and admission facilitated direct observation ⁽³⁸³⁾. The admission policy is mandatory at that time due to the continuing political insecurity, costs associated with travel and preserving laboratory facilities (360). Free food was provided to TB inpatients through the World Food Programme, resulting in enhancing case-detection rate. All patients were admitted to the hospital during the first 2 months of the intensive phase under DOT by health care workers in the NTP and the continuation phase, usually in the form of fixed-dose combination (FDC), was administered without DOT ⁽³⁾. After DOTS introduction, in 1995, the treatment success rate, cure rate and conversion rate of smear-positive PTB were sharply improve to 92% (for category 1), 84% and over 90%, respectively in 1995 ⁽³⁸³⁾. The DOTS strategy was then extended to all district hospitals in 1998 and all HC nationwide in 2004. In 2019, 1325 health facilities nationwide including 1205 HC were offering DOTS treatment as well as TB diagnostic service ⁽³⁷⁷⁾. In order to reach those who have limited access to the local health facilities, further decentralization

started with C-DOTS which engage community level volunteers to provide DOTS to TB patients, in 2002 ⁽³⁸²⁾. The main purpose of C-DOTS is to improve case finding through referral of TB suspects as well as to ensure daily DOTS for TB treatment at community levels ⁽³⁷⁷⁾. In 2019, C-DOTS has been implementing in 76 OD and 1,000 HC, resulting as a result, 9,665 TB cases, equivalent to 32% of total TB cases in the country, were detected.

This significant progress in DOTS expansion results in doubling of case-finding from about 20,000 in 2001 to more than 40,000 in 2011 and improving cure rate to more than 85% **(Figure I-13)** ^(372, 382). Since 2014, there is a substantial decline in overall number of cases from 44,000 to about 30,000 in 2019, about 6% decline every year ^(372, 382). During the last 21 years, the treatment success rate of TB has been maintained over 90%; 93% of treatment success rate in 2019 ⁽³⁷⁷⁾. In addition, this major progress also help prevent the spread of new infections through as a decline of 45% among bacteriologically positive prevalence rate was observed based on the two national prevalence surveys carried out after the implementation of DOTS in all referral hospital (2002) and the coverage of C-DOTS in most part of the country (2011) ^(382, 384).



Abbreviation: TB, tuberculosis; SM+, smear-positive TB; SM-, smear-negative TB; Extra-P, extra-pulmonary TB; DOTS, Directly-Observed Treatment Short-course.

Source: Adapted from CENAT, 2019 ⁽³⁷²⁾.

Figure I-13. Tuberculosis case notifications with important interventions, 1982- 2019.

2.7.2.3. PROGRAMMATIC MANAGEMENT OF DRUG RESISTANT TUBERCULOSIS (PMDT)

Prior to 2006, there was no mechanism for public sector MDR-TB testing or treatment in Cambodia ⁽³⁸⁵⁾. The PMDT in Cambodia was initiated in 2006 after treatment of the first case of MDR-TB. Since then, PMDT progressed substantially through the collaboration between CENAT and its partners, particularly the Cambodian Health Committee (CHC) and Médecin Sans Frontiers (MSF), followed by national scale up from 2011 ⁽³⁸⁵⁾. PMDT is managed by CENAT through the same infrastructure as TB. An MDR-TB focal point reports to the National Director of CENAT and supervised an Assistant Focal Point and the point persons for the other DR-TB control components. By the end of 2018, the Cambodian PMDT has a well-established community-based model of care approach through 11 PMDT treatment sites with 57 isolations rooms across Cambodia ^(372, 382). This well-established decentralised management network between PMDT treatment sites, health centres and community care and support services facilitates ambulatory based care of MDR-TB patients ⁽³⁸⁵⁾. Through PMDT, the NTP provides fully supervised standard treatment regimen for all MDR-TB patients identified under the programme. Eligible patients were provided the option to initiate or continue treatment as outpatients early in the course of treatment, and to receive home-based care with the support of community-based health staff ⁽³⁸⁵⁾.

2.7.2.4. TB/HIV COLLABORATIVE ACTIVITIES

To deal with the dual epidemic of TB and HIV, a collaborative TB/HIV activities was established in 2003 and implemented in all ODs through the strong collaboration between the NTP and national AIDS control programme ⁽³⁸²⁾. Through this collaboration, HIV screening was performed for TB patients in the TB services at all points of care, at health level since 2014, and TB screening was performed for PLHIV in all pre-ART and ART sites. In 2019, about 94% of TB patients have known HIV status and around 90% of newly HIV positive patients were referred for TB screening. INH Preventive Therapy was provided to all PLHIV AIDS who are not likely having TB disease since 2014 ⁽³⁷⁷⁾.

2.7.3. TB Laboratory diagnoses

In Cambodia, the NTP provide a free diagnostic service for TB patients registered under the program using the TB laboratory network which includes HC where sputum specimens are collected, microscopy centers, culture laboratories and the National TB Reference Laboratory (NRL) at CENAT in Phnom Penh. **Table I-4** provides an overview of available TB diagnosis and DST methods in Cambodia. The use of ineffective serological tests for TB was banded in Cambodia ⁽³⁸²⁾.

Table I-4. Overview of available TB diagnostics and DST methods in Cambodia

Diagnostic tests	No. of sites	Remarks
Microscopy	218*	Includes 32 fluorescent microscopes (NTP)
GeneXpert MTB/RIF	64*	78 instruments in total, all 4 module instruments (NTP)
TB culture	5	CENAT (NTP), Kampong Cham Hospital (NTP), Battambang Hospital (NTP), Sihanouk Hospital Center of HOPE (NGO) and Institut Pasteur du Cambodge (private)
First line drug susceptibility testing (FLD DST)	4	CENAT (NTP), Kampong Cham Hospital (NTP), Sihanouk Hospital Center of HOPE (NGO), Institut Pasteur du Cambodge (private)
Second line drug susceptibility testing (SLD DST)	3	CENAT (NTP), Sihanouk Hospital Center of HOPE (NGO), Institut Pasteur Phnom Penh (private)
Molecular testing: - Line Probe assay for FLD (HAIN GenoType MTBDR <i>plus</i>) - Line Probe assay for SLD (HAIN GenoType MTBDR <i>sl</i>) - Sequencing of drug resistant genes - Genotyping	1	Institut Pasteur du Cambodge (private)

* The data only include laboratories managed under NTP.

2.7.3.1. MICROSCOPY

Smear microscopy is routinely used to detect new TB patients and follow-up treatment in Cambodia. At present, the microscopy network is comprised of 218 microscopy centers (**Table I-4**), or 1.4 per 10,000 population, which are providing TB diagnosis services under the direct NTP laboratory network coverage. Most of microscopy centers use conventional microscopy method with ZN staining. Fluorescence microscopy has expanded to 32 facilities, starting in 2009. The positivity rate of smear examination was 4.3% in 2019⁽³⁷⁷⁾. To strengthen the quality sputum examination, the laboratory quality assurance has been implemented in almost all microscopy centers (97%) and NTP has conducted the crosschecking by re-examining the read slides. In 2019, the agreement rate was 99.4% and only about 0.6% of participant laboratory showed unaccepted results.

2.7.3.2. XPERT MTB/RIF

In 2011, the Cambodian NTP introduced GeneXpert® platform (Cepheid, USA) which could rapidly and accurately detect M.tb and its resistance to RIF ⁽⁷²⁾. The use of this new technology was rapidly expanded throughout the country. Currently, 78 four-module GeneXpert instruments are distributed in 64 different laboratories managed by NTP (**Table I-4**). The utilization of Xpert MTB/RIF test was expanded in 2017. Initially, the platform was only used as the initial screening test for PLHIV and people with presumptive DR TB ⁽³⁸⁶⁾. For PLHIV with one positive symptom of TB, rather than receiving clinical exam, chest x-ray, and sputum smear microscopy, would initially receive an Xpert assay for TB diagnosis. Patients with presumptive MDR-TB first receive an Xpert test and started on empirical treatment based on Xpert results while awaiting culture and DST results ⁽³⁸⁶⁾. Since 2017, the platforms were also used for presumptive TB patient who were new smear positive cases, children <15 years old, elderly over 55 years old, close contacts with smear positive PTB, diabetic, prisoner, injection drug users and those seeking care in hospital ⁽³⁷⁸⁾. In 2019, 71,488 tests were performed and of them the proportion of sample with M.tb detected and RIF resistance detected, M.tb detected and RIF not detected, M.tb detected and RIF resistance indeterminate and error result were 0.3%, 12.2%, 0.3% and 3.8%, respectively.

2.7.3.3. CULTURE AND DRUG SUSCEPTIBILITY TESTING

Since the mid-2000s, smear-positive TB cases started to fall, while smear-negative TB and extrapulmonary TB cases started to rise, indicating the need for more accurate and rapid diagnosis method and algorithm to address case detection particularly smear-negative cases ^(372, 382). In late 1999, with the technical support from JICA, NTP introduced TB culture using solid medium. Liquid medium (MGIT), rapid identification for M.tb and FLD DST were implemented later in 2011 at the NRL ^(377, 385). Currently, the NTP has established culture capabilities in three laboratories equipped with biosafety level 2 room: CENAT (NRL), Battambang Provincial Hospital TB laboratory, and Kampong Cham Provincial Hospital TB laboratory (**Table I-4**). The three laboratories perform routine culture for diagnosis and follow-up on specimens sent by designated provinces. All the sputum received for culture were processed and inoculated onto solid and liquid media. The cultures are incubated at 37°C for 6 to 8 weeks. All positive culture isolates are identified to exclude non-tuberculous mycobacteria. The following FLD are tested at: INH (concentration tested 0.1 µg/ml), RIF (1.0 µg/ml), STM (0.1 µg/ml) and EMB (5.0 µg/ml). Since 2011, CENAT perform FLD DST for isolates collected at CENAT and isolates sent from Battambang culture laboratory. The isolates collected at Kampong Cham culture laboratory were send to IPC for FLD DST as well as SLD DST until 2014 when Kampong Cham culture laboratory success to implement the test. The SLD DST were evaluated by the supranational TB reference laboratory, Japanese Research Institute of Tuberculosis (RIT), and put into service in 2014 at NRL (and since 2012 at IPC). The following SLD are tested at: Ofx (2.0 µg/ml),

Am (1.0 µg/ml), Km (2.5 µg/ml) and Cm (2.5 µg/ml). However, no DST is performed for PZA. The SLD DST is only available at CENAT (NRL). Beside the public laboratory, TB culture and DST are also available in two other laboratories: Sihanouk Hospital Center of HOPE (for its patients) and Institute Pasteur du Cambodge (private laboratory for general public) (**Table I-4**). There are also many private microscopy laboratories and some private laboratory equipped with GeneXpert system but none perform culture and DST except for IPC.

2.7.3.4. LINE PROBE ASSAY (LPA)

Line Probe Assay (HAIN GenoType) was used as rapid molecular testing for first- and second line DST by the NTP, however the test is only available at the IPC in Phnom Penh (**Table I-4**). Through on-demand testing, NTP sent the specimen usually from RIF-resistant patient to IPC for rapid molecular DST in order to decide the MDR-TB treatment regimen: 24-month standardized regimen versus shorter regimen (**Table I-5**).

2.7.4. Comparative performance of Loopamp Assay with microscopy, Xpert and culture for the diagnosis of Pulmonary Tuberculosis in Cambodia

(Appendix 2. Article 2: Evaluation of Loopamp Assay for the Diagnosis of Pulmonary Tuberculosis in Cambodia).

The Loopamp™ MTBC kit (TB-LAMP) is recommended by WHO for detection of MTBC in low income countries with a still low DR TB rate ⁽⁷⁶⁾. In collaboration with the National Center for Tuberculosis and Leprosy Control (CENAT), we conducted two cross-sectional studies (Smear Microscopy Center Study and Central-Level Laboratory Evaluation) to evaluate the performance of TB-LAMP and test its feasibility in Cambodia on sputa collected from presumptive TB patients ⁽³⁸⁷⁾. 499 samples prospectively collected from people undergoing TB testing in the framework of the NTP were tested at a smear microscopy center and a panel of 200 stored specimens were tested at a central-level mycobacteriology laboratory. Using mycobacterial cultures as reference, TB-LAMP results were compared with those of LED-FM and Xpert® MTB/RIF.

At the microscopy center, TB-LAMP sensitivity was higher than that of LED-FM (81.5% [95% CI, 74.5-87.6] versus 69.4% [95% CI, 62.2-76.6]), but lower than that of the Xpert assay (95.5% [95% CI 92.3-98.8]). At the central-level laboratory, TB-LAMP sensitivity (92.8% [95% CI, 87.6-97.9]) was comparable to that of Xpert (90.7% [95% CI, 85.0-96.5]) using stored sample. No significant difference in terms of specificity between TB-LAMP and Xpert assays was observed in both study sites. Our data demonstrate that TB-LAMP could be implemented at microscopy centers in Cambodia to detect TB patients and that TB-LAMP can be a better choice to replace smear microscopy for rapid TB diagnosis of new presumptive TB patients, in settings with relative low prevalence of DR TB and difficulties to implement Xpert assay.

2.7.5. TB treatment in Cambodia

A free treatment service using the FDC is provided for all TB patients registered under NTP in Cambodia. TB treatment is divided into two main groups of patients: patients infected with drug susceptible TB and patients infected with RR/MDR-TB.

2.7.5.1. TREATMENT REGIMENS FOR DRUG-SUSCEPTIBLE TUBERCULOSIS

All the TB patients are categorized based on history of previous TB treatment prior start of standard TB treatment ⁽³⁸⁸⁾. The standard treatment regimen consists of two phases: the intensive phase (initial phase) and the continuous phase. From 1980 to 1993, treatment strategy of long duration (3EMB+INH+PZA/9EMB+INH) was used. Due to the shortages of anti-TB drugs, the cure rate was not satisfactory at that time, only 40-50% ⁽³⁸³⁾. The NTP begin the Short-course chemotherapy with an eight-months regimen (2RIF+INH+PZA+EMB/6EMB+INH) in 1994. FDC tablets of INH and RMP were used, supplemented with PZA and EMB in loose tablets ⁽³⁾. All patients were admitted to the hospital during the first 2 months of the intensive phase under DOT by health care workers. The continuation phase, usually in the form of FDC, was administered without DOT. The standard WHO six-month regimen (2RIF+INH+PZA+EMB/4RIF+INH) (Table I-5) was applied in Cambodia in 2005. Patient registered in category 1 disease received the six-month regimen including an intensive phase of 2 months (60 days) duration with 4 drugs (RIF, INH, PZA, EMB) daily under direct observation by health staff or trained person/C-DOTS followed by a continuation phase of 4 months (120 days) duration with 2 drugs (RIF, INH) daily under direct observation by community member receiving instruction by health worker. At the end of the second month, if sputum smear examination is still positive then one month more of the initial phases has to be undertaken, making a total duration of 7 months (3RIF+INH+PZA+EMB/4 RIF+INH).

Table I-5. Treatment regimen for drug susceptible TB and RR/MDR-TB

Category	Intensive phase	Continuous phase
Drug susceptible TB treatment regimen		
Standard six-month regimen	2 RIF, INH, PZA, EMB	4 RIF, INH
RR/MDR-TB treatment regimen		
Standardized longer regimen	6-8 Km(Cm)-Lfx(Mfx)-Eto-Cs(PAS)- PZA-EMB*	12-18 Lfx(Mfx)-Eto- Cs(PAS)-PZA-EMB*
Shorter Treatment Regimen	4-6 Km-MfxHD-Cfz-Eto-PZA-INH _{HD} - EMB*	5 Mfx-Cfz-PZA-EMB*

Numbers before the letters indicate the duration in months of the phase of treatment.

INH: isoniazid; RIF: rifampicin; PZA: pyrazinamide; EMB: ethambutol; Km: kanamycin; Cm: capreomycin; Lfx: levofloxacin ; Mfx: moxifloxacin ; Eto: ethionamide ; PAS: para-aminosalicylic acid; Cs: Cycloserine ; Cfz: Clofazimine; HD, High dose.

* If still susceptible by drug susceptibility testing

2.7.5.2. TREATMENT REGIMENS FOR RR/MDR-TB PATIENTS

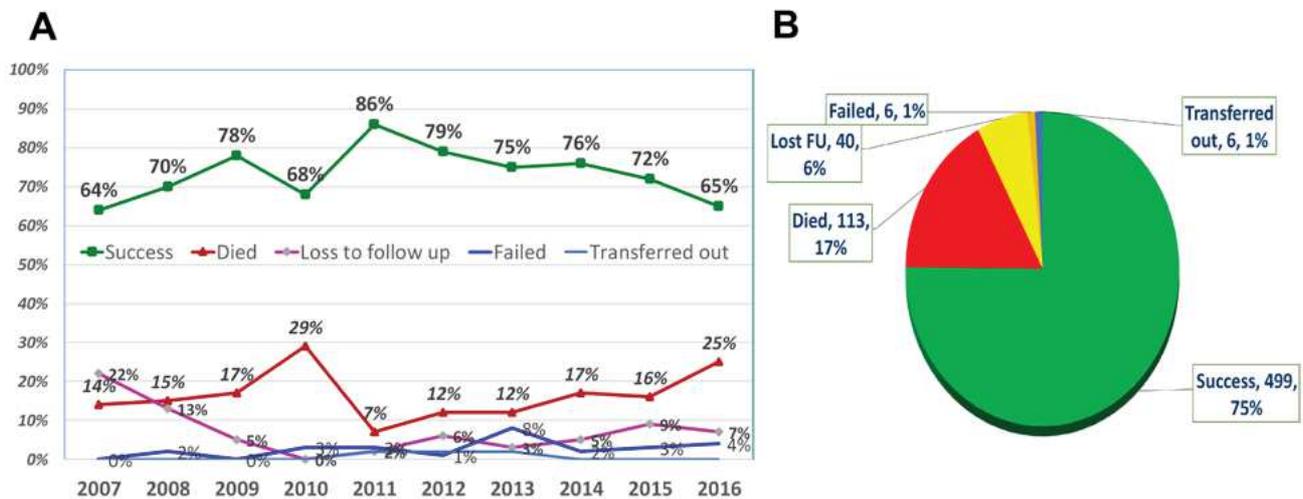
After the approval of the WHO Green Light Committee (GLC), the NTP started the treatment of the first MDR-TB case in 2007 with the standardized MDR-TB long regimen (**Table 1.5**)⁽³⁸⁵⁾. The standardized treatment regimen consists of 6 to 8 months intensive phase including injectable drugs and 12 to 18 months of continuous phase without any injectable drug. The medications are composed of FLD drugs such as PZA and EMB (if sensitive by DST) and SLD drugs including 1 FQ (Lfx or Mfx), 1 injectable drug (Km, Am or Cm), and 2 bacteriostatic drugs (Eto/Pto, Cs or PAS) (Table 1.6). In 2012, the injection phase of treatment was extended from 6 to 8 months for all patients based on WHO recommendations⁽³⁸⁵⁾.

Table I-6. Drugs used in the TB and MDR-TB treatment regimen

Drugs	Daily dosage	Dosage (mg) by patient weight (kg)				
		30-35	36-45	46-55	56-70	>70
First line drugs						
- Isoniazid	4-6 mg/kg, once daily	150	200	300	300	300
- Rifampicin	8-12 mg/kg, once daily	300	450	450	600	600
- Ethambutol	15-25 mg/kg, once daily	600	800	1000	1200	1200
- Pyrazinamide	20-30 mg/kg, once daily	800	1000	1200	1600	2000
Second line drugs						
- Second line injectable drugs :						
Kanamycin	15-20 mg/kg, once daily	500-625	625-750	750-875	1000	1000
Amikacin	15-20 mg/kg, once daily	500-625	625-750	750-875	1000	1000
Capreomycin	15-20 mg/kg, once daily	500-600	600-750	750-800	1000	1000
Streptomycin	12-15 mg/kg, once daily	500-600	600-700	700-800	900	1000
- Fluoroquinolones:						
Levofloxacin	750-1000 mg once daily	750	750	1000	1000	1000
Moxifloxacin	400 mg once daily	400	400	400	400	400
- Others second line drugs:						
Ethionamide	500-750 mg/day in 2 divided doses	500	500	750	750	1000
Prothionamide						
Cycloserine	500-750 mg/day in 2 divided doses	500	500	500	750	750
P-aminosalicylic acid (PAS)	8 g/day in 2 divided doses	8000	8000	8000	8000	8000-12000
Bedaquiline	400 mg per day for the first 2 weeks then 200 mg 3 times per week					
Delamanid	200 mg/day in 2 divided doses					
Linezolid	600 mg once daily	600	600	600	600	600
Clofazimine	200-300 mg per day for the first two months, then 100 mg per day					
High-dose isoniazid	16-20 mg/kg once daily	600-1000	1000-1500	1500	1500	1500

Source: Adapted from CENAT, 2016⁽³⁵⁸⁾.

Between 2007 and 2017, a total of 668 MDR/RR-TB patients diagnosed were enrolled on standardized MDR-TB treatment (long regimen) and 499 were treated successfully in 2018 (Figure I-14). The treatment success rate varies from year to year but the overall treatment success rate of MDR/RR-TB was 75% which was higher than an average of global level (54%).



Source: Adapted from Kim et al., 2019⁽³⁸⁹⁾.

(A) Data by year and (B) Overall data.

Figure I-14. RR/MDR-TB treatment outcomes 2006-2016.

Following the 2016 WHO recommendation on the use of the shorter treatment regimen (STR) over 9-11 months (Table I-5), Cambodia successfully transitioned to the STR from December 2017 for RR/MDR-TB patients who have never been previously treated with SLD and remain susceptible to FQ and SLID after LPA test⁽³⁷⁸⁾. The STR should not be prescribed in the following situations:

- Patient taking any drugs used in the STR for > 1 month
- Patient with intolerance to any of the drugs used in the STR or with known risk of toxicity
- Pregnant woman
- Patient with extrapulmonary TB

The first 10 MDR patient were enrolled in STR in 2017 and 8 (80%) were treated successfully in 2018, 1 (10%) was failed and switched to longer regimen and 1 (10%) was lost to follow-up⁽³⁸⁹⁾. Since implementation of the STR, the early cohort data have shown improved treatment outcomes compared with the adverse trends noted between 2011 and 2016. Although the results are preliminary, the improvement might suggest significant benefits from the reduced burden on the system related to the shorter treatment duration and from the increased attention to patient monitoring and drug safety. However, it is estimated that approximately 30% of RR/MDR-TB patient still need longer standardized or individualized treatment regimen including new drugs such as bedaquiline or Delamanid or Linezolid, especially for RR/MDR-TB patients in special situation that do not allow them to used STR or patients with resistance to second line drugs (Pre-XDR TB or XDR-TB⁽³⁷²⁾). Between 2016 and 2018, Bdq was used in 36 patients including 2 with XDR-TB and 24 with pre-XDR-TB in Cambodia⁽³⁷²⁾.

Chapter II: GENERAL MATERIALS AND METHODS

1. Study setting

Cambodia is a small country with 181,035 square kilometers in size populated by 15,288,489 people in 2019⁽³⁹⁰⁾. The country is divided into 25 provinces including the capital Phnom Penh which is the most densely populated region with internal migration of population from other 24 provinces of the country. Phnom Penh also has the highest number of TB cases⁽³⁹¹⁾.

We conducted a retrospective laboratory study on a collection of clinical M.tb cultured isolates stored between 2012 and 2017 in Medical Laboratory (Mycobacteriology) of the Institut Pasteur du Cambodge (IPC) and the National TB Reference Laboratory (NRL) of CENAT, Phnom Penh, Cambodia. Since its creation in 1995, the Medical Laboratory also known as “Laboratoire de Biologie Médicale” (LBM) aims at supporting health professional in Cambodia by offering them a variety of in vitro diagnostic tests covering all medical specialties including standard TB diagnosis. It has been an alternative TB and drug resistant TB diagnostic platform to the NRL. It is the first and the only laboratory in the country equipped with Biosafety Level 3 (BSL3) and able to provide molecular testing for TB including line probe assay and genotyping in addition to its standard TB diagnosis test. Through its routine activities, IPC has been involved in the surveillance of drug resistance TB in Cambodia since the first National Tuberculosis Drug Resistance Survey in 2000-2001⁽³⁾ and now the surveillance of second line drug resistance (pre- and XDR-TB) among RR/MDR-TB patients⁽³⁸⁵⁾.

2. Study population and methods used

The study included two collections of M.tb isolates: Human M.tb isolates (Sampling 1) and Bear isolates (Sampling 2).

2.1. Sampling 1: Human isolates

The collection 1 included a total of 710 M.tb isolates (single patient) with known drug susceptibility patterns for first line drugs (FLD) stored between 2012 and 2017 in NRL and LBM culture archives. The isolates were obtained via a platform established for routine screening of MDR-TB among high-risk groups by the National Tuberculosis Program (NTP).

National Tuberculosis Program routine MDR-TB screening

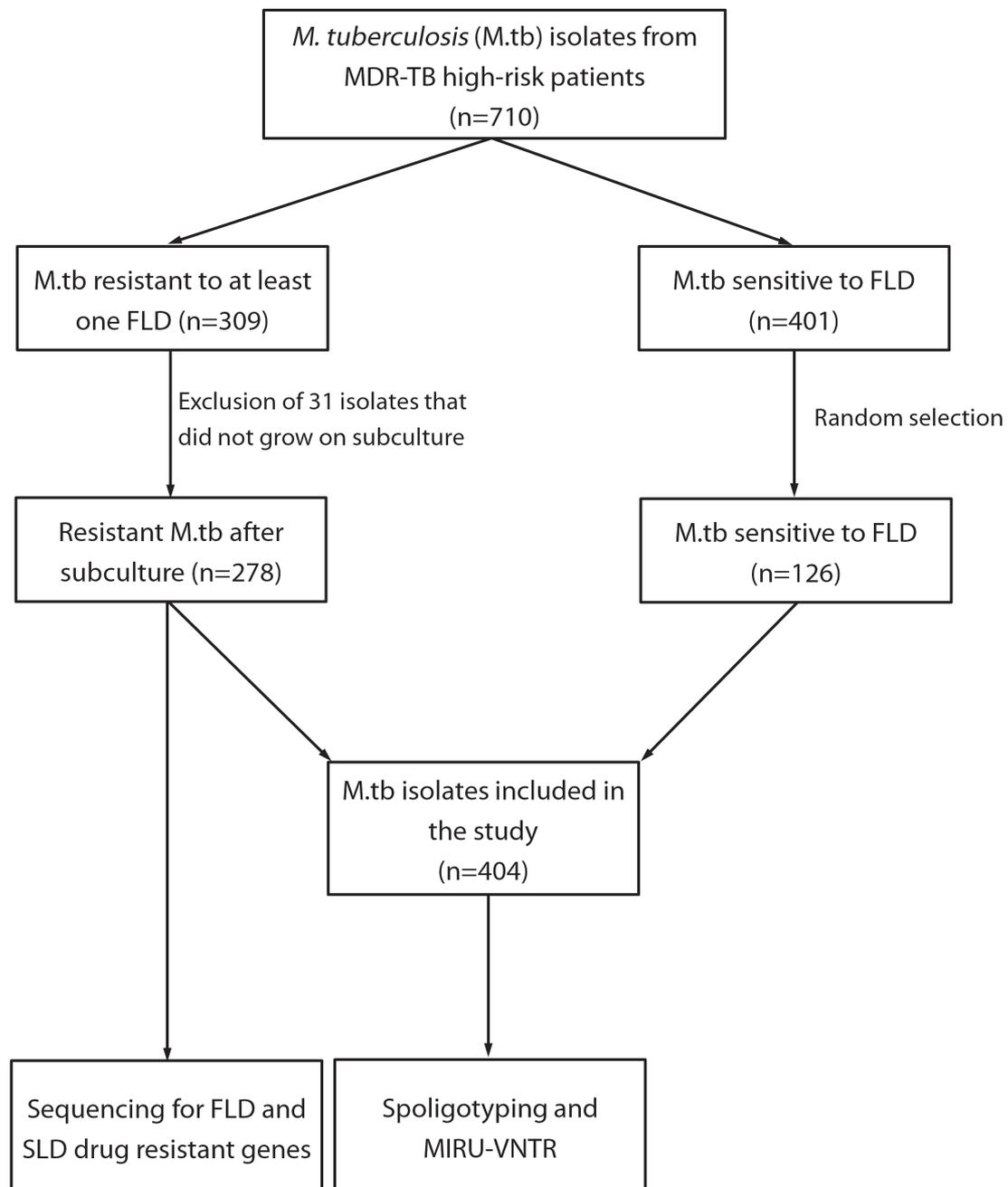
All MDR-TB high-risk groups including a) all pulmonary TB retreatment cases; b) patients with TB who are persistently smear positive after TB treatment at months 2 or 3; c) close contacts of MDR-TB cases with cough >2 weeks; and d) HIV-infected patient with any of TB symptoms (cough, weight loss, fever, night sweats) were screened using Xpert MTB/RIF (Xpert) at all GeneXpert laboratories in the country⁽³⁸⁵⁾. Sputum samples with Xpert MTB-positive results from group a), b) and c) and Xpert MTB-positive and RIF-resistant result from group d) were sent to zonal reference laboratories for culture and then DST for FLD. Between 2012 and 2014, IPC and NRL are the two main DST laboratories for the NTP. In 2015, the NTP implemented its second DST laboratory at Kampong Cham Provincial hospital's laboratory, which provides culture and DST services to seven provinces in the Northeastern part of the country. All the M.tb positive cultures isolated at IPC were stored in its respective culture archives, however only drug resistant strains were stored at the NRL. For this study, we included all drug resistant M.tb isolates available and stored between 2012 and 2017 and a panel of drug susceptible isolates, randomly selected, from the culture archives at the NRL and IPC.

Among the 710 M.tb isolates (**Figure II-1**), 309 (43.5%) were resistant to at least one FLD, defined as "drug resistant (DR)", and 401 (56.5%) were susceptible to all four FLDs tested, defined as "drug sensitive (DS)". All 309 DR isolates and 126 DS isolates randomly selected were initially included in the study. However, 31 (7.1%) DR isolates did not grow during subculture on LJ medium slants and were excluded from the study. The final sample consisted of 404 isolates (278 DR and 126 DS) and were undergone genotyping method using 43-spacer spoligotyping and 24-locus MIRUVNTR methods. All the DR isolates were sequencing for FLD and SLD drug resistant genes.

Available patient data including age, gender, geographic area of isolation, year of isolation and clinical information and first-line drug susceptibility profile of each isolate were collected from the laboratory records. Each sample evaluated was a single strain from a unique patient. Although serial strains are available in the strain bank, we were careful to only examine one strain per patient for this analysis.

2.2. Sampling 2: Bear isolates

The sampling 2 included 20 M.tb isolates collected between 2009 and 2017 from 19 sun bears (*Helartctos malayanus*) at one wildlife rescue center situated outside Phnom Penh and one staff member who was working in the rescue center and developed TB in 2011. Drug susceptibility testing to four first line drugs, 43-spacer spoligotyping and 24-locus MIRU-VNTR methods were performed for all isolates.



FLD: First line drugs; SLD: Second line drugs; DR: Drug resistant; DS: Drug sensitive

3. Ethical consideration

This study was endorsed by the Cambodian NTP and approved by the National Ethics Committee for Health Research. Since the study used only M.tb isolates that were collected from patients, patients' informed consent was not required.

4. Methods

4.1. Mycobacterial archived strains and DNA preparation

The original stocks of clinical MTB isolates stored at -80°C were re-cultured on Lowenstein-Jensen (LJ) medium slant in Mycobacteriology Biosafety Level 3 Laboratory at IPC. The isolates that did not grow were excluded from the study. The grown isolates with pure colonies were harvested for DNA extraction using thermolysis method. A loopful of culture scraped from LJ slant was suspended in 300 mL of TE buffer (10 mM Tris, 1 mM EDTA pH 8.5) and inactivated by heating for 20 minutes at 95°C in a dry bath to kill the bacteria and to release the DNA from the bacterial cells. The mixture was centrifuged at 13,200 rpms for 5 minutes and the supernatant that contains the DNA was used as PCR template and stored at -80°C.

4.2. Targeted genes sequencing

4.2.1. Drug resistance-associated mutations

The main genes associated with resistance to first line anti-TB drugs (FLD) and second line anti-TB drugs (SLD) were amplified by PCR and sequenced (Macrogen, Inc., Republic of Korea and Eurofins Genomics Germany GmbH, Germany). 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 (STM 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). Primer sequences are described in **Table II-1**.

Each sequence was treated independently using the Bioedit software (version 7.2.6) and QIAGEN CLC Main Workbench (version 5.5). The consensus sequence was generated. Multi-sequence alignment was then performed with Automated ClustalW alignment integrated in Bioedit software. Point mutations were identified by comparison with the sequence of the M.tb H37Rv reference strain available in GenBank (NC_000962.3). To describe the resistance-associated mutations in *rpoB* gene and *gyrB* gene, a numbering system based on the *Escherichia coli* sequence annotation and 2002 M.tb H37Rv sequence re-annotation has been used, respectively ^(107, 185). The interpretation of mutations related to resistance were based on resistance mutation sites reported in previous studies ^(184, 392-394).

Table II 1. Primers used for Sanger sequencing of genes involved in anti-TB drug resistance

Drug(s)	Gene/gene promoter	Primer sequence (5' to 3')	Annealing T (oC)	Length (bp)	Target region
RIF	<i>rpoB</i> -F1	F- <i>rpoB</i> 1: GTCGACGCTGACCGAAGAAG R- <i>rpoB</i> 1: TCTCGCCGTCGTACAGTACAG	62	1148	Clusters I (including RRDR), II, III
	<i>rpoB</i> -F2	F- <i>rpoB</i> 2: TAGTTGCGTGCCTGAGATCC R- <i>rpoB</i> 2: TGGTCTGACCTCGTCAAG	60	1174	Promoter and N-terminal region
INH	<i>katG</i>	F- <i>katG</i> 1: CCAACTCCTGGAAGGAATGC R- <i>katG</i> 1: AGAGGTCAGTGGCCAGCAT	58	1169	Full length gene
		F- <i>katG</i> 2: ACGAGTGGGAGCTGACGAA R- <i>katG</i> 2: AACCCGAATCAGCGCACGT	60	1229	
	<i>inhA</i>	F- <i>inhA</i> 1: GCGACATACCTGCTGCGCAA R- <i>inhA</i> 2: ATCCCCCGTTTCTCCGGT	60	300	Promoter region
		F- <i>inhA</i> 3: GACACAACACAAGGACGCA R- <i>inhA</i> 4: TGCCATTGATCGGTGATACC	60	1008	Full length gene
STM	<i>rpsL</i>	F- <i>rpsL</i> : GCGCCCAAGATAGAAAG R- <i>rpsL</i> : CAACTGCGATCCGTAGA	58	451	Full length gene
	<i>rrs</i> -F1	F-S1: GAGAGTTTGTACTCTGGCTCAG R-S1: CCAGGTAAGTTTCTTCGCGTTG	58	973	Loops 530 & 915
EMB	<i>embB</i>	F- <i>embB</i> : TGACCGACGCCGTGGTGATA R- <i>embB</i> : GCCATGAAACCGGCCACGAT	62	1312	ERDR & flanking sequences
PZA	<i>pncA</i>	F- <i>pncA</i> : GCTTGCGGCGAGCGCTCCA R- <i>pncA</i> : TCGCGATCGTCGCGGCGTC	63	709	Entire <i>pncA</i> gene and its upstream promoter
KAN, AMK & CAP	<i>rrs</i> -F2	F-S2: GCGCAGATATCAGGAGG R-S2: CGCCCACTACAGACAAG	58	918	1400-1500 region
FQ	<i>gyrA</i> & <i>gyrB</i>	F- <i>gyrAB</i> : GCAACACCGAGGTCAAATCG R- <i>gyrAB</i> : CTCAGCATCTCCATCGCCAA	62	1296	QRDR of <i>gyrA</i> & <i>gyrB</i>
Compensatory mutation	<i>ahpC</i> and its promoter (INH)	F- <i>ahpC</i> : CCGCAACGTCGACTGGCTCATA R- <i>ahpC</i> : TTCCCGGCCAACAGATCCCCGG	62	835	<i>ahpC</i> gene and its promoter
	<i>rpoA</i> (RIF)	F- <i>rpoA</i> : AACCGATCCCAGTTCGTGAT R- <i>rpoA</i> : GCAGCTTGATCTTCACCTCG	60	856	<i>rpoA</i> gene
	<i>rpoC</i> (RIF)	F- <i>rpoC</i> : AGTCGCTTCCGATCTGCTC R- <i>rpoC</i> : TTGAGCTTGTGACGGTCTG	60	952	<i>rpoA-rpoC</i> interaction region

We calculated the sensitivity and specificity values by comparison of phenotypic and genotypic data for FLD drug (except for PZA). The two tailed-Fisher's exact test was used to compare the mutation frequencies between DR patterns and between M.tb families. *p*-values < 0.05 were considered statistically significant. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated to quantify the association of FLD resistance patterns with mutation frequencies.

4.2.2. Compensatory mutations in RIF-resistant and INH-resistant isolates

The *ahpC* gene and its promoter was amplified and sequenced for all INH-resistant isolates and the *rpoA* and *rpoC* genes were amplified and sequenced in all RIF-resistant isolates using the primers, PCR conditions and DNA sequencing described in the **Table II-1**. The analysis of sequences and mutations were performed according to the procedure described in **Chapter II-4.2.1** using QIAGEN CLC Main Workbench (version 5.5) software and based on sequence comparison with the M.tb H37Rv reference strain (GenBank NC_000962.3).

4.3. Drug Susceptibility Testing

The phenotypic DST method using liquid culture system BACTEC™ MGIT™ 960 (BD, Sparks, MD, USA) is currently the standard for phenotypic DST of FLD and SLD, as it is accurate and reproducible^(64,395). The MGIT-based DST (MGIT-DST) is based on the growth of *M.tb* isolate in a drug-containing tube in comparison to a drug free tube (Growth Control). The BACTEC MGIT instrument monitors and analyses the increased fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube to determine susceptibility results. The instrument automatically interprets these results, and reports results, susceptible or resistant.

In our study, MGIT-based DST (MGIT-DST) method was performed according to the manufacturer's instructions (BD BACTEC™ MGIT™ 960 SIRE Kit)⁽³⁹⁶⁾ and using the WHO-recommended critical concentrations⁽³⁹⁷⁾. The FLD MGIT DST using MGIT SIRE kits allows detecting susceptibility of *M.tb* to STM, INH, RIF and EMB with a critical concentration of 1.0 µg/mL, 0.1 µg/mL, 1.0 µg/mL and 5.0 µg/mL, respectively. The critical concentration recommend by the WHO for SLD and PZA MGIT-DST respectively were: Ofloxacin (Ofx) 2.0 µg/mL; Amikacin (Am) 1.0 µg/mL; Kanamycin (Km) 2.5 µg/mL, Capreomycin (Cm) 2.5 µg/mL and Pyrazinamide (PZA) 100 µg/mL⁽³⁹⁷⁾.

4.4. Spoligotyping

The spacer oligotyping or spoligotyping is an amplification-based method used to exploits polymorphisms in spacer sequences found in the chromosomal direct repeat locus of the MTBC genome. The direct repeat locus contains a variable number of well-conserved 36-bp direct repeats interspersed with nonrepetitive spacers sequences of 34 to 41 bp long⁽²⁹²⁾. Currently, 94 different spacer sequences were identified of which 43 selected from *M.tb* H37Rv (spacers 1-19, 22-32, and 37-43) and *M. bovis* BCG (spacers 20-21 and 33-36) are used to differentiate MTBC strain^(315,398).

In this study, the oligonucleotides DRa (5'-biotin TCGGGTTTTGGTCTGA-3') and DRb (5'-CCGAGAGGGGACGGAAAC-3') targeting any DR in the DR regions were used as primers to amplify the whole DR region by PCR. The classical 43-spacer spoligotyping was performed by reverse line blot hybridization of biotinylated PCR products to a membrane with 43 covalently bound synthetic oligonucleotides⁽²⁹²⁾. *M.tb* H37Rv and *M. bovis* BCG DNA were included as positive controls and molecular biology-grade water was used as a negative control. A part of isolates with reference isolates (H37Rv and BCG) used as control were characterized using Luminex®-based method according to the protocol previously published⁽³⁹⁹⁾.

The results, absence (0) or presence (1), of the 43 spacer sequences were entered into Excel spreadsheets in a binary format. The data were then compared with the SITVIT2 MTBC Genotyping Database (an updated version of SpolDB4 and SITVITWEB) database, which is a publicly available international multimarker genotyping database focus on MTBC (available at <http://www.pasteur-guadeloupe.fr:8081/SITVIT2>), in order to identify the Spoligo International Type

(SIT) and family⁽³⁰⁸⁾. The strains with an undefined pattern declared as “orphans” were further compared with SPOTCLUST database (available at http://tbinsight.cs.rpi.edu/run_spotclust.html)^(309, 310). The family assignment was retained when the probability was $\geq 95\%$.

4.5. MIRU-VNTR typing

Multiple-locus variable number tandem-repeat analysis (MLVA) has become a widely used method for molecular typing of bacteria. It assesses the variation in the number of tandem repeated DNA sequences found in many different loci in the genome⁽⁴⁰⁰⁾. MIRU-VNTR typing used a MLVA format for typing M.tb isolates by targeting variable number tandem-repeat (VNTR) markers, including genetic elements called mycobacterial interspersed repetitive units (MIRU)⁽⁴⁰¹⁾. They represent DNA sequences of 40 - 100 bp that are directly repeated, similar to eukaryotic minisatellites, and scattered in 41 locations, mainly in intergenic regions, throughout the M.tb H37Rv genome^(293, 320). In MIRU-VNTR, the number of repeated units in a set of 12, 15 or 24 loci (alleles) were assessed by PCR analysis using specific primers complementary to the flanking regions followed by gel electrophoresis or an automated DNA sequencer for an accurate sizing of the PCR products^(326, 329). The size (bp) of the amplicon is used to calculate the number of repeat units in each locus. The calculated numbers of repeats of each VNTR locus (alleles) are combined into a string which consists of integers and is referred to as the MIRU-VNTR profile⁽³³¹⁾. A part of isolates with a reference isolate (H37Rv) used as control were characterized using an automatic sequencing at the GENSEQ platform (<http://www.labex-cemeb.org/fr/genomique-environnementale-2>) from the labEx CeMEB (Montpellier, France) according to the protocol previously published⁽³²⁷⁾.

For our study, we used 24 international standardized MIRU-VNTR loci to analyze genetic diversity of M.tb clinical isolates, as it is highly discriminatory and reproducible^(326, 402). A specific master mix was prepared for each analyzed locus (**Table II-2**). The patterns obtained for the 24 loci were used to create a 24-digit allelic profile for each isolate. The MIRU-VNTR pattern was analyzed using the MIRU-VNTRplus database (available at www.miru-vntrplus.org/). The resolution power of 24 MIRU-VNTR was determined based on the Hunter-Gaston-Discriminatory Index (HGDI)⁽⁴⁰³⁾. The method was classified as “highly discriminant” (HGDI, > 0.60), “moderately discriminant” (HGDI, $0.3-0.6$), and “poorly discriminant” (HGDI, < 0.3)⁽⁴⁰⁴⁾. The genetic diversity within sample (Hs) was calculated using FSTAT version 2.9.3.2⁽⁴⁰⁵⁾.

Table II 2. MIRU-VNTR primer sequences

Locus^a	Alias	Repeat unit length (bp)	PCR primer pairs (5' to 3')
154	MIRU 02	53	TGGA CTTCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAT
424	MTUB 04	51	CTTGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCCGGATTCTTC
577	ETR C	58	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACCGCAAATTGTGA
580	MIRU 04	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC
802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
1955	MTUB 21	57	AGATCCCAGTTGTGTCGTC CAACATCGCCTGGTTCTGTA
2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTA CTGTGA
2163b	QUB 11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
2347	MTUB 29	57	GCCAGCCGCCGTGCATAAACCT AGCCACCCGGTGTGCCTTGATGAC
2401	MTUB 30	58	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCATTAGTA
2461	ETR B	57	ATGGCCACCCGATACCGCTTCAGT CGACGGGCCATCTTGGATCAGCTAC
2531	MIRU 23	53	CTGTGATGGCCGCAACAAAACG AGCTCAACGGGTTCCGCCCTTTGTC
2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA
3171	MTUB 34	54	GGTGCGCACCTGCTCCAGATAA GGCTCTCATTGCTGGAGGGTTGTAC
3192	MIRU 31	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
3690	MTUB 39	58	CGGTGGAGGGGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
4052	QUB 26	111	AACGCTCAGCTGTCGGAT CGGCCGTGCCGCCAGGTCTTCCCGAT
4156	QUB 4156	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT

^a MIRU-VNTR loci are listed according to their position in kbp on the H37Rv genome

In order to reach the objectives of this PhD thesis, which aims to understand the emergence, spread and evolution of antibiotic resistance in *Mycobacterium tuberculosis* (M.tb) in Cambodia, this chapter has been subdivided in four parts, each addressing specific questions about diagnosis and drug resistance.

The first part describes genetic diversity of FLD drug resistant clinical isolates and their link with FLD resistance patterns by using genotyping methods and phylogenetic analysis. This finding is detailed in paper 2 entitled “Genetic diversity and multidrug-resistant in *Mycobacterium tuberculosis* isolates in Cambodia”.

The second part focuses on SLD drug resistant clinical M.tb isolates by phenotypic and genetic approach. We aimed to explore how SLD resistant isolates including pre- and XDR isolates have emerged and what are the genetic processes leading to these high levels of drug resistance? This study is detailed in paper 3 entitled “Resistance to second line anti-TB drugs in Cambodia: a phenotypic and genetic study” submitted to the Journal of Infection and Resistance (manuscript is accepted with minor revisions).

In the third part of this thesis, we aimed to address the following questions: What are the genetic determinants of FLD and SLD drug resistance in Cambodian M.tb isolates? How M.tb evolved from sensitive to highly drug resistant status including mono-resistance, MDR, pre-XDR and XDR? Do the clinical isolates display specific genetic characteristics explaining the successful spread of highly drug resistant strains in Cambodia? Can we predict the evolution of drug resistance from the genetic data?

The last part of the thesis deals with the human and animal transmission of drug susceptible and drug resistant M.tb in a rescue center in Cambodia. This study is detailed in “Zoo-anthroponosis: *Mycobacterium tuberculosis* in a population of captive sun bears”. The data was presented in a poster format at the 50th Union World Conference on Lung Health, 30 October to 2 November 2019, Hyderabad, India.

Result 1: GENETIC DIVERSITY AND MULTIDRUG-RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS POPULATION IN CAMBODIA

This manuscript (Article 3) will be submitted in December to PLOS One.

Running title: Genetic diversity and multidrug-resistance in *Mycobacterium tuberculosis* population in Cambodia

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Abstract

Background: Molecular epidemiology has been largely used in order to better understand the causes of emerging and spread of multidrug-resistant tuberculosis (MDR TB). However, there is still little information available in Cambodia, a country with high burden of TB. This study aims to explore the genetic structure and diversity of drug resistant *Mycobacterium tuberculosis* (M.tb) population circulating in Cambodia and its association with epidemiological factors.

Methods: A total of 404 M.tb isolates, including 278 drug resistant and 126 fully susceptible isolates, collected between 2012 and 2017 from patients with high-risk of having MDR-TB were characterized by spoligotyping and 24-MIRU VNTR typing.

Results: According to the spoligotyping analysis, the two major families which account for about 89.6% of all isolates were Beijing (182, 47.4%) and EAI (162, 42.2%) families. In combination with 24-MIRU VNTR data, thirty clusters shared by 69 isolates (two to three isolates per cluster; the clustering rate was 10.2%) were observed. Compared to EAI, Beijing isolates were associated with drug resistance phenotype, especially MDR (COR, 2.63; 95% CI, 1.51 to 4.61; $p = 0.001$) and quadruple drug resistance (COR, 9.31; 95% CI, 2.72 to 31.94; $p < 0.001$), and clustering (COR= 2.69; 95% CI 1.51 to 4.82; $p < 0.001$), suggesting recent transmissions. However, the transmission rate remains relatively low. More interestingly, we observed three clonal complexes among our sample; one clonal complex CC1 formed by 29 Beijing isolates with the majority of them were MDR with resistance to three or four drugs.

Conclusion: In conclusion, the data suggest that Beijing family, which is currently the most predominant family among drug resistance isolates circulating in the country, is associated with highly drug resistance and recent transmission. Rapid interventions in terms of diagnostic and treatment is essential to prevent the spread of drug resistant Beijing strains in the population.

Introduction

Tuberculosis (TB), the leading cause of death by a single infectious disease, remains a global issue (1). Antibiotic resistance is a growing threat and complicates the disease control. In 2019, WHO reported an estimated 1.7 billion people infected with *Mycobacterium tuberculosis* complex, 10.0 million people developed TB disease, 1.4 million died and 362 700 had multidrug-resistant TB [MDR-TB, TB resistant to isoniazid (INH) and rifampicin (RIF) which are the two most effective first-line anti-TB drugs] (1). Among MDR-TB cases, 6.0% had extensively drug-resistant TB [(XDR-TB, defined as MDR-TB plus resistance to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin)] (1). The treatment success rate among TB cases were 85% for susceptible TB but only 57% for MDR-TB and lower than 50% for XDR-TB (1).

Cambodia ranks 17th among 30 high burden countries in terms of TB incidence rate (287 per 100 000 population) (1). Although the incidence of MDR-TB is still low, the number of drug resistant TB (DR-TB) is increasing overtime. In the 2000 National Drug Resistant Survey (NDRS), the proportion of MDR-TB were found in 0.0% and 3.1% among new cases and retreatment cases, respectively (2). In 2006, during the second NDRS, the proportion of MDR-TB were found in 1.4% among new cases and 10.5% among retreatment cases (3). Ten years after the second survey, one case of extensively drug-resistant tuberculosis (XDR-TB, MDR-TB resistant to any fluoroquinolones and at least to one injectable second-line drugs), was reported for the first time in 2017 (4). Currently, WHO estimated 1000 MDR-TB or Rifampicin resistant TB (RR-TB) cases among notified pulmonary TB cases (1.8% among new cases and 8.2% among retreatment cases) in the country but only 135 (13.5%) cases were identified (5). The proportion of MDR-TB was found even higher among new cases (3.7%) and

previously treated cases (28.9%) in TB/HIV co-infected patients in one study conducted between 2007 and 2009 by Walls et al (6). The authors also suggested that the prevalence of drug resistant TB in Cambodia might be higher than previously reported (6).

Molecular epidemiology has been largely used in order to better understand the causes of emerging and spread of drug-resistant TB. However, there is still little information available in Cambodia, a country with high burden of TB. In this framework, the genetic structure and diversity of drug resistant *Mycobacterium tuberculosis* (M.tb) population was investigated. The evolution of MDR M.tb population over a six year-period and the associated epidemiological factors were also explored.

Material and methods

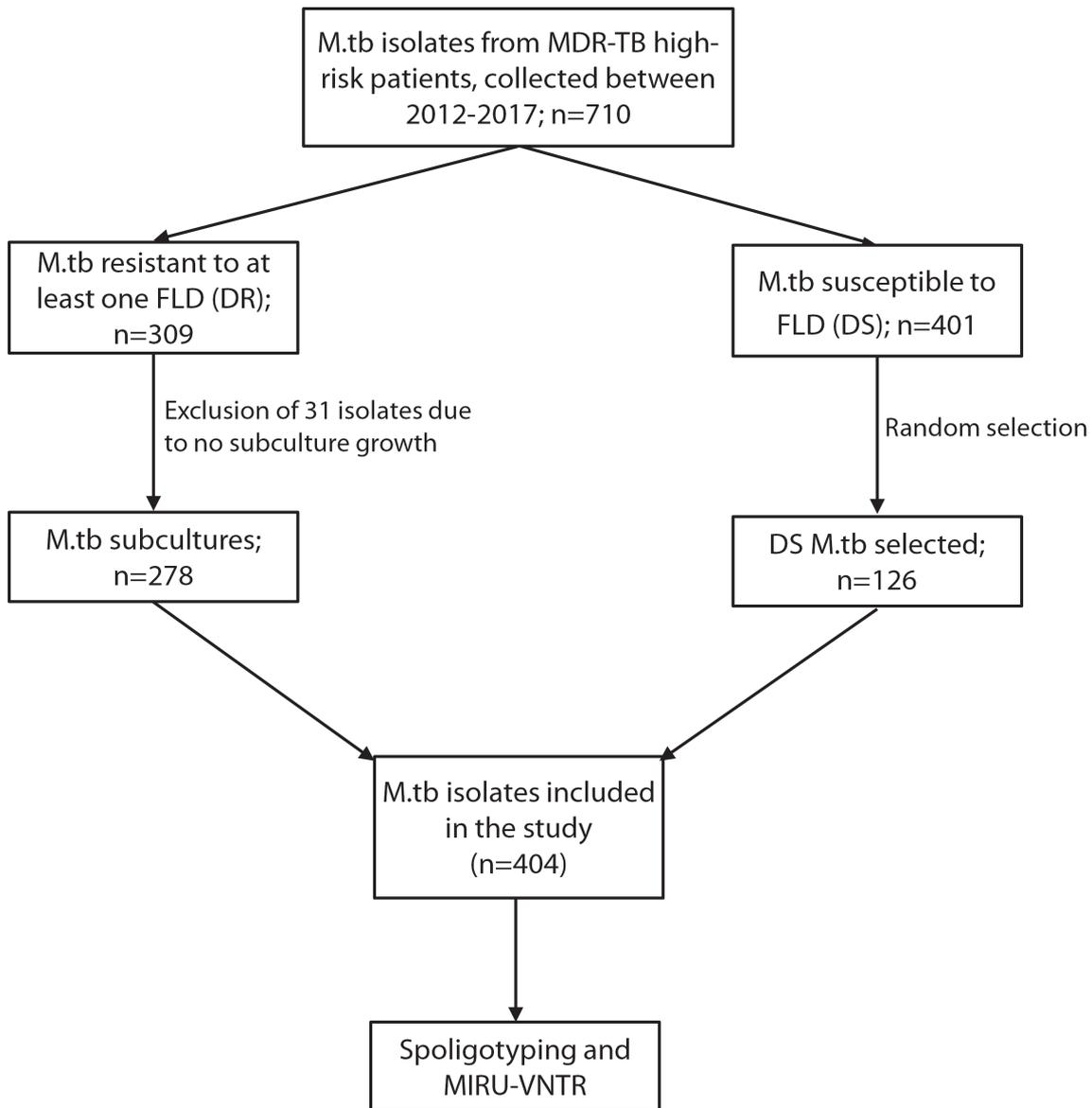
Ethics statement

This study was endorsed by the National Tuberculosis Control Program (NTP) and the ethical clearance was provided by the Ethical Committee for Health Research, Phnom Penh, Cambodia. The data in this study were anonymously analyzed.

***M. tuberculosis* collection and NTP routine for MDR-TB screening**

This is a retrospective laboratory study on first line drugs (FLD) resistant M.tb clinical isolates stored between 2012 and 2017 at the two main laboratories in Cambodia; the National TB Reference Laboratory (NRL) of the National Centre for Tuberculosis and Leprosy Control (CENAT) and the Mycobacteriology Laboratory of the Institut Pasteur in Cambodia (IPC). The collection was obtained via a platform established for routine screening of MDR-TB among high-risk groups by the NTP (7). All MDR-TB high-risk groups included were screened using Xpert MTB/RIF, followed by culture and phenotypic drug susceptibility testing (pDST) for FLD including rifampicin (RIF; 1.0 µg/mL), isoniazid (INH; 0.1 µg/mL), ethambutol (EMB; 5.0 µg/mL) and streptomycin (STM; 1.0µg/mL) using the automated BACTEC MGIT 960 (MGIT AST SIRE kit, Becton Dickinson (BD), Sparks, MD, USA) (8). STM is considered in this study as one of the FLD since it was used in first-line TB treatment for retreated patients until the end of 2017 (9). The DST results were not available for pyrazinamide (PZA).

A total of 710 isolates with known DST for FLD was retrieved from NRL and IPC collections during the six years period (2012 to 2017) (Fig 1). Among them, 309 (43.5%) were resistant to at least one FLD, defined as “drug resistant (DR)”, and 401 (56.5%) were susceptible to all four FLDs tested, defined as “drug susceptible (DS)”. Our study included all 309 DR isolates and a panel of randomly selected DS isolates representing half of the number of DR isolates by year. However, 31 (7.1%) drug-resistant isolates could not be subcultured on Lowenstein-Jensen (LJ) media and were excluded from the study. The final sample consisted of 404 isolates (278 DR and 126 DS).



Abbreviations: DR, drug resistant; DS, drug susceptible; FLD, first line drugs; M.tb, *Mycobacterium tuberculosis*

Fig 1. *M. tuberculosis* isolates collection and processing.

Each isolate was from a unique patient. Available patient data including age, gender, geographic area of isolation, year of isolation and clinical information and first-line drug susceptibility profile of each isolate were collected from the laboratory record.

Culture and DNA Extraction

All the isolates were cultured on LJ slants. Mycobacterial colonies on positive LJ media were suspended in 300 µl of TE buffer (10mM Tris-HCl, 1mM EDTA) and heated at 95°C for 30 minutes. Heated samples were centrifuged at 12000 rpm for 5 minutes. The bacterial DNA-containing supernatant was retrieved and used for molecular analysis. All extracted DNA were characterized by the combination of 43-spacers spoligotyping and 24-locus MIRU-VNTR typing.

Spoligotyping

The direct repeat (DR) region was amplified with a pair of primers (Dra: 5'-biotin TCGGGGTTTTGGGTCTGA-3' and DRb: 5'-CCGAGAGGGGACGGAAAC-3') (10). The amplified product was hybridized to a set of 43 oligonucleotides probes, which were immobilized on a nylon membrane, each representing a unique spacer DNA sequence within the DR regions. The hybridized DNA was detected on an X-ray film by chemiluminescence. A part of isolates was characterized using Luminex®-based method according to the protocol previously published (11). Two genomic DNA of *M. tuberculosis* H37Rv and *M. bovis* BCG were included as positive controls.

MIRU-VNTR Typing

MIRU-VNTR typing was performed by PCR amplification of 24 loci set as described by Supply *et al.* (12). Each locus was amplified individually and PCR fragments were separated by electrophoresis using a 1.5% agarose gel. The amplicon size was estimated by comparison with 100 bp molecular marker and independently verified by two separate individuals. The number of repeats at each locus was calculated using an allele-calling table described by Supply *et al.* (12) and used to create a 24-digit allelic profile for each isolate. A part of isolates with a reference isolate (H37Rv) used as control were characterized using an automatic sequencing at the GENSEQ platform (<http://www.labex-cemeb.org/fr/genomique-environnementale-2>) from the labEx CeMEB (Montpellier, France) according to the protocol previously published (13).

Data management and analysis

Spoligotype patterns were coded as a binary number and then entered in the 6th version of the international genotyping database SITVIT2 (available at <http://www.pasteur-guadeloupe.fr:8081/SITVIT2/>) in order to identify the spoligo-international type (SIT) (14). The isolates with an undefined pattern declared as "orphans" were further compared with SPOTCLUST database (available at http://tbinsight.cs.rpi.edu/run_spotclust.html) (15, 16). The family assignment was retained when the probability was $\geq 95\%$. The 24 international standardized MIRU-VNTR loci were coded as numerical value reflecting the number of repeats in tandem at each locus. The result was analyzed using the MIRU-VNTRplus database (available at www.miru-vntrplus.org/) (17). The allelic diversity (h) and discriminatory power of each MIRU-VNTR locus was determined using the Hunter-Gaston-Discriminatory Index (HGDI) (18). The calculation was performed by using the web base tool (available at http://insilico.ehu.eus/mini_tools/discriminatory_power). We considered $h > 0.6$ as highly discriminative, $0.3 \leq h \leq 0.6$ as moderately discriminative, and $h < 0.3$ as poorly discriminative (19). The genetic diversity within sample (H_s) and the genetic differentiation index (F_{st}) were calculated using FSTAT version 2.9.3.2 (20). Phylogenetic analyses were performed based on a combined spoligotypes and 24 loci MIRU-VNTR profiles and a Neighbor-Joining (NJ) tree was build using the Chord distance (Cavalli-Sforza and Edwards 1967) setting in the MIRU-VNTRplus web-based tool to calculate the distance matrix (12, 21). *Mycobacterium canettii* from MIRU-VNTR Plus reference strain

was used as outgroup. A cluster was defined as a group of two or more isolates sharing the same combined spoligotype and 24 MIRU-VNTR patterns. The clustering rate was calculated using the formula: (number of clustered isolates - number of isolate clusters) / total number of isolates) (22).

To assess the associations between variables (drug resistance, demographic data, genotype and clustering), the χ^2 test, crude odds ratio (COR) and 95% confidence interval (95% CI) were calculated. The Fishers' exact was used if any expected counts were less than 5. A *p*-value of < 0.05 was considered statistically significant. All statistical data analyzed with STATA (Version 13.0, StataCorp, College Station, Texas).

Results

Patient characteristics and *M. tuberculosis* population

A total of 278 drug resistant and 126 fully susceptible *M.tb* isolates obtained from 404 MDR-TB high-risk patients during 2012 to 2017 were included in this study. The demographic and clinical data linked to the *M.tb* isolates are summarized in Table 1. For some patients, the epidemiological data could not be collected as mentioned in the table. The age of the patients ranged from 15 to 86 years, with a mean age of 44.7 years. The male/female ratio in the study population was 1.78. The provenance of the patients was grouped into two: (1) the "Capital group" with 194 patients from Phnom Penh capital city and (2) the "Provincial group" with 210 patients distributed in 20 provinces varied from 1 to 72 patients per province (mean number 10.5).

Table 1. Patient demographic and clinical characteristics linked to 404 *M. tuberculosis* isolates

Characteristics		Total n (%) ^a	Drug resistant TB, n (%) ^a	Drug susceptible TB, n (%) ^a	<i>p</i> -value
Gender	Total	384 ^b	264	120	
	Male	242 (63.0)	169 (64.0)	73	0.5494
	Female	142 (37.0)	95 (36.0)	47	
Age (years)	Total	384 ^b	264	120	
	15-34	127 (33.1)	81 (30.7)	46 (38.3)	0.285
	35-64	143 (37.2)	104 (39.4)	39 (32.5)	
	>64	114 (29.7)	79 (29.9)	35 (29.2)	
Residence	Total	404	278	126	
	Capital	194 (48.0)	107 (38.5)	87 (69.0)	< 0.001
	Provinces ^c	210 (52.0)	171 (61.5)	39 (31.0)	
Type of TB disease	Total	404	278	126	
	Pulmonary	396 (98.0)	273 (98.2)	123 (97.6)	0.709
	Extra-pulmonary	8 (2.0)	5 (1.8)	3 (2.4)	
Smear result	Total	309 ^b	213	96	
	Positive	202 (65.4)	145 (68.1)	57 (59.4)	0.156
	Negative	107 (34.6)	68 (31.9)	39 (40.6)	
Previous anti-TB treatment	Total	362 ^b	258	104	
	Yes	333 (91.9)	245 (95.0)	90 (84.9)	0.002
	No ^d	29 (8.1)	13 (5.0)	16 (15.1)	
Period of collection	Total	404	278	126	
	2012-2013	148 (36.6)	104 (37.4)	44 (34.9)	0.652
	2014-2015	165 (40.8)	115 (41.4)	50 (39.7)	
	2016-2017	91 (22.5)	59 (21.2)	32 (25.4)	

a % calculated by column.

b Data were not available for some of the patients

c Twenty provinces including Kampong Cham (n=72), Kandal (n=30), Takeo (n=19), Prey Veng (n=18), Kampot (n=13), Svay Rieng (n=12), Kampong Speu (n=9), Kampong Thom (n=6), Kratie (n=5), Preah Sihanouk (n=5), Banteay Meanchey (n=4), Kampong Chhnang (n=4), Battambang (n=3), Preah Vihear (n=3), Koh Kong (n=2), Kep (n=1), Kampong Speu (n=1), Pursat (n=1), Siem Reap (n=1) and Tboung Khmum (n=1).

d Including 7 cases among people living with HIV

The majority of patients (98.0%, 396/404) had pulmonary TB, while 8 (2.0%) patients had extra-pulmonary TB (meninges, n=3; lymph nodes, n=2; pleura, n=2, and skin, n=1). The TB treatment history was available for 362 patients: 333 (91.9%) were retreated patients, and the remaining 29 (8.1%) were new TB patients, including 7 (4.3%) patients living with HIV. 65.4% (202/309) of patients had positive smear. The occurrence of drug resistance was significantly higher in patients living in provincial areas and in previously treated patients. The number of DR isolates was relatively distributed by year of collection, except for 2016, in which only 8.6% (24/278) of DR isolates was collected. The low number of DR isolates available in 2016 was due to a technical problem, which caused the overall DST activities at NRL partially interrupted.

Resistance profile to first line anti-TB drugs

Besides the 126 (31.2%) fully susceptible isolates (resistant to the four FLDs; RIF, INH, EMB, STM) randomly selected, the 278 (68.8%) DR *M.tb* isolates displayed various patterns of FLD resistance.

Among the 278 DR isolates, 113 (40.7%) were mono-resistant (resistant to only one FLD), 44 (15.8%) were poly-resistant (resistant to more than one FLD but not to INH and RIF at the same time) and 121 (43.5%) were MDR (resistant to at least INH and RIF at the same time). The resistances to INH and STM were the most commonly found among drug resistant isolates, representing 75.5% (210/278) and 61.5% (171/278), respectively. RIF resistance was detected in 50.4% (140/278) isolates, of them 18 (6.5%) were mono-RIF resistance. Resistance to EMB was less common, observed mostly among MDR isolates (17.3%, 48/278). The isolates resistant to all four FLDs, defined in the study as “Quadruple resistant” (QDR), were found in 14.4% (40/278) of all drug resistant isolates (Table 2).

Table 2. Phenotypic resistance to first line anti-TB drugs among the 278 DR *M. tuberculosis* strains investigated.

	Frequency n (%)
Any resistance	278 (100)
Any INH resistance	210 (75.5)
Any STM resistance	171 (61.5)
Any RIF resistance	140 (50.4)
Any EMB resistance	55 (19.8)
Mono-drug resistance	113 (40.7)
STM only	48 (17.3)
INH only	46 (16.5)
RIF only	18 (6.5)
EMB only	1 (0.4)
Poly-drug resistance but not MDR	44 (15.8)
INH + STM	37 (13.3)
INH + EMB	3 (1.1)
RIF + STM	1 (0.4)
INH + STM + EMB	3 (1.1)
Multidrug resistance (MDR)	121 (43.5)
INH + RIF	31 (11.1)
INH + RIF + STM	42 (15.1)
INH + RIF + EMB	8 (2.9)
INH + RIF + STM + EMB (Quadruple resistance)	40 (14.4)

Abbreviation: INH, isoniazid; RIF, rifampicin; STM, Streptomycin; EMB, ethambutol

***M. tuberculosis* family identification**

Spoligotyping and 24-locus MIRU-VNTR typing results were obtained for 403 among the 404 isolates (S1 Table). One isolate did not give any spoligotype pattern. Among the 403 isolates, 13 revealed double alleles at a single MIRU-VNTR locus and six isolates revealed mixed genotype patterns (double alleles in at least two MIRU-VNTR loci). These 19 isolates were excluded from the analysis (S1 Table). Consequently, the data were analyzed for 384 *M.tb* isolates, including 121 DS and 263 DR.

Spoligotyping analysis

Analysis of the spoligotyping data was done by assigning spoligo-international type (SIT) numbers and genotypic spoligotype family designations in comparison with SITVIT2 and SPOTCLUST database. A total of 84 distinct spoligotype patterns were found, with 87.2% (335/384) grouped in

35 similar spoligotype patterns containing two to 169 isolates while 12.8% (49/384) had a unique pattern (Fig 2). Using SITVIT2 database, family/subfamily could be identified for 54 patterns. The remaining 22 patterns had no matching pattern and was determined as “not defined”. The 22 not defined patterns were then analyzed using SPOTCLUST database. Among them, 17 patterns could be assigned to a family with a probability higher than 95%.

Finally, the global sample containing 7 (1.8%, grouped in 5 patterns) isolates was “not defined”. The remaining, 377 isolates belonged to nine families and 19 sub-families. The two major families which account for about 89.6% of all isolates were Beijing (182, 47.4%) and East African-Indian (EAI) (162, 42.2%) families. Within the EAI family, six EAI subfamilies were identified: EAI5 (84, 51.9%), EAI1-SOM (55, 34.0%), EAI2-Nonhaburi (11, 6.8%), EAI4-VNM (8, 4.9%), EAI2-Manila (3, 1.8%) and EAI3-IND (1, 0.6%). Other minor families were found in low proportion: T (11, 2.9%), Unknown (U) (9, 2.3%), Haarlem (H) (4, 1.0%), Manu-ancestor (3, 0.8%), Zero-copy (3, 0.8%), Cameroon (2, 0.5%) and Latin-American and Mediterranean (LAM) (1, 0.3%). No Central Asian Strain (CAS) family was found in our sample.

Regarding the frequencies of the major shared types, SIT1 (Beijing) was predominant in this study with 170/384 (44.3%), followed by SIT48 (EAI1-SOM), SIT236 (EAI5), SIT204 (EAI5), SIT459 (EAI1-SOM) and SIT89 (EAI2-Nonhaburi), each consisting of 36, 21, 13, 12 and 10 isolates, respectively.

MIRU-VNTR analysis

Full 24-MIRU-VNTR loci results were obtained for 374 among the 384 isolates (97.4%). For 10 isolates, one or two loci among QUB11b ($n=4$), MIRU20 ($n=3$), ETRA ($n=3$) and MIRU 39 ($n=1$) could not be amplified after three repetitions, these results were notified as missing data at the respective loci.

The 24 loci MIRU-VNTR typing of the 384 M.tb isolates allowed distinguishing 341 patterns, including 307 unique patterns and 34 patterns shared by 78 isolates (ranged from 2 to 4 isolates per pattern). The genotypic diversity (G_d) and the mean genetic diversity (H_s) were 0.888 and 0.367, respectively. The Hunter-Gaston discriminatory Indexes (HGDI, h) for the 24 MIRU-VNTR data were 0.9993, 0.9980 and 0.9985 for the total population, Beijing family and EAI family, respectively. The average of the numbers of alleles per locus was 7.46, ranging between three for MIRU2 loci and 19 for MIRU4 loci. The allele diversity index of each of the 24 MIRU-VNTR loci was calculated and presented in S2 Table. Considering all isolates, ten loci were highly discriminative ($h > 0.6$), six were moderately discriminative ($0.3 \leq h \leq 0.6$) and eight were poorly discriminative ($h < 0.3$). The discriminatory power of each locus was different between Beijing, EAI, and the other genotypes. In the Beijing population, Mtub21 and QUB11b showed a high degree of discrimination, whereas 15 loci were poorly discriminative, including even loci with discriminatory index < 0.1 . Twelve loci showed high discrimination for genotypes other than Beijing and EAI genotypes (other genotypes), three of which, including Mtub21, ETRA, and Mtub39, exhibited a high discriminatory power for the EAI genotypes; except for MIRU4 which showed low to moderate discriminatory power for EAI genotypes. The allelic profile of each MIRU-VNTR locus (copy number of tandem repeats) in Beijing, EAI and other families is shown in S1 Fig.

Combination of spoligotyping and MIRU-VNTR results

The dendrogram generated by Neighbor-Joining (NJ) algorithm based on Spoligotyping and MIRU-VNTR data allowed to determine phylogenetic relationships between the 384 M.tb isolates (S2 Fig). The Beijing family isolates were clearly differentiated from others families on the tree, while EAI family was mainly subdivided in two groups, which correspond to EAI2-nonthaburi family and other EAI families. The genetic differentiation between Beijing, EAI and other families was supported by a value of F_{st} equal to 0.486 and a $p = 0.016$. The F_{st} within EAI family also supported the differentiation between EAI2 sub-families (EAI2-manila and EAI2-nonthaburi) and the other EAI sub-families ($F_{st} > 0.5, p < 0.05$), while EAI1-SOM and EAI5 showed a lower differentiation ($F_{st}=0.069, p = 0.035$).

Cluster analysis

The NJ tree was also used to determine the clusters defined as group of isolates having the same MIRU-VNTR and spoligotype profile. Thirty clusters (labeled from C1 to C30) shared by 69 isolates (two to three isolates per cluster; the clustering rate was 10.2%) were observed (Fig 3), while the sole MIRU-VNTR data revealed 34 shared patterns. In four MIRU-VNTR shared patterns, isolates were sharing the same MIRU-VNTR pattern but different spoligotypes (S1 Table). Among the 30 clusters, 21 clusters of 47 isolates belonged to Beijing family (clustering rate = 14.3%), eight clusters of 19 isolates belonged to EAI family (clustering rate = 6.8%), and one cluster of two isolates belonged to Manu-ancestor family (clustering rate = 33.3%) (Table 3). The proportion of Beijing and EAI isolates in clusters were 25.8% and 11.7%, respectively. Compared to EAI family, the isolates belonging to Beijing family had a significantly higher clustering proportion (COR= 2.69; 95% CI 1.51 to 4.82; $p < 0.001$). More interestingly, 13 Beijing clusters of 29 isolates (C6, C8, C11, C17, C20, C21, C22, C23, C24, C25, C26, C27 and C29) formed a clonal complex (CC1) by sharing alleles at 22 of 24 MIRU-VNTR loci and 21/29 (72.4%) isolates were MDR with resistance to three or all the four FLD. Two smaller clonal complexes were also observed in EAI clusters; cluster C1 and C10 formed CC2 and cluster C3 and C14 formed CC3 with 5 isolates each (Fig 3).

Data were not available to assess potential epidemiologic links between the subjects in clusters. However, 15 among 30 clusters (9 Beijing clusters, 5 EAI clusters and 1 Manu-ancestor cluster) could be geographically linked since isolates were from patients living in the same provinces.

Table 3: Estimation of clustering rate for overall sample, Beijing, EAI and Manu-ancestor population

Characteristics	Total	Beijing	East-African Indian	Manu-ancestor
Total number of isolates	384	182	162	3
Number of unique isolates	315	135	143	2
Number of clusters	30	21	8	1
Number of clustered isolates	69	47	19	2
Cluster size (range)	2 - 3	2 - 3	2 - 3	2
Clustering rates (%)	10.2	14.3	6.8	33.3

Regarding drug resistant isolates, 26 clusters contained at least one drug resistant isolate, of them 18 clusters were formed of at least one MDR isolate. MDR isolates presented a significant higher proportion of clustering than those non-MDR-TB (26.9% vs. 16.9%; COR 1.82; 95% CI 1.00 to 3.29; $p = 0.048$). Additionally, six clusters including 4 MDR clusters (C8, C11, C15 and C18) contained isolates having the same phenotypic drug resistant profile; C8 and C11 belong to CC1 while C15 and C18 do not belong to any clonal complex (Fig 3).

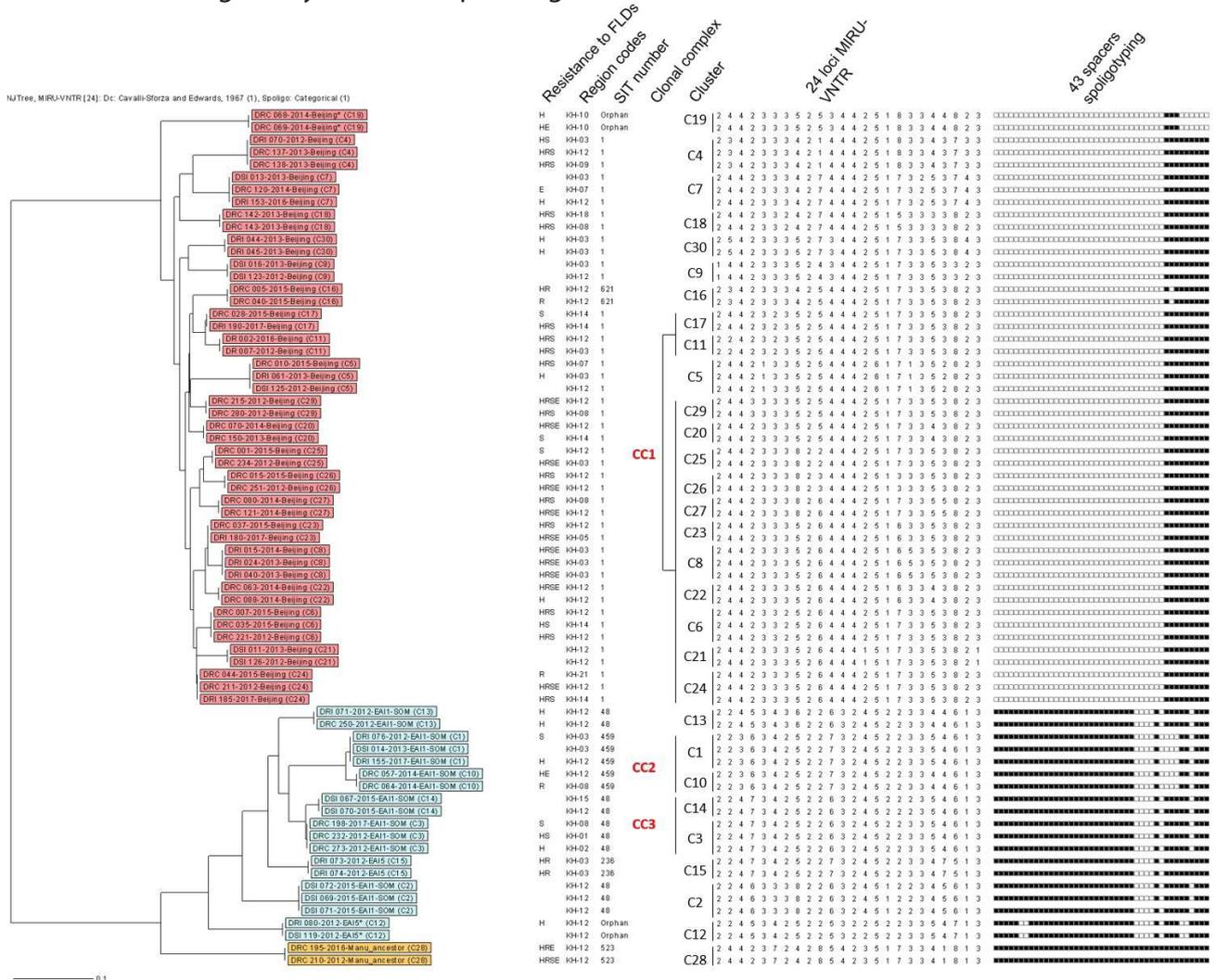


Fig 3. Neighbor-joining tree based on the MIRU-VNTR and spoligotyping data for 69 clustered isolates.

From left to right: i) NJ tree based on the 24-locus MIRU-VNTR and spoligotyping data for the 69 isolates grouped in 30 clusters; ii) Resistant profiles to first line anti-TB drugs (FLD: H, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol); iii) Regional codes of provinces of Cambodia (KH-01, Banteay Meanchey; KH-02, Battambang; KH-03, Kampong Cham; KH-05, Kampong Speu; KH-07, Kampot; KH-08, Kandal; KH-09, Koh Kong; KH-10, Kratie; KH-12, Phnom Penh; KH-14, Prey Veng; KH-15, Pursat; KH-18, Sihanoukville; KH-21, Takeo); iv) Spoligotype international type (SIT) number based on SIVIT2 database; v) Clonal complex defined by at least two strains linked by no more than two loci changes; vi) cluster identification; vii) Number of repetitions of each of the 24 MIRU VNTR loci; and viii) 43-spacer spoligotypes (black spots represent the presence and white spot represent the absence of 1–43 spacers).

Association of M.tb genotypes with drug resistance, demographic and clinical data

The proportion of drug resistance, as well as demographic and clinical data were calculated for the two main families; Beijing and EAI (Table 4). The proportion of drug-resistant isolates among patient infected with Beijing and EAI family was 80.8% and 55.6%, respectively. In addition, 76 (51.7%) isolates belonging to Beijing family were MDR, while only 28.7% belonged to EAI family. Comparatively to EAI isolates, Beijing isolates were associated with drug resistance phenotype (COR 3.36; 95% CI 2.02 to 5.61; $p < 0.001$) and this association was stronger for MDR (COR, 6.01; 95% CI, 3.29 to 10.97; $p < 0.001$) and QDR (COR, 21.26; 95% CI, 6.08 to 74.34; $p < 0.001$). In addition, 80% (84/105) of MDR-TB patients had positive smear. MDR-TB (COR 2.43; 95% CI, 1.25 to 4.72; $p = 0.009$) and particularly QDR (COR 3.97; 95% CI, 2.72 to 31.94; $p < 0.001$), is likely to be linked with positive smear. None of the demographic and clinical characteristics studied including positive smear were found to be significantly associated with Beijing genotype.

Table 4. General characteristics of patients infected with Beijing (n=182) or EAI (n=162) isolates.

Characteristics		Beijing isolates N (%a)	EAI isolates N (%a)	COR (95% CI)	p-value ^b
Sex	Men	115 (65.0)	90 (60.0)	1	
	Women	62 (35.0)	60 (40.0)	1.23 (0.77-1.99)	0.354
Age group	15-34	65 (36.7)	44 (29.3)	1	
	35-54	69 (39.0)	56 (37.3)	1.01 (0.61-1.71)	0.945
	>54	43 (24.3)	50 (33.3)	1.53 (0.88-2.66)	0.134
Place of residence	Capital	83 (45.6)	80 (49.4)	1	
	Province	99 (54.4)	82 (50.6)	0.85 (0.55-1.34)	0.484
Previous treatment history	Yes	160 (91.1)	131 (89.7)	1	
	No	10 (5.9)	15 (10.3)	1.83 (0.74-4.71)	0.109
Smear result	Positive	88 (67.7)	77 (62.1)	1	
	Negative	46 (34.3)	47 (37.9)	1.17 (0.68-2.00)	0.320
Year of isolation	2012-2013	78 (42.8)	54 (33.3)	1	
	2014-2015	66 (36.3)	70 (43.2)	0.65 (0.40-1.06)	0.084
	2016-2017	38 (20.9)	38 (23.5)	0.69 (0.39-1.22)	0.204
Drug resistance	No	35 (19.2)	72 (44.4)	1	
	Yes	147 (80.8)	90 (55.6)	3.36 (2.02-5.61)	< 0.001
Drug resistant patterns	Mono-resistance	45 (24.7)	47 (29.1)	1.97 (1.11-3.49)	0.021
	Poly-resistance	26 (14.3)	17 (10.5)	3.15 (1.51-6.55)	0.002
	Multi-drug resistance	76 (41.8)	26 (16.1)	6.01 (3.29-10.97)	< 0.001
	Quadruple resistance	3 (1.9)	31 (17.0)	21.26 (6.08-74.34)	< 0.001

^a % calculated by column.

^b p-value from the Chi-square test.

Discussions and conclusion

As worldwide, the number of DR-TB is increasing overtime in Cambodia (1-3, 6). The first report of XDR-TB has been declared to the WHO in 2016 (4). In addition, since Indonesia, Myanmar, the Philippines, Thailand, and Vietnam are among countries with high burden of TB and/or MDR-TB and the population movements are continuously increasing between Cambodia and these countries,

it is essential to monitor the circulation of the highly resistant and hypervirulent M.tb genotypes in order to optimize the disease control strategy (1). The present study gives the first outline of the diversity of the M.tb population structure among patients suspected of having drug resistant TB in Cambodia over a period of 6 years. Our results show that Beijing (47.4%) and EAI (42.2%) families are the two most predominant genotypes among our sample. Similar to the first assessment conducted by Zhang *et al.* (2015) on a restricted sample collected between 2007 and 2008, both Beijing and EAI family represent almost 90% in our sample. Other families like T, Haarlem, Latin-American and Mediterranean (LAM), Cameroon, Manu_ancestor and U (unknown), represent only a small proportion of the population. Central Asian (CAS) family remains absent in Cambodia (23). The Zero_copy family (SIT 405) characterized by the absence of IS6110 insertion elements (24), was reported for the first time in the present study, but weakly prevalent (n=3; 0.8%).

As largely described, the Beijing family is predominant in Eastern Asia and often associated with drug resistance (23, 25-27), hypervirulence (25, 28), younger ages (27) and outbreak (29, 30) and has spread worldwide in recent decades (25, 31, 32). Compared to Zhang *et al.* study in which the proportion of Beijing family was 30%, the proportion in our study increased to 47.4% and now became the most predominant family (23). In addition, as expected, the Beijing family was associated with drug resistant patterns and especially with MDR (COR, 2.63; 95% CI, 1.51 to 4.61; $p = 0.001$) and QDR (COR, 9.31; 95% CI, 2.72 to 31.94; $p < 0.001$).

The cluster analysis based on the combination of MIRU-VNTR and spoligotype data revealed 30 clusters shared by 69 isolates (21 clusters belonged to Beijing family and 18 clusters were formed of at least one MDR isolate). Beijing family was, thus, more likely regrouped in clusters (COR= 2.69; 95% CI 1.51 to 4.82; $p < 0.001$) suggesting its probable involvement in recent transmission events. It is worth noting that even if these results are informative regarding the spread of M.tb in the population under study and the risk of transmission, for more detailed molecular epidemiology study the clustering data should be confirmed by whole genome sequencing which gives the highest genetic resolution.

Though the transmission rate found among our sample was relatively low (clustering rate of 14.3% with only 2 to 3 isolates per cluster), the increase of Beijing family circulating in the country could have a major impact on the TB epidemiology due to its high transmission ability as well as its association with drug resistance (27, 28, 30, 33).

None of the demographic and clinical characteristics of patients collected including sex, age, place of residence, smear results, previous treatment history and year of isolation were associated with Beijing family in contrast to previous reports (27). The only signal concerned 15 (9 Beijing clusters, 5 EAI clusters and 1 Manu-ancestor cluster) among the 30 clusters for which the isolates of a same cluster were from patients living in a same province (Fig 3). Except for four clusters, 21 isolates in eleven clusters was identified almost all in the same period with maximum one-year interval. The global lack of association can be due to a sampling bias since our study included isolates from presumed MDR-TB patients recruited by the NTP. The sampling is not thus representative of the M.tb population circulating in Cambodia and the clustering rate could be underestimated.

Regarding the phylogenetic relationships, three clonal complexes (CC) were identified. One clonal complex CC1 was formed by 29 Beijing isolates with the majority of them being MDR with resistance to three or four FLDs. The 29 isolates were distributed through the six-year of study period between 2012 to 2017 and the majority of them was from patient living in Phnom Penh capital city (KH-12; n=15), followed by Kampong Cham province (KH-03) and Prey Veng province (KH-14) with five isolates each (Fig 3 and S1 Table). These data reflect the spread of MDR Beijing strains that evolved overtime in Cambodia, particularly in Phnom Penh which is the most populated area (34).

In conclusion, the data suggest that Beijing family is currently the most predominant genotype among drug resistant isolates involved in highly drug resistance and recent transmission. Further studies should be conducted to further understand the molecular epidemiology of Beijing family in Cambodia and in provincial areas to determine the routes of transmission and the factors of MDR and XDR emergence. Rapid interventions in terms of diagnostic and treatment are essential to prevent the spread of drug resistant Beijing strain in the population.

Conflicts of Interest

The authors have declared that no competing interests exist.

Data Availability Statement

All relevant data are within the manuscript and its supporting information files.

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Supporting information

S1 Table. Demographic data, resistance patterns, MIRU-VNTR and spoligotype patterns of the 404 *Mycobacterium tuberculosis* isolates

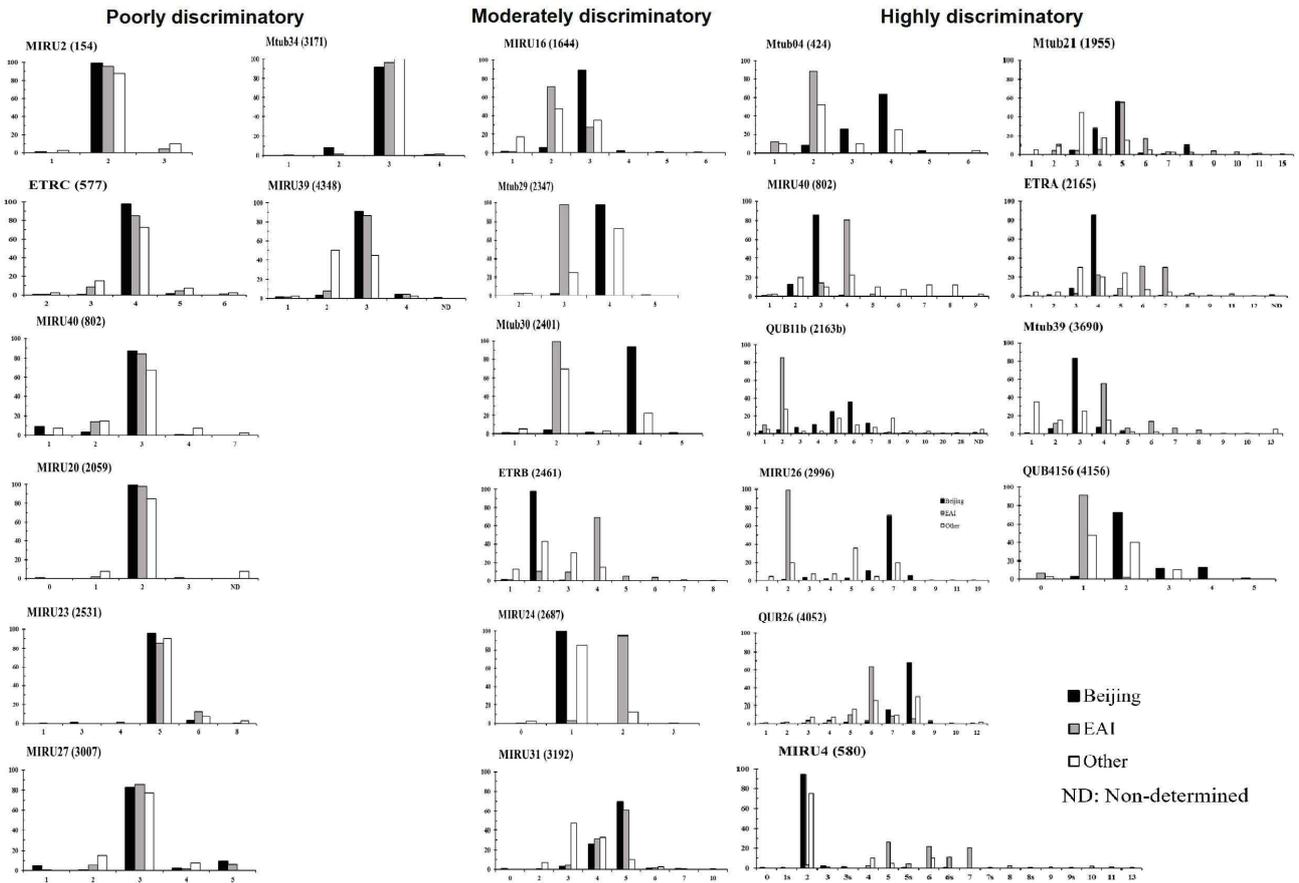
The data was available in the google drive through link below:

https://drive.google.com/file/d/1PByS-LGT_Jp8HlXT_eQDrwwnyQs6yEE/view?usp=sharing

S2 Table. HGDI of different MIRU-VNTR loci in total population and population belong to Beijing, EAI and other genotypes

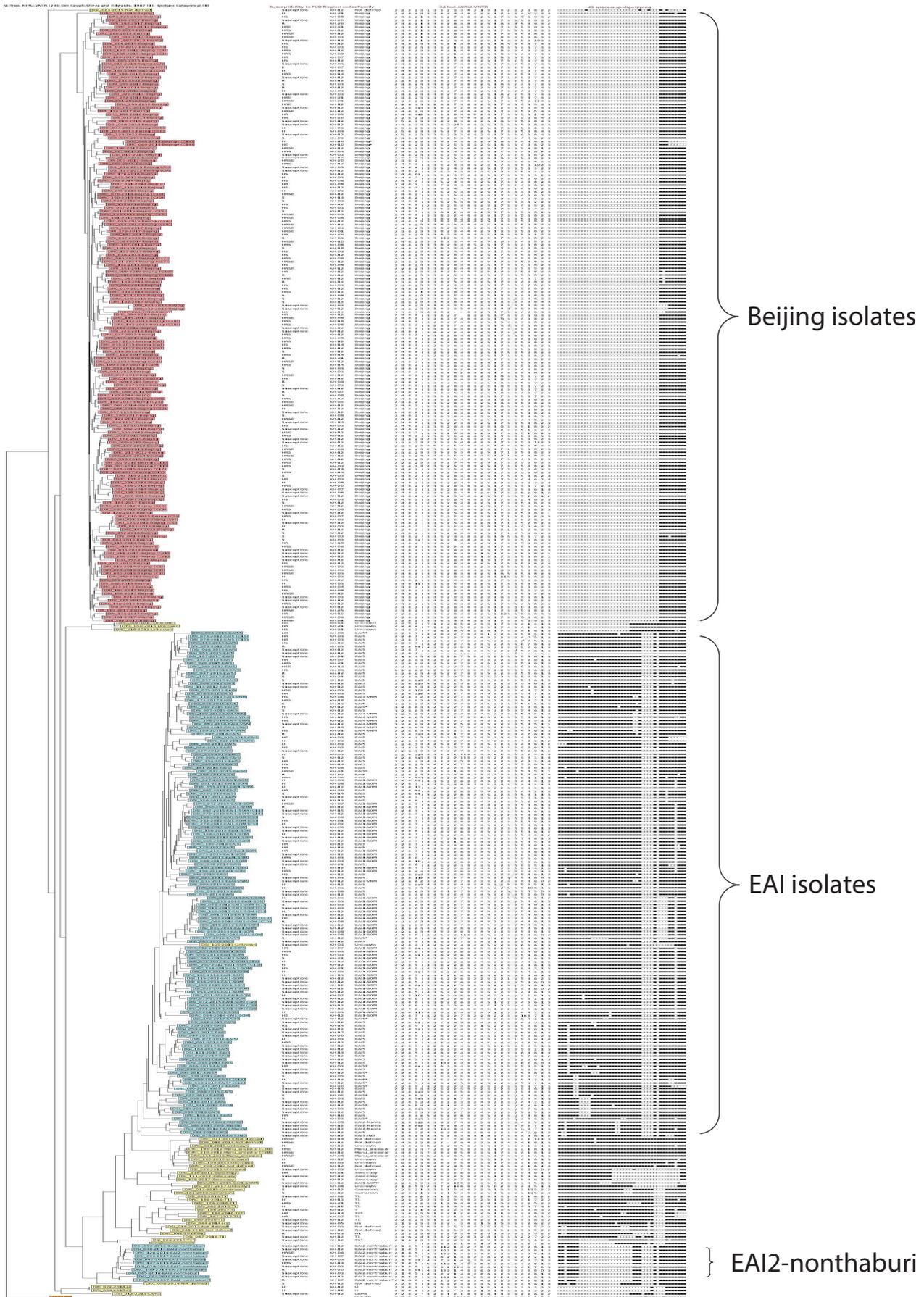
MIRU-VNTR locus		Total (n=384)		Beijing genotype (n=182)		EAI genotype (n=162)		Other genotypes (n=40)	
Locus	Alias	n. of allele	h	n. of allele	h	n. of allele	h	n. of allele	h
MIRU 2	154	3	0.0709	2	0.0219	2	0.0832	3	0.2295
Mtub 04	424	6	0.6554	4	0.5227	2	0.2083	5	0.6577
ETRC	577	5	0.1892	4	0.0435	5	0.2665	5	0.4564
MIRU 4	580	19	0.6759	7	0.1067	16	0.8292	4	0.4256
MIRU 40	802	5	0.2820	4	0.2292	4	0.2662	5	0.5231
MIRU 10	960	9	0.6298	4	0.2506	5	0.3270	9	0.8731
MIRU 16	1644	6	0.5284	6	0.2049	3	0.4214	3	0.6372
Mtub 21	1955	12	0.6919	7	0.6059	11	0.6636	7	0.7474
MIRU 20	2059	4	0.0414	3	0.0219	2	0.0366	2	0.1532
QUB-11b	2163b	12	0.7660	10	0.7773	8	0.2657	10	0.8478
ETRA	2165	11	0.6754	6	0.2333	9	0.7559	8	0.8141
Mtub 29	2347	4	0.5127	3	0.0540	2	0.0485	3	0.4218
Mtub 30	2401	5	0.5209	5	0.1162	2	0.0123	4	0.4679
ETRB	2461	8	0.5985	3	0.0434	8	0.5026	4	0.7090
MIRU 23	2531	6	0.1734	3	0.0852	5	0.2605	3	0.1885
MIRU 24	2687	4	0.4964	1	0.0000	4	0.0840	3	0.2679
MIRU 26	2996	11	0.6642	10	0.4818	2	0.0123	7	0.8013
MIRU 27	3007	5	0.2939	5	0.3016	5	0.2581	3	0.3808
Mtub 34	3171	4	0.1046	3	0.1622	4	0.0608	1	0.0000
MIRU 31	3192	8	0.5568	6	0.4556	7	0.5322	5	0.6692
Mtub 39	3690	11	0.7245	5	0.3040	9	0.6535	7	0.7859
QUB-26	4052	11	0.7400	11	0.5080	8	0.5740	7	0.8154
QUB-4156	4156	6	0.6354	5	0.4464	3	0.1508	4	0.6192
MIRU 39	4348	4	0.2735	4	0.1670	4	0.2364	4	0.5603

*Bold value represented diversity index (h) > 0.6000.



S1 Fig. Allelic distribution of 24 MIRU-VNTR loci in Beijing, EAI and other genotype population.

Black, gray and white bars represent the repeat numbers of each locus in Beijing, EAI and other genotype, respectively. X-axis: %, the proportion of repeat numbers of the locus; Y-axis: the repeat numbers of each locus.



S2 Fig. Neighbor-Joining tree built using Cavalli-Sforza and Edwards distance matrix calculated from the combination of MIRU-VNTR and spoligotyping data of 384 *M. tuberculosis* isolates. The 30 clusters are identified on the tree as well as the spoligotype families, drug resistance patterns, the MIRU-VNTR and spoligotyping profiles.

Result 2: Focus on *Mycobacterium tuberculosis* resistant to second line anti-tb drugs in Cambodia

*Manuscript (Article 4) is accepted with minor revisions for publication
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ORIGINAL RESEARCH

Sokleaph Cheng et al

Running title: Resistance to second-line anti-TB drugs in Cambodia: a phenotypic and genetic study

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Abstract

Background: Due to the emergence of *Mycobacterium tuberculosis* (M.tb) clinical isolates resistant to most potent first line drugs (FLD), second-line drugs (SLD) are being prescribed more frequently. We explore the genetic characteristics and molecular mechanisms of M.tb isolates phenotypically resistant to SLD, including pre-extensively drug-resistant (pre-XDR) and extensively drug-resistant (XDR) isolates.

Methods: Drug-resistant (DR) M.tb isolates collected from 2012 to 2017 were tested using sequencing and phenotypic drug susceptibility testing. Genotypes were determined to explore their links with SLD resistance patterns.

Results: Of the 278 DR M.tb isolates, 6 non-multidrug resistant (non-MDR) isolates were fluoroquinolones (FQ)-resistant, 3 were XDR and 16 were pre-XDR (14 resistant to FQ and 2 to second-line injectable drugs). The most frequent mutations in FQ-resistant and second-line injectable drugs resistant isolates were *gyrA* D94G (15/23) and *rrs* a1401g (3/5), respectively. 75% of pre-XDR isolates and 100% of XDR isolates harbored mutations conferring resistance to pyrazinamide. All XDR isolates belonged to the Beijing genotype of which one, named XDR+, was resistant to all drugs tested. One cluster including pre-XDR and XDR isolates was observed.

Conclusion: This is the first description of SLD resistance in Cambodia. The data suggest that the proportion of XDR and pre-XDR isolates remains low but is on the rise compared to previous reports. The characterization of the XDR+ isolate in a patient who refused treatment underlines the risk of transmission in the population. In addition, genotypic results show, as expected, that the Beijing family is the main involved in pre-XDR and XDR isolates and that the spread of the Beijing pre-XDR strain is capable of evolving into XDR strain. This study strongly indicates the need for rapid interventions in terms of diagnostic and treatment to prevent the spread of the pre-XDR and XDR strains and the emergence of more resistant ones.

Keywords: *Mycobacterium tuberculosis*; Extensively drug-resistant tuberculosis; Pre-extensively drug-resistant tuberculosis; Fluoroquinolone; Second-line injectable drugs

Introduction

Nowadays, tuberculosis (TB) is the leading cause of death from a single infectious agent, *Mycobacterium tuberculosis* (M.tb), ranging above HIV.¹ In 2018, an estimated 10.0 million people developed TB and around 1.5 million people died of this disease, including 1.25 million deaths from HIV-negative individuals and 251,000 deaths from HIV-associated TB.¹ Drug-resistant TB (DR-TB) is of major concern to TB control worldwide, as no country or region is spared. The emergence of multidrug-resistant (MDR) (TB-resistant to at least rifampicin and isoniazid) and extensively drug-resistant (XDR) (MDR plus resistance to any fluoroquinolone and any second-line injectable drug) M.tb isolates, along with the lack of new effective drugs to fight the disease, is a growing problem.

An estimated 484,000 MDR-TB cases emerged in 2018 and among the incident of TB cases, 3.4% of new cases and 18% of previously treated cases were estimated to have MDR-TB.¹ As of 2018, XDR-TB, has been identified in 127 countries, with an average proportion of XDR-TB of 8.5% among MDR-TB cases². Totally drug-resistant (TDR) TB have also been described, but the term is not yet recognized by the World Health Organization (WHO).³⁻⁵

Multiple factors have led to the emergence of XDR M.tb isolates, including underdeveloped laboratory capacities that prevent timely diagnosis of MDR-TB and limited access to second-line drugs to treat MDR-TB.⁶ Evidence-based treatment is considered as the best treatment option to prevent the development of drug-resistant TB.⁷ However, due to the delay of the results, limited access to conventional drug susceptibility testing (DST) and the unreliable results for some of the anti-TB drugs such as pyrazinamide (PZA), the treatment is usually initiated without knowledge of the drugs susceptibility to the bacilli in many developing countries including Cambodia.⁸⁻¹⁰

Currently, first-line drugs (FLD), including isoniazid (INH), rifampin (RIF), ethambutol (EMB) and PZA, form the core of treatment regimen for drug susceptible TB, while fluoroquinolones (FQ) and second-line injectable agents (SLID) are the two main classes of drugs used in the MDR-TB treatment regimen.^{9,11} Prior to 2017, streptomycin (STM) was also included in the first-line TB treatment for retreated patients (WHO category II regimen) in many countries where conventional or molecular DST were not routinely available.^{12,13} However, due to its resistance and toxicity, STM is now a reserved second-line injectable agent and used as a substitute for amikacin in the MDR-TB regimen.⁹ Some FLD such as INH, EMB and PZA also play an important role in the chemotherapy of drug-resistant TB.⁹ Compared to drug susceptible TB, treatment of MDR-TB and XDR-TB requires prolonged expensive chemotherapy, often associated with important side effects and a low success rate, 56% for MDR-TB and 39% for XDR-TB.¹ More extreme drug resistance patterns to all available FLD and SLD drugs, ie totally drug-resistant TB (TDR),⁴ also known as extremely drug-resistant (XXDR) by Migliori et al,¹⁴ have already been reported and were untreatable with currently available drugs. For this reason, it is of critical importance to understand how drug resistance arises, evolves and how it may be prevented. The aim of this study is to acquire phenotypic and genetic knowledge specifically on second-line anti-TB drug (SLD) resistance in Cambodia. This study was focused on M.tb clinical isolates phenotypically resistant to SLD, including pre-XDR and XDR.

Materials and methods

M.tb strain collection and NTP routine for MDR-TB screening

This study included FLD resistant M.tb isolates taken from the collection of clinical isolates obtained via a platform established for routine screening of MDR-TB among high-risk groups by the National Tuberculosis Program (NTP).¹⁵ Since 2012, people at risk of developing MDR-TB were screened using Xpert MTB/RIF, followed by culture and phenotypic drug susceptibility testing (pDST)

for FLD. All confirmed RIF resistant (RR) or MDR-TB cases received a 24-month standardized regimen in two phases: an 8-month intensive phase and 16-month continuation phase. The treatment contained PZA and at least 4 effective SLD including one SLID, one FQ and two oral bacteriostatic drugs selected among Ethionamide (Eto), Cycloserine (Cs) or P-aminosalicylic acid (PAS).¹⁶ The Cambodian NTP adopted the WHO-recommended MDR-TB shorter regimen in 2017 and the rapid molecular DST (GenoType MTBDRs/, Hain LifeScience, Germany) was systematically used to screen for resistance to FQ and SLID for all eligible patients for the shorter regimen.¹⁷

The National TB Reference Laboratory (NRL) of the National Centre for Tuberculosis and Leprosy Control (CENAT) and the Mycobacteriology Laboratory of the Institut Pasteur in Cambodia (IPC) are the two main DST laboratories in Cambodia. During their routine screening activities to detect drug-resistant TB among MDR-TB high-risk groups, M.tb were isolated, identified, and cryo-preserved in the strain bank at the IPC and NRL. All the strains were thus tested for their susceptibility to first line anti-TB drugs (FLD) including rifampicin (RIF; 1.0 µg/mL), isoniazid (INH; 0.1 µg/mL), ethambutol (EMB; 5.0 µg/mL), streptomycin (STM; 1.0µg/mL) by pDST using the automated BACTEC MGIT 960 (MGIT AST SIRE kit, Becton Dickinson (BD), Sparks, MD, USA) (see Table S1).¹⁸ STM is considered as one of the FLD since it was used in first-line TB treatment for retreated patients until the end of 2017.¹¹ The DST results were not available for PZA.

Patient data including age, gender, geographic area and year of registration, clinical information, FLD susceptibility profile and treatment outcomes were collected from the patients' laboratory and available medical records.

DNA preparation

The original stocks of clinical M.tb isolates stored at -80°C were re-cultured on Löwenstein-Jensen (LJ) medium slants. A loopful of M.tb colonies recovered from LJ medium slants were suspended in 300 mL of TE buffer (10 mM Tris, 1 mM EDTA pH 8.5) and boiled for 30 min. The supernatant containing bacterial DNA was used as PCR template.

Phenotypic drug susceptibility testing (pDST)

The pDST of M.tb to second-line anti-TB drugs was carried out in a Biosafety Level 3 laboratory at the IPC using the BACTEC™ MGIT™ 960 liquid culture system (BD, Sparks, MD, USA). The MGIT DST method was used according to the standard critical antibiotic concentrations recommended by the WHO: ofloxacin (Ofx; 2.0 µg/mL); amikacin (Am; 1.0 µg/mL); kanamycin (Km; 2.5 µg/mL) and capreomycin (Cm; 2.5 µg/mL).¹⁹ Based on the FLD and SLD phenotypic DST profiles, the M.tb isolates under this study were categorized into MDR, non-MDR, fluoroquinolone-resistance (FQ-R), second-line injectable drug (SLID) resistance, pre-XDR and XDR (see Box 1).

Sequencing

The main genes associated with resistance to FLD and SLD were amplified by PCR using specific primers reported in Table S2 and sequenced (Macrogen Inc., Republic of Korea and Eurofins Genomics Germany GmbH, Germany). For FLD resistance, the following genes and gene fragments were studied: the *katG* gene, the *inhA* gene coding and the *inhA* promoter (INH resistance); the *rpoB* gene (RIF resistance); the *rpsL* gene and *rrs*-F1 fragment targeting *rrs* 530 and 915 loops (STM resistance); the *embB* gene (EMB resistance); the *pncA* gene and its promoter (PZA resistance). For SLD resistance (including FQ and SLID), the following genes or gene fragments were analyzed: the *gyrA* and *gyrB* genes (FQ resistance); the *rrs*-F2 fragment targeting the *rrs* 1400-1500 region (SLID resistance).²⁰ The sequences were aligned with the M.tb H37Rv reference (GenBank NC000962.3) using the Bioedit software v7.2.6.²¹ To describe the mutations associated with resistance in the *rpoB* and *gyrB* genes, a numbering system based on the *Escherichia coli* sequence annotation and 2002 M.tb H37Rv sequence re-annotation was used.^{22,23} The interpretation of mutations related to resistance was based on resistance mutation sites reported in previous studies.^{20,24-26} When the results between DNA sequencing and pDST were not consistent, both methods were repeated. If the repeated result conflicted with the original data, a third round of testing was accepted as the final result.

Spoligotyping

Spoligotyping was carried out following previously described standard techniques.^{27,28} The spoligotype international type (SIT) was determined using the 6th version of the international genotyping database SITVIT2.²⁹ The isolates with an undefined pattern declared as "orphans or New" were further compared with SPOTCLUST database.³⁰ The family assignment was retained when the probability was $\geq 95\%$.

MIRU-VNTR

Standardized 24-locus mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing was performed by following the recommendations of Supply *et al.*³¹ The allelic profiles were analyzed using the MIRU-VNTRplus database (available at www.miru-vntrplus.org/).

The resolution power of 24 MIRU-VNTR was determined based on the Hunter-Gaston-Discriminatory Index (HGDI).³² The method was classified as "highly discriminant" (HGDI ranged between 0.90-0.99), "moderately discriminant" (HGDI ranged between 0.6-0.9) and "poorly discriminant" (HGDI <0.6).³³ The genetic diversity within sample (H_s) was calculated using FSTAT version 2.9.3.2.³⁴

Phylogenetic reconstruction

Phylogenetic analyses were performed combining spoligotype and 24 loci MIRU-VNTR profiles and a Neighbor-Joining (NJ) tree was built using the Chord distance (Cavalli-Sforza and Edwards 1967) setting in the MIRU-VNTRplus web-based tool to calculate the distance matrix.^{35,36} We included one *M. canettii* reference database isolate as outgroup. A cluster was defined as a group of two or more isolates sharing the same combined spoligotype and 24 MIRU-VNTR patterns.

Results

***Mycobacterium tuberculosis* population under study**

Among a total of 710 *M.tb* isolates (1 isolate per patient) collected from 2012 to 2017 and stored at the IPC and NRL banks, 309 were phenotypically resistant to at least one FLD and 401 were pan-susceptible (Figure 1). Among the 309 drug-resistant *M.tb* isolates, 278 were successfully subcultured on Lowenstein-Jensen (LJ) medium slants and underwent sequencing of the *gyrA/B* and/or *rrs-F2* genes involving resistance to FQ and SLID, respectively. However, the sequencing results were obtained for only 272 isolates including 118 (43.4%) MDR isolates and 154 (56.6%) non-MDR isolates. Among these, 37 isolates carried mutations in the *gyrA/B* and *rrs-F2* genes (see Table S1). The data on these 37 isolates are detailed below. Out of these 37 isolates, the phenotypic resistance to any SLD including Ofx, Am, Km and Cm was found only for 25 isolates which were then used for genotypic study.

Characteristics of patients

Patient data including age, gender, geographic area, year of registration and clinical information linked to the 37 isolates carrying mutations in the *gyrA/B* and/or *rrs-F2* genes were collected, results are shown in Table 1. Absence of recorded data were considered as missing data. From the available data, most of the study subjects were males (23/36; 63.9%). The median age at the time of diagnosis was 47.1 (IQR 30.0-55.0), it ranged from 23 to 80 years. The majority of patients had pulmonary TB (33/34; 97.1%). One patient had lymph node TB, the most common extrapulmonary TB. Among the 31 pulmonary TB patients, 74.2% (23/31) were smear-positive. Most study subjects were TB cases in re-treatment (33/35; 94.3%) with two HIV-positive patients (2/13; 15.4%). A higher number of SLD resistance TB cases were observed among males (68%), the 35-54 years of age group (60%) and those who lived in urban settings (48%).

Resistance to first-line drugs

Phenotypic resistance to FLD: INH, RIF, STM, EMB (see Table S1)

The phenotypic resistance to FLD was available for INH, RIF, STM and EMB. For susceptibility to PZA, only molecular data were acquired in the framework of this study and are detailed in the

following paragraph. Among the 37 isolates, 24 (64.9%) were MDR and 13 (35.1%) were non-MDR. The phenotypic resistance rate of all the clinical isolates to each FLD was as follows: INH, 83.8% (n=31); RIF, 70.3% (n=26); STM, 64.9% (n=24) and EMB, 27.0% (n=10). Among the 24 MDR isolates, 21.6% (n=8) were resistant to all four FLD, defined here as “Quadruple drug-resistant” (QDR) isolates.

Mutations in the *katG*, *inhA*, *rpoB*, *rrs-F1*, *rpsL* and *embB* genes

The mutations detected in genes involving resistance to FLD are reported in Table 2. Among the 31 INH-resistant isolates, 24 (77.4%) harbored mutations in the *katG* gene, 5 (16.1%) in the *inhA* core gene and/or its promotor, and 1 (3.2%) in both genes. One (3.2%) INH-resistant isolate had no mutation in either *katG* or *inhA*. The most common mutations were *katG* S315T (20/30, 66.7%), followed by *inhA* c-15t+S94A (3/30, 10%) and *inhA* c-15t (2/30, 6.7%). Other mutation points were found: *katG* S315N; *katG* W149R; *katG* W477L+A479P and *katG* F720S+*inhA* c-15t in one isolate each. For one (3.2%) isolate, *katG* could not be amplified after repetitions using two pairs of primers targeting two different regions on the gene. The result suggests that the partial or completed *katG* gene was truncated, as reported by Ramaswamy *et al.*³⁷ One out of 6 INH-susceptible isolates had mutation G560S in the *katG* gene.

Among the 26 RIF-resistant isolates, 25 (96.2%) had mutations at least at one codon in the 81-bp *rpoB* rifampicin resistance-determining region (RRDR) and one isolate had no mutation in the *rpoB* gene. The most prevalent drug-resistant mutations were S531L, H526D/R/Y and D516V, which accounted for 50.0%, 19.2% and 3.8%, respectively. Five isolates carried double mutations; three (11.5%) carried L511P + H526Q and two (7.7%) were found with L511R + D516Y mutations. One isolate carried *rpoB* Q513-F514ins. Among the 11 RIF-susceptible isolates, one harbored mutation I572F, which was reported to appear among the mutations conferring low-level RIF resistance.^{38,39}

All the 24 isolates resistant to STM exhibited mutations in *rpsL* or *rrs-F1*. The mutation in *rpsL* K43R was the most prevalent and accounted for 50%, followed by the mutations *rpsL* K88R and *rrs-F1* a514c accounting for 29.2% and 12.5%, respectively. Two other mutations, *rpsL* K88T and *rrs-F1* c905a, were found in one STM-resistant isolate each.

Resistance to EMB was observed in ten isolates, among them eight (80%) isolates harbored mutations in the *embB* gene. The most common drug-resistant mutations were observed in the *embB* codon M306L/V/I and *embB* codon F330S/V, found in 5 and 2 of the ten resistant isolates, respectively. However, four single mutations at the *embB* codon Q497L/R; D354A and G406A were detected in five of the 27 EMB-susceptible isolates. Overall, 7 of the 37 isolates had contradictory results between EMB resistance phenotype and *embB* gene sequencing (see Table S3).

Overall, among the 37 isolates, one INH-resistant, one RIF-resistant and two EMB-resistant isolates had no mutation in the studied genes. In contrast, mutations in the *katG* (G560S), *rpoB* (I572F) and *embB* (D354A, G406A, Q497L and Q497R) genes were found in one INH-susceptible, one RIF-susceptible and 5 EMB-susceptible isolates, respectively.

Mutation in the *pncA* gene (PZA resistance)

Overall, mutations in the *pncA* gene and its promoter were found in 18/37 (48.6%) of the isolates, including 16/24 (66.7%) MDR isolates and 2/13 (15.4%) non-MDR isolates. Seven out of eight (87.5%) QDR isolates had mutations in the *pncA* gene. Sequencing revealed high SNP (single-nucleotide polymorphism) diversity in the *pncA* gene (see Table 2). In total, 13 different mutation patterns were identified, among them 12 mutation points were found in 17 isolates and one isolate with a partial *pncA* gene. All the 12 mutations and the deletion had been previously documented in PZA resistant isolates.^{20,26} The most common mutation was A171E found in three isolates (16.7%), followed by V7G, V131G and a-11g in two isolates each (11.1%).

Resistance to second-line drugs (SLD)

Mutations in the *gyrA* and *gyrB* genes

Among the 37 isolates under study, mutations in the *gyrA* gene were found in 25 isolates (67.6%) (see Table 3 and Table S1). Twenty-three out of 25 (92.0%) isolates had a single mutation in the quinolone resistance-determining region (QRDR) of *gyrA* and the most recorded mutation was D94G (15/23, 62.5%), followed by A90V (5/23, 21.7%), D94A (1/23, 4.3%), S91P (1/23, 4.3%) and G88A (1/23, 4.3%). Among two other isolates, one harbored a double mutation in the QRDR region of *gyrA* (D94G and H87Q) and another a single point D7N mutation outside the QRDR region.

Mutations in the *gyrB* gene were observed in 11 isolates (29.7%) and all of them were located outside the QRDR region. The most common mutation observed in the *gyrB* gene was A644D (5/11, 45.5%). Other point mutations were found: G407C, S413A, A432P, G512R, K600T and I601L in one isolate each. The *gyrB* A644D mutation was found together with mutations in *gyrA* in three isolates (see Table 3).

Mutations in the *rrs-F2* gene

Among the 37 isolates under study, six isolates exhibited mutations in the *rrs-F2* gene (see Table S1). Among them, mutation a1401g was found in three (50%) isolates and three other mutations including g1177a, 1204 ins a and c1489t, that have not been reported elsewhere, were found in one isolate each.

DST for SLD on the 37 strains with mutations in the *gyrA/B* and/or *rrs-F2* genes

In total, 37 isolates carried mutations in genes linked to SLD. The 37 isolates under study were tested for their susceptibility to second-line drugs including FQ (Ofx) and SLID (Km, Am and Cm); the results are shown in Table S1. Among them, only 25 isolates including 19 MDR and 6 non-MDR exhibited resistance to at least one SLD and were used for genotypic analysis. Among non-MDR isolates, Ofx-resistance was found in all the 6 isolates including isolates with mono-resistance to STM ($n=3$) and to RIF ($n=2$) and poly-resistance to INH and STM ($n=1$). Resistance to any of the SLID was

not observed among non-MDR isolates. Among the 19 MDR isolates, 16 (66.7%) were resistant to either Ofx (14; 87.5%) or at least one SLID (2; 12.5%) corresponding to the pre-XDR profile, and three (12.5%) MDR isolates were resistant to Ofx and at least one SLID simultaneously corresponding to XDR isolates.

All 23 isolates with a single mutation in the QRDR region of the *gyrA* gene were resistant to Ofx. A double mutation (D94G and H87Q) and a single point D7N mutation outside the QRDR region were found in FQ-susceptible isolates. Ten out of 11 isolates with the *gyrB* mutation were susceptible to Ofx. One isolate with the *gyrB* A644D mutation was found in one Ofx-resistant isolate, together with mutations in *gyrA* D94G, known to be FQ determinant.

Three isolates with the *rrs*-F2 a1401g mutation were resistant to at least one SLID by pDST; two isolates were resistant to Am, Km and Cm and one resistant to Am and Km (see Table S1). Among the isolates with the 3 new mutations, the isolate with the c1489t substitution was resistant to Am and Km, while the isolates with the two other substitutions (g1177a and 1204 ins a) were found susceptible to the three SLID. Among the 37 isolates, one isolate was Km-resistant and did not have any mutations in the *rrs* gene.

It is worth noting that, 3/3 (100%) XDR isolates and 12/16 (75%) pre-XDR isolates carried mutations in the *pncA* gene conferring resistance to PZA. In addition, one of the three (33.3%) XDR isolates, referred as "XDR+" in this study, was totally resistant to all 4 FLD (INH, RIF, EMB and STM) and 4 SLD (Ofx, Am, Km and Cm) tested, as well as resistant to PZA by carrying mutation T49P in the *pncA* gene.

Genotyping

Genotypic analysis was performed on the 25 isolates phenotypically resistant to at least one SLD and for which molecular data and pDST were in agreement. Spoligotype patterns of 24 isolates were found in the international database (SITVIT2) and one unique pattern (4%) was declared a new type. Four families were identified among the 24 isolates, including these Beijing, EAI2-nonthaburi/EAI5, Manu-ancestor and Zero-copy families. The Beijing family was the most predominant, representing 79.2% (n=19), followed by EAI (8%, n=2), Manu ancestor (8%, n=2) and Zero-copy (4%, n=1). Seventeen isolates shared the Beijing-SIT1 spoligotype pattern, accounting for 70.8% of all isolates in the study (see Table S4). When comparing spoligotyping results with drug resistance patterns, the predominant Beijing family was found in 80.0% (4/5), 85.7% (18/21), 81.3% (13/16) and 100% (3/3) of SLID-resistant, Ofx-resistant, pre-XDR and XDR M.tb isolates, respectively. Manu-ancestor and Zero-copy isolates were found in 12.5% (2/16) and 8.3% (1/16) of pre-XDR isolates respectively, while the 2 isolates of the EAI-2/5 family were found in non-MDR isolates.

Among the 25 isolates, MIRU-VNTR analysis showed that 21 (72.6%) isolates had unique MIRU-VNTR genotypes, and two (21%) of the isolates shared the same genotype. Two isolates showed mixed MIRU-VNTR genotypes (double allele in one loci) and were excluded from the analysis. The 24 MIRU-VNTR loci Hunter-Gaston Discriminatory Index reached 0.9967.

Genetic variability was analyzed using MIRU-VNTR loci among the 23 sample sets and within the Beijing group (n=19). The genotypic diversity of all the 23 isolates was 0.956. The average of allele numbers per locus was 3.29, ranging from 1 to 7, and 2 loci (MIRU-20 and MIRU-39) were monomorphic. Among the 19 Beijing isolates, the genotypic diversity was 0.947 and the average of allele numbers per locus was 2.29, ranging from 1 to 5. Eight (MIRU-02, MIRU-04, MIRU-16, MIRU-20, Mtub-29, ETRB, MIRU-24 and MIRU-39) of the 24 loci were monomorphic. The genetic diversity index H_s was 0.378 for the entire sample and 0.211 for Beijing isolates.

A dendrogram was constructed based on both spoligotyping and MIRU-VNTR results (Figure 2) and was used to determine clusters. A cluster is defined as a group of isolates having the same MIRU-VNTR and spoligotype profiles. We observed only one cluster (Cluster I) of 2 isolates (DR_002 and DR_007) which belonged to the Beijing family. In addition, three Beijing isolates, including the two isolates of Cluster I and DRC_003, formed a clonal complex (CC I) by sharing 23 of the 24 MIRU-VNTR loci. Interestingly, except for the phenotypic resistance to Km for isolate DR_002 and mutation G400A of the *embB* gene for isolate DRC_003, the three isolates had the same pDST (resistance to INH, RIF, STM and Ofx) and mutation patterns: *katG*_WT, *inhA*_c-15t&S94A, *rpoB*_L511P&H526Q, *rpsL*_L88R, *pncA*_A171E, *gyrA*_A90V, *gyrB*_WT and *rrs*_WT. DR_007 was isolated in 2012 from a patient in Kompong Cham province and DRC_003 and DR_002 were identified from patients in Phnom Penh, three and four years later, with an additional mutation named G406A in the *embB* gene and an additional resistance to Km, respectively.

Treatment outcome of patients with pre-XDR and XDR isolates (see Table S4)

Among the 19 pre-XDR and XDR isolates identified in our study, the treatment information was available for 16 patients including two of the three patients with XDR isolates and 14 of the 16 patients with pre-XDR isolates (13 pre-XDR_{FQ} and 1 pre-XDR_{SLID} patients). For two pre-XDR patients (1 pre-XDR_{FQ} and 1 pre-XDR_{SLID}), the information was missing and the patient with the XDR+ (resistant to 5 FLD and 4 SLD) isolate refused treatment (see Table S4). The treatment information was not available for patients with SLD-resistant non-MDR isolates.

All the 16 patients initially received the 20 to 24 months standardized MDR-TB treatment regimen which is composed of a combination of FLD and SLD including SLID (Km or Cm), FQ (Levofloxacin; Lfx or Moxifloxacin; Mfx), Eto, Cs, PZA and EMB. The regimen was later adjusted according to clinical evaluation and available phenotypic or molecular DST. Four patients including 2 XDR (DR_001 and DR_002) and 2 pre-XDR_{FQ} (DR_003 and DR_004) did not respond to the standardized treatment regimen and revealed FQ resistance. Thus, these four patients switched to a 24-month regimen containing Bedaquiline and SLID for 9 to 12 months as well as Cs, Clofazimine, Linezolid, PAS, PZA and EMB.

Among the 16 patients, 12 (75.0%) including 10 pre-XDR_{FQ} and 2 XDR were cured, 1 pre-XDR_{SLID} (6.3%) was lost during follow-up and 3 pre-XDR_{FQ} (18.7%) died during the intensive phase of treatment. Two of them died after receiving the treatment for less than one month and one died

several months after the discontinuation of the treatment due to other medical issues (see Table S4). Of the 12 patients for whom the treatment was successful, respectively nine, two, and one had isolates belonging to the Beijing, Manu_ancestor and undefined family. The four patients who died during the treatment and the one who was lost during follow-up presented isolates belonging to the Beijing family (see Table S4). Overall, there was a 71.4% (10/14) and 100.0% (2/2) treatment success rate for pre-XDR and XDR patients, respectively.

Discussion

Although XDR TB cases have been reported worldwide, Cambodia, with nearly 35 000 cases of TB and 150 MDR-TB cases annually, reported the first XDR-TB case to the WHO in 2016.² Due to the absence of surveillance programs and systematic screening for SLD drug resistance among MDR patients, the burden of pre-XDR and XDR-TB has been under reported in Cambodia and is still unknown. However, in an initial previous report, the proportions of pre-XDR and XDR, based on molecular tests among MDR isolates identified between 2007 and 2009, were 14% and 1%, respectively.⁴⁰ Another study reported four XDR-TB cases (0.7%) and only one pre-XDR-TB case (0.1%) confirmed by DST among MDR-TB patients between 2006 and 2016, but only the patients who did not respond to the treatment regimen were tested for SLD resistance.¹⁵ The present study reports the first description of SLD drug resistance in *M.tb* in Cambodia using a combination of molecular screening for mutations in the *gyrA/B* gene conferring FQ resistance and the *rrs-F2* gene conferring SLID resistance followed by pDST confirmation. Overall, only 25 of the 37 *M.tb* isolates harboring mutations in the *gyrA/B* and/or *rrs-F2* genes were resistant to FQ or SLID by pDST. The proportion of pre-XDR and XDR cases among MDR isolates in our study were 13.6% and 2.5%, respectively. These proportions remain lower than the global estimation of 6.2% for XDR-TB and 20.8% for FQ-resistance among RR/MDR-TB¹ but higher than the two previous reports.^{15,40} It should be noted that, for the first time in Cambodia, one XDR isolate (DRI_151), named XDR+ in our study, was resistant to the 4 SLD and 5 FLD tested.

Among the isolates resistant to SLD in our study, the resistance to fluoroquinolones (FQ), observed in the MDR group (14.4%; $n=17$) as well as non-MDR group (3.9%, $n=6$), was more common than the resistance to SLID. This result was expected since FQ is one of the most commonly used antibiotics in non-TB infections in Cambodia and the drug is accessible without prescription at the counter of pharmacies.⁴¹ High incidence of FQ-resistance has also been reported in many parts of the world, particularly in countries with a high TB burden.⁴² FQ is used in both the standardized and shorter MDR-TB regimen and is considered as an essential part of the MDR-TB regimen.⁹ Considering the high incidence of FQ-R described in our study, resistance to FQ should be determined right after patients are diagnosed with MDR-TB.

All 23 FQ-resistant isolates had mutations in the QRDR region of the *gyrA* gene represented by 5 SNP (see Table 3). Mutations at codon 94 were the most frequent (69.6%), followed by codon 90 (21.7%), codon 91 (4.3%) and codon 88 (4.3%). These findings are in accordance with previous

reports, according to which 60.0%-90% of FQ-resistant isolates had mutations at codons 88-94.⁴³⁻⁴⁵ The five SNP detected in our study (D94G, D94A, S91P, A90V and G88A) have also been reported to cause cross resistance among FQ compounds (Ofx, Lfx and Mfx).²⁰ The D94G mutation was also observed in the XDR+ isolate.

Unlike the *gyrA* gene, mutations in the *gyrB* gene are less commonly associated with resistance to FQ in *M.tb*.⁴³ In our study, all mutations in the *gyrB* gene, 7 SNP in 10 isolates, were located outside the QRDR region and most of them (10/11) were observed in FQ-susceptible isolates (see Table 3). The A644D mutation in the *gyrB* was only found in 1/23 FQ-resistant isolates but was observed together with the D94G mutation in *gyrA*, known to be FQ determinant. No *gyrB* mutation was observed in the XDR+ isolate. Overall, all the 9 isolates with mutations only found outside the QRDR region of *gyrA/B* were found susceptible to FQ, suggesting no impact on FQ resistance. However, one FQ-susceptible isolate harbored a double mutation in the QRDR region of the *gyrA* gene (see Table 3): mutation D94G, known to be FQ determinant, and mutation H87Q, reported in one study but not associated with FQ-resistance.⁴⁶ This double *gyrA* mutation has never been reported elsewhere before. The presence of a double *gyrA* mutation in a FQ-susceptible isolate might suggest: i) hetero-resistance with a lack of detection of the resistant population by the pDST method; ii) deactivation of FQ resistance caused by the D94G mutation due to the presence of mutation H87Q or iii) other mechanisms involved.

Resistance to SLID was found in 4.2% (5/118) of MDR isolates, three times less than the rate of FQ resistance. This can be explained by the fact that in Cambodia, injectable agents are not usually used to treat common infections compared to FQ. Mutation a1401g in the *rrs-F2* gene is reported as the most prominent mutation causing cross-resistance among SLID.^{22,47,48} This mutation was also found in the XDR+ isolate conferring resistance to all the 3 SLID tested. In our study, cross-resistance was detected in 4/5 SLID-resistant isolates and 3 of them carried the a1401g mutation, in agreement with previous reports. One isolate (DR_001), carrying a new mutation named c1489t, also exhibited cross-resistance to Am and Km. Another isolate (DR_002) resistant only to Km did not reveal any mutations in the *rrs* gene, suggesting the contribution of other mechanisms. It is worth noting that two isolates with mutations (g1177a and 1204 ins a) located outside the 1400-1500 region of the *rrs* gene were susceptible to all the 3 SLID tested, suggesting no impact on SLID resistance.

Briefly, out of the 37 isolates harboring mutations in genes associated with SLD resistance (ie *rrs-F2*, *gyrA* and *gyrB*), 12 were SLD susceptible by pDST. Among these 12 SLD susceptible isolates, 11 isolates carried mutations not conferring resistance and one isolate carried the D94G mutation linked to FQ resistance in association with other mutations (see above). Even if the sampling is small, these results suggest that molecular detection of SLD resistance is beneficial in our study in complement to pDST and is time saving.

All pre-XDR and XDR isolates harbored genetic mutations conferring RIF and INH resistance. The most prevalent mutations were located in codon *rpoB* S531L (52.6%; 10/19) and *katG* S315T (73.7%; 14/19) in agreement with previous reports.^{45,49} Globally, more than 75% and 90% of the pre-

XDR and XDR isolates, respectively, have been reported with phenotypic resistance to PZA.⁵⁰ In our study, mutations conferring PZA resistance were observed in all XDR isolates and 70% of pre-XDR isolates. Since PZA resistance is not routinely tested in Cambodia, the drug is systematically added in the treatment regimen for drug-resistant TB patients when there is no clinical contraindication for its use. Our findings underline that the resistance to PZA should be assessed among MDR-TB before using this drug in pre-XDR and XDR-TB treatment regimens.

Regarding the spoligotyping and MIRU-VNTR typing, the dominant genotype was Beijing, representing 81.3% of pre-XDR and 100% of XDR isolates, in agreement with previous reports in Cambodia and other parts of the world.^{40,44,45,48} MIRU-VNTR profiles were heterogeneous with 21 genotypes out of 23 isolates. One cluster of two isolates belonged to the Beijing family (DR_002-XDR-2016 and DR_007-pre-XDR-2012). With another additional isolate (DRC_003-pre-XDR-2015), they form a clonal complex by sharing 23 of the 24 MIRU-VNTR loci and 8 out of 9 genetic patterns of drug resistance genes. This finding suggests that resistant isolates propagate in Cambodia and, particularly in our study, are able to evolve from pre-XDR to XDR. This underlines the absolute necessity of a better control strategy in terms of diagnostic and treatment to avoid the spread of highly resistant strains such as the XDR+ strain (DRI_151).

The treatment success rate in our study was high, 71.4% for pre-XDR TB and 100.0% for XDR-TB, compared to the WHO latest treatment outcomes of 56% for MDR-TB and 39% for XDR-TB.¹ However, among the twelve pre-XDR-TB patients who received the standardized MDR-TB treatment regimen, only 57.1% (8/12) were cured. It is worth noting that the patient with the highly resistant XDR+ Beijing isolate (DRI_151) refused treatment. This XDR+ strain was isolated in 2017 from a smear-positive sputum sample from a patient who lived in Phnom Penh capital city, suggesting a high risk of transmission of this strain in the population and a possible evolution towards more antibiotic resistance.⁵¹

Since we used a limited set of genes to screen for resistance of M.tb isolates to SLD, we could not determine the resistance mechanisms for the Km resistance in one out of five isolates resistant to SLID. Furthermore, pDST was only performed on mutant isolates in our study. Therefore, it is likely that we underestimated the proportion of FQ- and SLID-resistant isolates for which the resistance mechanism involved other resistant genes, such as *eis* promotor, *whiB*, *tlyA* or genes encoding mycobacterial efflux pumps.^{20,52}

Conclusion

The present report is the first description of SLD resistance including pre-XDR and XDR M.tb isolates in Cambodia using both molecular and phenotypic methods. Our data suggest that the proportion of XDR and pre-XDR among MDR isolates remains low in Cambodia but on the rise compared to previous reports. The increasing proportion of pre-XDR and XDR M.tb isolates, the detection of an XDR+ isolate and the absence of systematic susceptibility testing raise concern for the patients. Indeed, pre-XDR and XDR patients were treated as MDR-TB patients and thus received

suboptimal treatment, which is likely to contribute to poor patient outcomes, DR resistant strain transmission and the acquisition of additional drug resistances. Taking this current evidence into consideration, PZA should not be used in the treatment regimen for pre-XDR TB and XDR-TB unless the resistance has been ruled out. DST using phenotypic or molecular methods for PZA should be performed for all MDR-TB patients in order to avoid a treatment failure and the risk of transmission of highly resistant strains. The genotypic results confirmed that pre-XDR Beijing strains circulate in the population and can evolve into highly resistant forms of XDR isolates. These results indicate the need for rapid interventions to prevent the spread of these highly resistant isolates and the emergence of extremely resistant isolates. Further study should be performed prospectively to better understand the current burden of SLD resistance in *M.tb* in Cambodia and the mechanisms responsible for XDR and XDR+ resistance.

Abbreviations

Am, amikacin; CC, clonal complex; Cm, capreomycin; Cs, Cycloserine; DNA, Deoxyribonucleic acid; DR, Drug-resistant; DST, drug susceptibility testing ; EAI, East African-Indian; EMB, ethambutol; Eto, Ethionamide; FLD, first line drugs; FQ, fluoroquinolones; HGDI, Hunter-Gaston- Discriminatory Index; HIV, human immunodeficiency virus; INH, isoniazid; Km, kanamycin; Lfx, Levofloxacin; LJ, Löwenstein-Jensen; *M.tb*, *Mycobacterium tuberculosis*; MDR, multidrug-resistant; Mfx, Moxifloxacin; NTP, National Tuberculosis Program; Ofx, ofloxacin; PCR, polymerase chain reaction; pDST, phenotypic drug susceptibility testing; Pre-XDR, pre-extensively drug-resistant; PSA, P-aminosalicylic acid; PZA, pyrazinamide; QDR, Quadruple drug resistance; QRDR, quinolone resistance-determining region; R, resistance; RIF, rifampin; RR, RIF resistant; RRDR, rifampicin resistance-determining region; S, Susceptible; SIT, spoligotype international type; SLD, second-line drugs; SLID, second-line injectable drugs; SNP, single-nucleotide polymorphism; STM, streptomycin; TB, tuberculosis; WHO, World Health Organization; XDR, extensively drug-resistant.

Ethics Statement

This study protocol was approved by the National Ethics Committee for Health Research, Phnom Penh, Cambodia. Since the study only used *M.tb* isolates that were collected from patients for diagnosis or follow-up purposes, the patients' informed consents were not required.

Data availability statement

The data supporting the findings of this study are available within the article and its supplementary materials.

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Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Box 1 Definition of drug-resistant categories based on phenotypic DST profiles

First-line drug resistance (FLD-R)*: Resistance to any first-line drugs (FLD) tested including INH, RIF, EMB and STM.

Second-line drug resistance (SLD-R): Resistance to any second-line drugs tested including fluoroquinolones (Ofx) and second-line injectable drugs (Am, Km and Cm).

Multidrug-resistance (MDR): Resistance to at least two of the most effective anti-TB drugs, INH and RIF.

Non-MDR: Resistance to one or more FLD but not to INH and RIF at the same time.

Quadruple drug resistance (QDR): Resistance to all four first line drugs tested including INH, RIF, EMB and STM.

Fluoroquinolone resistance (FQ-R): Phenotypic resistance to any fluoroquinolone (ie Ofx in this study).

Second-line injectable drug (SLID) resistance: Phenotypic resistance to at least one of three SLID including Am, Km and Cm.

Extensively drug-resistant (XDR): MDR isolates simultaneously resistant to any fluoroquinolone (ie Ofx in this study) and at least to one SLID (Am, Km and Cm).

Pre-extensively drug-resistant (pre-XDR): MDR isolates resistant either to any FQ (ie Ofx in this study) or one SLID (Am, Km and Cm).

Note: *Phenotypic DST is not available for pyrazinamide.

Abbreviation: Am, amikacin; Cm, capreomycin; DST, Drug susceptibility testing; EMB, ethambutol; INH, isoniazid; Km, kanamycin; Ofx, ofloxacin; R, resistance; RIF, rifampicin; STM, streptomycin.

Table 1 Demographic and clinical information of the study population (n=37 isolates) and second-line drug (SLD) resistance confirmed cases by phenotypic drug susceptibility testing (n=25 isolates)

Characteristics		Study population, number (%)	SLD resistance cases, number (%)
Gender	Total	36	25
	Male	23 (63.9)	17 (68.0)
	Female	13 (36.1)	8 (32.0)
Age (years)	Total	36	25
	15-34	11 (30.6)	8 (32.0)
	35-54	16 (44.4)	15 (60.0)
	≥55	9 (25.0)	2 (8.0)
	Median age and range (years)	47.1 (23-80)	43.2 (23-76)
Household location	Total	37	25
	Phnom Penh City	14 (37.8)	12 (48.0)
	Other provinces	23 (62.2)	13 (52.0)
Type of tuberculosis disease	Total	34	25
	Pulmonary TB	33 (97.1)	25 (100)
	Lymph node TB	1 (3.0)	0
Year of registration	Total	37	25
	2012	4 (10.8)	3 (12.0)
	2013	8 (21.6)	2 (8.0)
	2014	10 (27.0)	9 (36.0)
	2015	6 (16.2)	4 (16.0)
	2016	5 (13.5)	4 (16.0)
	2017	4 (10.8)	3 (12.0)
Smear-positive sample		23/31 (74.2)	19/24 (79.2)
Previous treatment for TB disease		33/35 (94.3)	22/24 (91.7)
HIV/TB co-infection		2/13 (15.4)	2/13 (15.4)

Abbreviations: HIV, human immunodeficiency virus; SLD, second-line drugs; TB, tuberculosis.

Table 2 Mutation patterns in genes involved in resistance to first-line drugs (INH, RIF, EMB, STM and PZA)

Drug	Gene	Mutation patterns in drug-resistant isolates ^a	Mutation patterns in drug-susceptible isolates ^a
RIF	<i>rpoB</i>	D516V; H526D; H526R; H526Y; S531L; L511P & H526Q; H526D & M736T; L511R & D516Y; Q513-F514ins; S531L & L635P	I572F
INH	<i>katG</i>	W149R; S315T; S315N; W477L & A479P; Gene deletion	G560S
	<i>inhA</i> gene and promoter region	c-15t; c-15t & S94A	
	<i>katG</i> + <i>inhA</i>	F720S + c-15t	
EMB	<i>embB</i>	M306I; M306L; M306V; D328Y; F330S; F330V	D354A; G406A; Q497R; Q497L
STM	<i>rpsL</i>	K43R; K88R; K88T	
	<i>rrs</i> -F1 ^b	a514c; c905a	
PZA ^c	<i>pncA</i> gene and promoter region	a-11g; V7G; D8E; L19P; L35P; T47P; H71Y; Y103C; S104R; V131G; A171E; A171V; partial <i>pncA</i> gene deletion	

Notes: ^aDrug susceptibility patterns were obtained using the BACTEC MGIT 960 method used according to the standard critical antibiotic concentrations recommended by the WHO: RIF (1.0 µg/ml), INH (0.1 µg/ml), EMB (5.0 µg/ml), STM (0.1 µg/ml); ^bSequencing targeting Loops 530 & 915 of the *rrs* gene; ^cSusceptibility to PZA is not available.

Abbreviations: EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RIF, rifampicin; STM, streptomycin.

Table 3 Mutation patterns of target genes for fluoroquinolones and second-line injectable drugs

Gene	Mutation patterns	Nb of isolates (pDST)		Drug to which the isolate was resistant	Total isolates
		Susceptible	Resistant		
<i>gyrA</i>	G88A	0	1	Ofx	1
	A90V	0	5	Ofx	5
	S91P	0	1	Ofx	1
	D94A	0	1	Ofx	1
	D94G	0	14	Ofx	14
<i>gyrB</i>	G407C ^a	1	0		1
	S413A ^a	1	0		1
	A432P ^a	1	0		1
	G512R ^a	1	0		1
	K600T ^a	1	0		1
	I601L ^a	1	0		1
	A644D ^a	2	0		2
<i>gyA + gyrB</i>	D7N ^a + A644D ^a	1	0		1
	H87Q & D94G + A644D ^a	1	0		1
	D94G + A644D ^a	0	1	Ofx	1
<i>rrs-F2^b</i>	a1401g	0	2	Am, Km, Cm	2
	a1401g	0	1	Am, Km	1
	c1489t	0	1	Am, Km	1
	g1177a	1	0		1
	1204 ins a	1	0		1
	No mutation	0	1	Km	1

Notes: ^aMutation outside quinolone resistance-determining regions; ^bSequencing targeting the 1400-1500 regions of *rrs* gene.

Abbreviations: Am, amikacin; Cm, capreomycin; DST, drug susceptibility testing; Km, kanamycin; Ofx, ofloxacin.

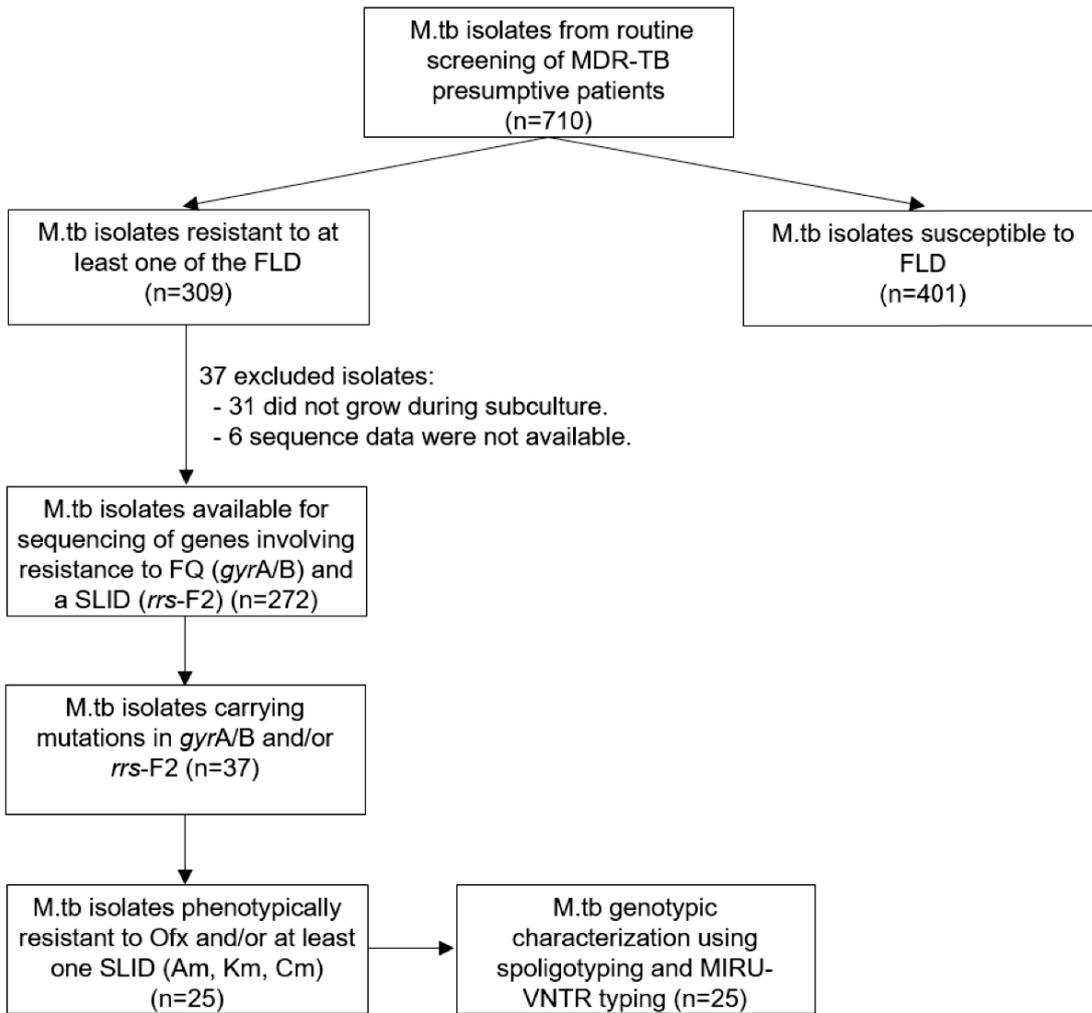
Table 4 Proportion of resistance phenotypes among Beijing and non-Beijing genotypes

Phenotypic drug resistance	Beijing, n (%) ^a	non-Beijing, n (%) ^a	Total, n (%) ^a
XDR-TB	3 (100.0)	0	3
FQ-resistant; Pre-XDR-TB	11 (84.6)	2 (15.4)	13
FQ-resistant; neither XDR- nor pre-XDR-TB	4 (80.0)	1 (20.0)	5
SLID-resistant; Pre-XDR-TB	1 (50.0)	1 (50.0)	2
Total	19 (82.6)	4 (17.4)	23

Notes: ^aProportion calculated by column.

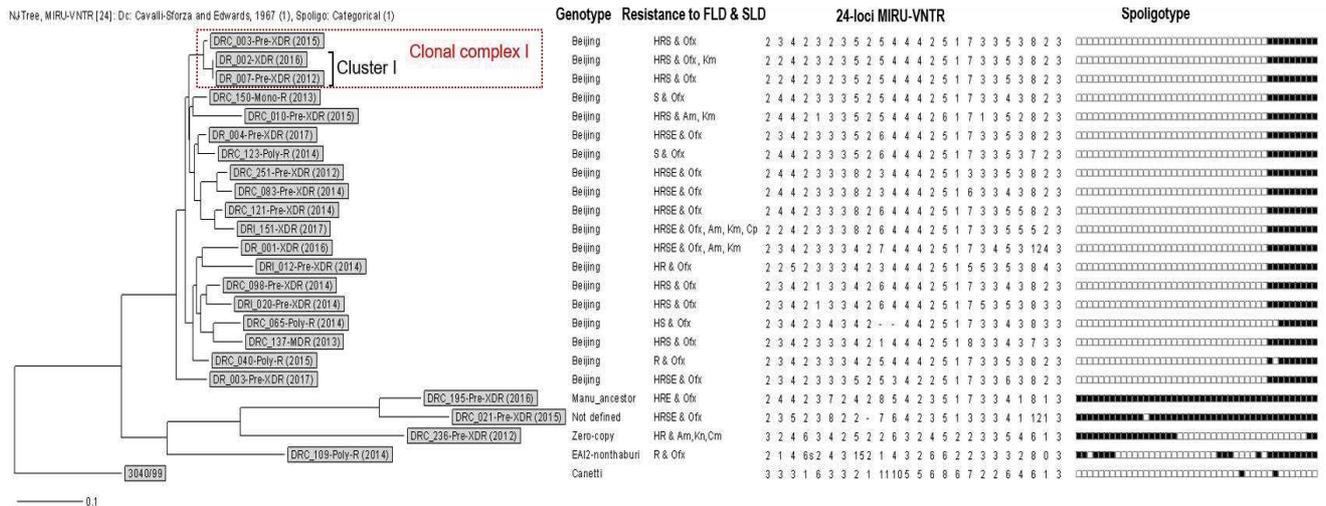
Abbreviations: FQ, fluoroquinolone; SLID, Second-line injectable drugs.

Figure 1 *Mycobacterium tuberculosis* collection and analytic methods.



Abbreviations: Am, amikacin; Cm, capreomycin; FLD, first line drugs; FQ, fluoroquinolones; Km, kanamycin; M.tb, *Mycobacterium tuberculosis*; MDR, multidrug-resistant; Ofx, Ofloxacin; SLID, second-line injectable drugs; TB, tuberculosis.

Figure 2 Phylogenetic analysis of spoligotyping and 24 loci MIRU-VNTR results of 25 *Mycobacterium tuberculosis* isolates resistant to SLD.



Abbreviations: Am, amikacin; Cm, capreomycin; E, ethambutol; FLD, first line drugs; H, isoniazid; Km, kanamycin; Ofx, ofloxacin; R, rifampicin; S, streptomycin; SLD, second-line drugs.

Supplementary data

Table S1 Phenotypes and mutations associated with anti-TB drug resistance in 272 M.tb isolates

The data are available in the google drive through link bellow:

https://drive.google.com/file/d/1vdeGo6W0w9gbzNLCP_eujDRXSJ2b4aMk/view?usp=sharing

Table S2 Primer sequences and positions used to amplify the relevant genes involved in FLD and SLD drug resistance in *M. tuberculosis*

The data are available in the google drive through link bellow:

<https://drive.google.com/file/d/14ro2UdEEgO99znfl9jYpi2cl1UpBz9cP/view?usp=sharing>

Table S3 Demographic and clinical data, phenotype and mutations associated with the anti-TB resistance of the 37 studied isolates

Isolates ID	Registration date	Sex	Age	TB treatment history	Smear result	Phenotypic resistance		Drug resistance mutations (genes)*						
						FLD	SLD	INH (<i>katG; inhA</i>)	RIF (<i>rpoB</i>)	STM (<i>rpsL; rrs-F1</i>)	EMB (<i>embB</i>)	PZA (<i>pncA</i>)	FQ (<i>gyrA; gyrB</i>)	SLID (<i>rrs-F2</i>)
DR_001	2016	M	28	Re-treatment	POS	HRSE	Ofx, Am, Km	S315T; WT	Q513-F514ins	L43R; WT	D328Y	>300bp del	D94G; WT	c1489t
DR_002	2016	F	23	Re-treatment	POS	HRS	Ofx, Km	WT; c-15t & S94A	L511P & H526Q	L88R; WT	WT	A171E	A90V; WT	WT
DR_003	2017	F	51	Re-treatment	POS	HRSE	Ofx	S315T; WT	S531L & L635P	L43R; WT	M306V	WT	D94A; WT	WT
DR_004	2017	M	50	Re-treatment	POS	HRSE	Ofx	S315T; WT	S531L	L43R; WT	F330V	L19P	D94G; WT	WT
DR_007	2012	M	67	Re-treatment	NA	HRS	Ofx	WT; c-15t & S94A	L511P & H526Q	L88R; WT	WT	A171E	A90V; WT	WT
DRC_003	2015	F	30	Re-treatment	NEG	HRS	Ofx	WT; c-15t & S94A	L511P & H526Q	L88R; WT	G406A	A171E	A90V; WT	WT
DRC_010	2015	M	40	HIV/TB	POS	HRS	Am, Km	S315T; WT	H526R	L43R; WT	WT	WT	WT; WT	a1401g
DRC_021	2015	M	25	Re-treatment	POS	HRSE	Ofx	S315T; WT	H526D	WT; a514c	M306I	A171V	A90V; WT	WT
DRC_040	2015	M	41	Re-treatment	POS	R	Ofx	WT; WT	H526Y	WT; WT	WT	WT	D94G; WT	WT
DRC_065	2014	F	32	Re-treatment	POS	HS	Ofx	S315T; WT	I572F	L88T; WT	Q497R	H71Y	S91P; WT	WT
DRC_083	2014	M	52	Re-treatment	POS	HRSE	Ofx	W477L & A479P; WT	S531L	WT; a514c	WT	Y103C	D94G; WT	WT
DRC_098	2014	M	53	Re-treatment	POS	HRS	Ofx	S315T; WT	S531L	L43R; WT	Q497L	V131G	D94G; WT	WT

DRC_109	2014	M	27	Re-treatment	POS	R	Ofx	WT; WT	S531L	WT; WT	WT (L355L)	WT	G88A; WT	WT
DRC_121	2014	M	46	Re-treatment	POS	HRSE	Ofx	S315T; WT	S531L	L88R; WT	WT	L35P	D94G; WT	WT
DRC_123	2014	F	23	Re-treatment	NEG	S	Ofx	WT; WT	WT	L43R; WT	WT	WT	D94G; WT	WT
DRC_137	2013	F	76	Re-Treatment	NEG	HRS	Ofx	S315T; WT	S531L	L43R; WT	WT	a-11g	D94G; A644D	WT
DRC_150	2013	M	50	Re-treatment	POS	S	Ofx	WT; WT	WT	L43R; WT	WT	WT	D94G; WT	WT
DRC_177	2016	M	48	Re-treatment	NEG	HRE	Ofx	S315T; WT	L511R & D516Y	WT; WT	M306L	V7G	D94G; WT	WT
DRC_195	2016	F	51	Re-treatment	POS	HRE	Ofx	S315T; WT	L511R & D516Y	WT; WT	M306L	V7G	D94G; WT	WT
DRC_236	2012	M	30	HIV/TB	POS	HR	Am, Kn, Cm	S315T; WT	S531L	WT; WT	WT	WT	WT; WT	a1401g
DRC_251	2012	M	44	Re-treatment	POS	HRSE	Ofx	S315T; WT	S531L	WT; a514c	M306V	D8E	D94G; WT	WT
DRI_012	2014	F	43	Re-treatment	POS	HR	Ofx	Deletion; WT	H526D	WT; WT	WT	WT	A90V; WT	WT
DRI_020	2014	M	53	Re-treatment	POS	HRS	Ofx	S315T; WT	S531L	L43R; WT	Q497L	V131G	D94G (S104S); WT	WT
DRI_021	2014	M	52	NA	NEG	S	Ofx	WT; WT	WT	L88R; WT	WT	WT	D94G; WT	WT
DRI_151 (XDR+)	2017	M	46	Re-treatment	POS	HRSE	Ofx, Am, Km, Cm	S315T; WT	S531L	L88R; WT	F330S	T47P	D94G; WT	a1401g

Notes: * Synonymous mutation is indicated by brackets.

Abbreviations: Am, amikacin; Cm, capreomycin; E or EMB, ethambutol; F, female; FQs, fluoroquinolones; H or INH, isoniazid; Km, kanamycin; M, male; NA, not available; NEG, negative; POS, positive; R or RIF, rifampicin; S or STM, streptomycin; TB, tuberculosis; WT, wild type (absence of non-synonymous mutation); Z or PZA: pyrazinamide.

Result 3: Evolution of drug resistance in *Mycobacterium tuberculosis* in Cambodia

1 Objectives

Cambodia ranked 17th globally among the 30 countries with the highest incidence of TB (1). WHO estimated that 1.8% of new TB cases and 11% of retreatment TB cases in Cambodia are MDR/RR-TB (WHO data). Compared to the first national drug resistance survey, in which no MDR-TB case was observed among new cases and only 3.2% among retreatment cases, the increasing emergence of MDR-TB will pose a significant threat to the control and treatment of the disease in the near future (First DR Survey). This indicates the need to understand the molecular mechanisms underlying this escalation of resistance in Cambodia where little information is available, in order to prevent a further increase in the burden of MDR-TB. Therefore, the present work aimed to determine the mechanisms of resistance by the characterization of mutations associated with resistance to FLD and SLD among 278 resistant *Mycobacterium tuberculosis* clinical isolates collected from Cambodia between 2012 and 2017 and to study the evolution of drug resistance in M.tb.

The materials and methods as well as the characteristics of the 278 isolates used in this work are described in the Chapter II. A total of 29 (10.4%) isolates were excluded from the analysis; 9 with clonal variant or mix MIRU-VNTR genotype patterns and 20 isolates with missing sequencing results for all the 9 studied genes. Therefore, only 249 isolates were included in the analysis.

2 Results

2.1 Genotypic and phenotypic characterization of the 249 study isolates

2.1.1 Drug resistance profiles for FLD: INH/RIF/STM/EMB

Among the 249 included isolates, except for PZA resistance, the phenotypic resistance rate for each FLD drug was as follows: 77.1% ($n= 192$) for INH, 51.0% ($n= 127$) to RIF, 61.8% ($n= 154$) for STM, and 19.7 ($n= 49$) to EMB. Twelve different phenotypic resistant patterns were observed and regrouped in different categories (**Table III-1**). 109 (56.2%) were MDR and 140 (43.8%) were non-MDR. Based on the number of FLD to which the isolates were resistant, the isolates were grouped as mono-FLD resistant (39.4%, 4 patterns), double-FLD resistant (26.9%, 4 patterns), triple-FLD resistant (18.5%, 3 patterns) and quadruple-FLD resistant (15.3%).

Table III-1. Distribution of the 249 M.tb isolates by FLD resistance profiles and genotypes

Resistance categories	Drug resistance	<i>M. tuberculosis</i> genotypes			Total
		Beijing	EAI	Other	
Categories by number of FLD drug resistance					
Mono resistance	INH	12	24	6	42
	RIF	9	7	1	17
	STM	22	13	3	38
	EMB	1	0	0	1
Double resistance	INH + RIF (MDR)	10	13	5	28
	INH + STM	22	12	1	35
	RIF + STM	0	1	0	1
	INH + EMB	1	2	0	3
Triple resistance	INH + RIF + STM (MDR)	30	8	1	39
	INH + RIF + EMB (MDR)	3	0	1	4
	INH + STM + EMB	1	2	0	3
Quadruple resistance	INH + RIF + STM + EMB (MDR)	30	3	5	38
Categories by MDR and non-MDR					
MDR		73	24	12	109
Non-MDR		68	61	11	140
Total		141	85	23	249

FLD, First line drugs; INH, isoniazid; RIF, rifampicin; STM, streptomycin; EMB, ethambutol; MDR, multidrug resistance

***M. tuberculosis* genotypes: spoligotyping and 24-loci MIRU-VNTR**

The genotyping results of the 249 included isolates was briefly described in **Table III-2**. Based on spoligotyping, we detected 53 different spoligotypes corresponding to 240 M.tb isolates. Using respectively, SITVIT2 and SPOTCLUTS database, 42 spoligotypes corresponding to 236 isolates and additional 9 spoligotypes corresponding to 10 isolates has been identified as family/subfamily. In total, 141 (56.6%) isolates were Beijing, 85 (34.1%) isolates were EAI and 23 (9.2%) isolates were other minor families including T (4, 1.6%), H (3, 1.2%), Manu-ancestor (3, 1.2%), Zero-copy (2, 0.8%), Cameroon (2, 0.8%), Unknown (6, 2.4%) and not defined families (3, 1.2%). The distribution of each FLD drug resistant pattern according to the M.tb families is described in **Table III-1**.

The 24-locus MIRU-VNTR typing revealed 217 distinct genotypes including 163 unique patterns and 26 small clusters shared by 58 isolates (containing 2-3 isolates per cluster). Of 26 clusters, 18 belonged to Beijing family; six belonged to EAI family and one cluster belonged to Manu_ancestor family. One cluster of two isolates shared the same MIRU-VNTR genotype but belonged to different spoligotype families (EAI4-VNM versus Zero-copy family).

2.2 Analysis of mutations in *rpoB*, *katG*, *inhA*, *rpsL*, *rrs-F1* and *embB*

2.2.1. Mutations in *rpoB* gene for RIF resistance

A 1149-bp fragment of the *rpoB* gene, from nucleotides 972 to 2120 including clusters I (residues 507–533, *E. coli* RNAP numbering, containing the 81-bp RIF resistance-determining region or RRDR), II (residues 563–572) and III (residue 687), associated with Rif-resistance were sequenced for all studied isolates. To study the N-terminal cluster (residue 146), the second primer were used to sequence the RIF-resistant isolates without mutation in the cluster I – III regions. The RIF resistance-associated mutations in *rpoB* are described in the **Table III-3**.

The sequencing results of *rpoB* gene revealed that 119/127 (93.7%) RIF-resistant isolates harbored mutations within the studied gene fragment. Eight (6.3%) RIF-resistant isolates did not contain any mutation. Twenty-eight different kinds of mutation, 14 single point mutations, 12 double mutations and two indels were observed. Overall, 116 (91.3%) out of the 127 RIF-resistant isolates harbored at least one mutation within the 81-bp RRDR between *rpoB* codons 507 and 533 of *M.tb* and 2.4% (3 isolates) had only mutation outside the RRDR region (including cluster II and N-terminal region). No mutation was found in cluster III of this gene.

The three most common mutations were located at codon 531 (55.1%, n=70 isolates), codon 526 (22.8%, n=29 isolates) and codon 516 (9.4%, n=12 isolates), all accounting for 87.4% (n=111 isolates) of RIF-resistant isolates. Two different amino acid replacements were observed in codon 531 (Ser531Leu/Trp) and codon 516 (Asp516Val/Tyr), of which Ser531Leu and Asp516Val was detected in 49.6% and 6.3%, respectively of the RIF-resistant isolates. Mutations at codon 526 were the most variable with 8 different amino acid replacements (His526Tyr/Arg/Asp/Gly/Leu/Thr/Gln/Asn), in which His526Tyr accounted for 7.9% of the RIF-resistant isolates.

Among RIF susceptible isolates, 13 out of 122 (1.8%) isolates harbored mutations within RRDR and 2 had mutations outside RRDR. Nine different types of mutations were detected. Of them, 5 mutation types including Leu511Pro, Asp516Tyr, His526Leu, Leu533Pro and Ile572Phe were previously linked to low-level of RIF resistance which are often missed by standard MGIT-DST^(408, 409), three mutations (His526Cys, Met736Thr and Thr508Ser & Leu511Pro) only found in RIF-susceptible isolates and one mutation (His526Gly) found in one RIF-resistant and one RIF-susceptible isolate.

Table III-3. Mutations in *rpoB* gene and their distribution according to *M.tb* genotypes (Beijing versus EAI versus Other)

Codon position(s)	Mutation(s)	RIF-resistant isolates			RIF-susceptible isolates			Total
		Beijing	EAI	Other	Beijing	EAI	Other	
146	Val146Phe		1				1	
251	Val251Phe	1					1	
511	Leu511Pro					2	2	
513	Gln513Leu		1				1	
516	Asp516Tyr	2	1			2	5	
516	Asp516Val	6		2			8	
526	His526Arg	5					5	
526	His526Asp	3		1			4	
526	His526Cys					1	1	
526	His526Gly		1			1	2	
526	His526Leu		1		2		3	
526	His526Thr	1					1	
526	His526Tyr	8	2				10	
531	Ser531Leu	42	13	8			63	
531	Ser531Trp	1	1				2	
533	Leu533Pro				2	1	1	4
563	Thr563Ile*	1					1	
572	Ile572Phe				1		1	
736	Met736Thr*					1	1	
480 & 531	Thr480Ile & Ser531Leu	1					1	
508 & 509	Thr508_Ser509 del			1			1	
508 & 511	Thr508Ser & Leu511Pro				1		1	
511 & 516	Leu511Arg & Asp516Tyr			1			1	
511 & 526	Leu511Pro & His526Gln	3					3	
513 & 514	Gln513_Phe514 ins	1					1	
522 & 567	Ser522Leu & Pro567Thr	1					1	
526 & 526	His526Tyr & His526Asp		1				1	
526 & 529	His526Arg & Arg529Gln		2				2	
526 & 531	His526Asn & Ser531Leu		1				1	
526 & 736	His526Asp & Met736Thr		1				1	
526 & 757	His526Asn & Thr757Ala	1					1	
531 & 584	Ser531Leu & Phe584Ser	1					1	
531 & 635	Ser531Leu & Leu635Pro	1					1	
531 & 663	Ser531Leu & Ser663Ala		1				1	
Absence of non-synonymous mutation		3	5		53	45	9	115
Total		82	32	13	59	53	10	249

* Mutation that has never been reported elsewhere

2.2.2. Mutations in *katG* and *inhA* genes for INH resistance

A total of 167/192 (86.9%) INH-resistant isolates harboring mutations in either *katG* and/or *inhA* conferring resistance to INH and the mutations are detailed in the **Table III-4**. Of them, the *katG* mutation and *inhA* mutation was detected in 123/192 (64.1%) and in 36/192 (18.8%) isolates, respectively. Coexistent *katG* and *inhA* mutations were detected in 8/192 (4.2%) INH-resistant isolates. Twenty-five (13.0%) INH-resistant isolates had a wild-type sequence in both *katG* and *inhA*.

Table III-4. Mutations in *katG* and *inhA* promoter/core gene and their distribution according to *M.tb* genotypes

Gene (s)	Codon position(s)	Mutation(s)	INH-resistant isolates			INH-susceptible isolates			Total
			Beijing	EAI	Other	Beijing	EAI	Other	
<i>katG</i>	99	Gly99Ala*				1			1
	125	Gly125Val*	1						1
	149	Trp149Arg	1						1
	155	Tyr155Cys	2						2
	198	Trp198Stop			1				1
	268	Gly268Asp*	1						1
	277	Gly277Ala*	1						1
	315	Ser315Asn	1	3					4
	315	Ser315Gly	3						3
	315	Ser315Thr	62	23	16				101
	351	Trp351Cys*	1						1
	394	Thr394Met*	1						1
	421	Gly421Ser	1						1
	467	Ala467Ser*		1			1		2
	479	Ala479Glu	1						1
	491	Gly491Cys						1	1
	570	Gly570Asp	1						1
	580	Asp580Asn		1					1
	720	Phe720Ser*	1						1
	140 & 315	Ser140Asn & Ser315Asn		2					2
	149 & 523 & 705	Trp149Arg & Glu523Lys & Arg705Gln					1		1
	315 & 494	Ser315Gly & Gly494Ser	1						1
	315 & 614	Ser315Thr & Ala614Thr	2						2
	360_365 or 364_369	360_365 or 364_369 del cggcgc*	1						1
	477 & 479	Trp477Leu & Ala479Pro	1						1
	Entire gene	Deletion	1						1
Absence of non-synonymous mutation			25	34	2	30	20	3	114
<i>inhA</i>	-15	C-15T	11	17	2		1		31
	-15 & 16	C-15T & Ile16Thr		1					1
	-15 & 62	C-15T & Ile62Thr		1					1
	-15 & 94	C-15T & Ser94Ala	8	1					9
	94	Ser94Ala		1					1
	94 & 190	Ser94Ala & Ala190Ser		2					2
	Absence of non-synonymous mutation			90	41	17	32	20	4

* Mutation that has never been reported elsewhere

Among *katG* mutations, the most frequent mutations were observed in codon 315 including Asp315Thr (78.6%), Asp315Asn (4.6%) and Asp315Gly (3.1%), accounting for 86.3% (113/131). One INH-R isolate had *katG* gene truncated. The most predominant mutation in *inhA* was observed at position -15 (C-T), counting for 93.2% (41/44). Five out of 57 INH-sensitive isolates had either mutations in *katG* or in *inhA* gene, of which four had *katG* mutation (Trp149Arg & Glu523Lys & Arg705Gln, Ala467Ser, Gly491Cys and Gly99Ala) and one had *inhA* -C15T mutation. Among these mutations, *katG* Gly491Cys was previously reported in INH-R isolate, *inhA* -C15T mutation was known to be associated with low level resistance to INH, and three other *katG* mutations have never been reported elsewhere (**Table III-4**). Only two mutations were observed in both INH-R and INH-S isolates (*katG* Ala467Ser and *inhA* -C15T).

2.2.3. Mutations in *rpsL* and *rrs-F1* gene for STM resistance

The mutations in *rpsL* and *rrs-F1* genes involving resistance to STM are described in the **Table III-5**. A total of 127 (81%) out of 154 STM-resistant isolates revealed mutations in *rpsL* or *rrs-F1*. Four types of point mutations were identified in the *rpsL* gene of 104 (104/154, 67.5%) STM-resistance isolates. The mutated positions at codon Lys43 (Lys-Arg, 70/154, 45.5%) and Lys88 (33/154, 21.4%; Lys-Arg, 30/154, 19.5% & Lys-Thr, 3/154, 1.9%) were the two most frequent sites, while the new mutation at codon Val37Leu (1/154, 0.6%) was observed in one STM-R isolate. Two out of 95 (2.1%) STM-susceptible isolates also harbored mutations in *rpsL* (codons Lys88Arg and Val23Ala).

Table III-5. Mutations in *rpsL* and *rrs-F1* gene and their distribution according to *M.tb* genotypes

Gene(s)	Codon (<i>rpsL</i>)/ nucleotide (<i>rrs</i>) position	Mutation	STM-resistant isolates			STM-susceptible isolates			Total
			Beijing	EAI	Other	Beijing	EAI	Other	
<i>rpsL</i>	23	Val23Ala*					1		1
	37	Val37Leu*		1					1
	43	Lys43Arg	63	7					70
	88	Lys88Arg	26	3	1	1			31
	88	Lys88Thr	1	2					3
	Absence of non-synonymous mutation			15	26	9	35	45	13
Total			105	39	10	36	46	13	249
<i>rrs-F1</i>	514	A514C	12	1	3				16
	517	C517T		3					3
	878	G878A		2					2
	905	C905A		1					1
	908	A908C		1					1
	Absence of non-synonymous mutation			93	31	7	36	46	13
Total			105	39	10	36	46	13	249

* Mutation that has never been reported elsewhere

For *rrs*-F1, nucleotide changes were observed in 23 (23/154, 14.9%) STM-resistance isolates (**Table III-5**). Base substitutions at nucleotide positions 514 (A-C) and 517 (C-T) of *rrs* 530 loop were observed in 16 (16/154, 10.4%) isolates and three (3/154, 1.9%) isolates, respectively. The mutations of the 915-loop region occurred at low frequency: substitution at nucleotide position 878 (G-A) in two isolates, 905 (C-A) in one isolate and 908 (A-C) in one isolate. Mutations in *rrs*-F1 and *rpsL* did not occur simultaneously in both STM-resistant and STM-susceptible isolates. Twenty-seven STM-resistant isolates did not present any mutation in any of these genes.

2.2.4. Mutations in *embB* gene for EMB resistance

Of the 49 EMB-resistant isolates, a total of 38 (77.6%) isolates had *embB* mutations (**Table III-6**). The most prevalent mutation was at codon 306 (Met306Val, $n=17$; Met306Ile, $n=4$ and Met306Leu, $n=2$) in 23 (46.9%) isolates, followed by mutations at codons 354 and 497 in 4 (8.2%) isolates each. Mutations at these positions accounted in total for 63.3% of EMB-resistant isolates. Eleven (22.4%) out of 49 EMB-resistant isolates had no mutation in *embB* gene.

Table III-6. Mutations in the *embB* gene and their distribution according to *M.tb* genotypes

Codon position(s)	Mutation(s)	EMB-resistant isolates			EMB-susceptible isolates			Total
		Beijing	EAI	Other	Beijing	EAI	Other	
306	Met306Ile	3		1	2	3	2	11
306	Met306Leu			2	1			3
306	Met306Val	14	2	1	2	1		20
309	Val309Phe*					1		1
318	Asn318Asp*		1					1
319	Tyr319Ser					1		1
328	Asp328Tyr	1						1
330	Phe330Ser*	1						1
330	Phe330Val	1						1
354	Asp354Ala	4				1		5
406	Gly406Ala				1			1
406	Gly406Asp	1						1
406	Gly406Cys	1			1			2
406	Gly406Ser	1						1
497	Gln497Arg		1	1	1	1		4
497	Gln497Leu	2			2			4
561	Leu561Arg*						1	1
Absence of non-synonymous mutation		7	3	1	95	70	14	190
Total		36	7	6	105	78	17	249

* Mutation that has never been reported elsewhere

Twenty-one (10.5%) out of the 200 EMB-S isolates revealed *embB* mutations, of them mutations in 17 isolates was also observed in EMB-R isolates (**Table III-6**). Overall, four novel mutations were found in four isolates: Val309Phe and Leu561Arg in EMB-S isolates and Asn318Asp and Phe330Ser in EMB-R isolates.

2.2.5. Determining drug resistance based on DNA sequencing results

For the four FLD, including INH, RIF, STM and EMB, the association between phenotypic resistance and the DNA sequencing data was analyzed to determine the sensitivity and specificity for detecting phenotypic resistance from DNA sequencing data. The results are showed in **Table III-7**. The sensitivity is the proportion of drug resistant isolates with mutations among all drug resistant isolates and the specificity is the proportion of drug susceptible isolates without any mutation among all drug susceptible isolates. Only known mutations conferring drug resistance were included in the analysis.

Table III-7. Comparison between phenotypic and genotypic data

Drug (s)	Locus/gene	Drug resistant	Drug susceptible	Sensitivity (%)	Specificity (%)
		(With mutation /without mutation)	(With mutation /without mutation)		
INH	<i>katG</i> *	124/68	2/55	64.6	96.5
	<i>inhA</i> and its promoter	44/148	1/56	22.9	98.2
	<i>katG</i> * and <i>inhA</i> (gene & promoter)	164/28	3/54	85.4	94.7
RIF	RRDR of <i>rpoB</i>	116/11	13/109	91.3	89.3
	<i>rpoB</i> gene	118/9	14/108	92.9	88.5
STM	<i>rpsL</i>	103/51	1/94	66.9	98.9
	<i>rrs-F1</i>	21/133	0/95	13.6	100
	<i>rpsL</i> and <i>rrs-F1</i>	124/30	1/94	80.5	98.9
EMB	<i>embB</i>	36/13	19/181	73.5	90.5

* The new mutations and the phylogenetic polymorphisms (*katG463*) were not included in the analysis.

Mutations in *katG* and *inhA* showed a sensitivity of 64.6% and 22.9%, respectively, to predict INH resistance, while a combined analysis of mutations in both genes had a high predictability of INH resistance (sensitivity of 85.4% and specificity of 94.7%). Among all the studied genes, *rpoB* (whole gene or RRDR) showed the highest sensitivity (>90%) but the lowest specificity (<90%) for detecting RIF resistance. In contrast, mutation in *rrs-F1* (100%) and *rpsL* (98.9%) have the highest specificity to determine STM resistance. In term of sensitivity, the *rrs-F1* gene had the lowest sensitivity (13.6%); while a combined analysis with *rpsL* gene revealed a higher prediction with a sensitivity of 80.5%. Mutations in *embB* gene had a predictability to detect EMB resistance with a sensitivity of 73.5% and a specificity of 90.5%.

2.3 Mutations associated with resistance to PZA and PZA resistance

The sequencing results of *pncA* gene and its putative promoter region were showed in **Table III-8**. Forty-nine (16.7%) of the 249 isolates had single point mutations in the *pncA* gene; 42 with single point mutations and seven had indels. The mutation patterns are dispersed throughout the gene. In total, 33 (67.3%) different mutation patterns (two in promoter region and 31 in coding gene) were identified, of them 26 had been previously documented and seven were new ^(184, 392, 410-416). The mutation in promoter region A-11G is the most recorded mutation present in 5 isolates, followed by Cys138Gly in 4 isolates, Ala171Glu in three isolates, Val131Gly in 3 isolates and 16 del T, Leu35Pro, Val157Gly, Thr167Ile, Ala171Gly in two isolates each. One hetero-resistant (mixed isolates with both resistant and susceptible strains) mutation was detected in the *pncA* gene and no double mutations were observed. One single nucleotide substitution at codon 64 resulted in premature stop codons. One isolate had nucleotide deletion in promoter region and 5 isolates had nucleotide deletions or insertions led to a shift in the reading frame and resulted in abnormal or early-truncated polypeptides.

Table III-8. Mutations in the *pncA* gene and their distribution according to *M.tb* genotypes

Codon position(s)	Mutation(s)	Genotypes			Phenotypic resistance to PZA	Total
		Beijing	EAI	Other		
-11	-11 del a *	1			1/1	1
-11	a-11g	2	2	1	3/3	5
4	Leu4Ser			1	1/1	1
7	Val7Gly			1	1/1	1
8	Asp8Glu	1			1/1	1
10	Gln10Pro	1			1/1	1
12	Asp12Ala	1			1/1	1
16	16 del T *		2		1/1	2
19	Leu19Pro	1			1/1	1
20	Val20Gly *	1			1/1	1
35	Leu35Pro	2			1/1	2
37	37 ins AGCGG *			1	1/1	1
42	42 ins ATCACGTC *	1			1/1	1
47	Thr47Pro	1			1/1	1
64	Tyr64Stop	1			1/1	1
67	Ser67Pro	1			1/1	1
71	His71Tyr	1			1/1	1
72	Cys72Tyr	1			1/1	1
96	Lys96Glu	1			1/1	1
103	Tyr103Cys	1			1/1	1
113	113 del G *		1		1/1	1
131	Val131Gly	3			3/3	3
136	Asp136Gly	1			1/1	1
138	Cys138Gly	4			3/3	4
139	Val139Gly		1		NA	1
155	Val155Gly		1		1/1	1
157	Val157Gly	2			1/1	2
167	Thr167Ile	2			2/2	2
171	Ala171Glu	3			3/3	3
171	Ala171Gly		2		1/1	2
171	Ala171Val			1	1/1	1
183	Val183Phe *		1		NA	1
-	>200 bp deletion	1			1/1	1
Absence of non-synonymous mutation		107	75	18	NA	200
Total		141	85	23	40	249

* Mutation that has never been reported elsewhere

Forty isolates with different mutation patterns (31 of the 33 patterns) in *pncA* gene were tested for PZA DST using Bactec MGIT 960 method (**Table III-8**). All the 40 isolates were confirmed to have resistance to PZA. The DST results were not available for two isolates; one with Val139Gly mutation and another one had Val183Phe mutation (new mutation).

2.4 Resistance to second line drugs: mutations and phenotypic data

2.4.1 Mutations in *gyrA* and *gyrB* genes for FQ resistance

Among the 249 M.tb isolates, 20 (8.0%) carried mutations in *gyrA* gene, 7 (2.8%) had mutations in *gyrB* gene while three (1.2%) had mutations in *gyrA* and *gyrB* genes; the results were showed in **Table III-9**. Regarding the mutations in *gyrA* gene, all the 20 isolates had single mutation point in the QRDR of *gyrA* gene, and the most recorded mutation was Asp94Gly (12/20, 60.0%), followed by Ala90Val (5/20, 20.0%). The two mutations represented 80.9% of *gyrA* mutations. Mutations Gly88Ala, Ser91Pro and Asp94Ala were found in one isolate each. The single point mutation in *gyrB* gene found in 7 isolates were all located outside the QRDR of *gyrB* gene. Of them, 6 mutation patterns were observed and only the mutation Ala644Asp was detected in two isolates and other mutations (Gly407Cys, Ala432Pro, Gly512Arg, Lys600Thr and Ile601Leu) were found in one isolate each. Three isolates had mutation in *gyrA* and *gyrB* simultaneously, of them two isolates had mutation in the QRDR region of *gyrA* (one isolate with a single mutation Asp94Gly and another one with double mutations Asp94Gly + His87Gln) and one isolate had mutation outside QRDR of *gyrA* Asp7Asn. All the three isolates had *gyrB* mutation Ala644Asp, which was predominant among all *gyrB* mutations, found in total 5/10 (50.0%) isolates. Overall, 23 (9.2%) and 10 (4.0%) isolates had mutations in *gyrA* and *gyrB* gene, respectively. Mutations in *gyrA* were located in the QRDR region for 22 out of the 23 isolates (one had double mutations), while all mutations in *gyrB* were located outside QRDR region. Besides, all 249 isolates carried polymorphisms Glu21Qln and Ser95Thr which were not linked to drug resistance.

Table III-9. Mutations in the *gyrA*, *gyrB* and *rrs*-F2 genes and their distribution according to *M.tb* genotypes

Drug	Gene	Mutation	Genotypes			Drug to which the isolate was resistant	Total isolates	
			Beijing	EAI	Other			
FQ	<i>gyrA</i>	Gly88Ala		1		Ofx	1	
		Ala90Val	4		1	Ofx	5	
		Ser91Pro	1			Ofx	1	
		Asp94Ala	1			Ofx	1	
		Asp94Gly	11		1	Ofx	12	
	<i>gyrB</i>	Gly407Cys*			1		Susceptible	1
		Ala432Pro*			1		Susceptible	1
		Gly512Arg*	1				Susceptible	1
		Lys600Thr*	1				Susceptible	1
		Ile601Leu*	1				Susceptible	1
		Ala644Asp*	2				Susceptible	2
	<i>gyrA</i> + <i>gyrB</i>	Asp94Gly + Ala644Asp*	1				Ofx	1
		Asp7Asn* + Ala644Asp*	1				Susceptible	1
		His87Gln & Asp94Gly + Ala644Asp*	1				Susceptible	1
	Absence of non-synonymous mutation	1	2	1		Susceptible	4	
			115	80	NA	NA	215	
SLID	<i>rrs</i> -F2	G1177A*		1		Susceptible	1	
		1204 ins A*		1		Susceptible	1	
		A1401G	1			Am, Km	1	
					1	Am, Km, Cm	1	
			1			Am, Km, Cm	1	
		C1489T*	1			Ofx, Am, Km	1	
		Absence of non-synonymous mutation	1			Km	1	
		22	3	2	Susceptible	27		
	155	80	20	NA	215			

* Mutation that has never been reported elsewhere

2.4.2 Mutations in *rrs* genes for SLID resistance

Six isolates (16.2%) exhibited mutations in *rrs*-F2 gene (see **Table III-9**). Of them, mutation A1401G found in three (50%) isolates, while three other mutations including g1177a, 1204 ins a and c1489t that have not been reported elsewhere, were found in one isolate each.

2.4.3 Comparison of mutations and phenotypic data for SLD, pre-XDR and XDR sample

Among the 249 M.tb isolates, a total of 34 isolates harboring mutations in *gyrA/B* and *rrs-F2* genes, were characterized using phenotypic DST for SLD including Ofx, Km, Am and Cm. The results are showed in **Table III-9**. Overall, 23 isolates including 18 MDR and 5 non-MDR exhibited resistance to at least one SLD. Among non-MDR isolates, Ofx-resistance was found in all the 5 (5/140; 3.6% of all non-MDR isolates) isolates including isolates with mono-resistance to STM ($n=2$) and to RIF ($n=2$) and poly-resistance to INH and STM ($n=1$). Resistance to any SLID was not observed among non-MDR isolates. Among the 18 MDR isolates, 15 (15/109; 13.8% of all MDR isolates) were resistant to either Ofx (13/109; 11.9%) or at least one SLID (2/109; 1.8%), corresponding to pre-XDR profiles and three (3/109; 2.8% of all MDR isolates) MDR isolates were resistant to Ofx and at least one SLID simultaneously corresponding to XDR isolates. It is worth noting that, one of the three (33.3%) XDR isolates was totally resistant to all 4 FLD (INH, RIF, EMB and STM) and 4 SLD (Ofx, Am, Km and Cm) tested, as well as resistant to PZA by carrying mutation T49P in the *pncA* gene. The isolate was named XDR+ isolate.

All the 20 isolates with single mutation in the QRDR region of *gyrA* gene were resistant to Ofx and all the 7 isolates with single mutation outside the QRDR region of *gyrB* mutation were susceptible to Ofx. Among the three isolates with mutations in *gyrA* and *gyrB*, only one isolate with *gyrA* Asp94Gly mutation, known to be FQ resistant determinant, together with *gyrB* Ala644Asp mutation were resistant to Ofx, while the two other isolates were susceptible to Ofx; one harboring double mutation (Asp94Gly and His87Gln) in *gyrA* and *gyrB* Ala644Asp mutation and the other one had mutations outside the QRDR of *gyrA* and *gyrB* (*gyrA* Asp7Asn and *gyrB* Ala644Asp).

Among the five isolates resistant to SLID, three isolates had *rrs-F2* A1401G mutation; one with *rrs-F2* C1489T mutation and the other one had no mutation in *rrs-F2* gene. Among the three isolates with A1401G mutation, two were resistant to Am, Km and Cm and one was resistant to Am and Km. One isolate with C1489T substitution was resistant to Am and Km, while one isolate that did not have any mutations in the *rrs* gene was resistant to Km. Two isolates with substitution G1177A and 1204 ins A were found susceptible to the three SLID.

2.5. Link between mutations and drug resistant profiles

We explored whether the different FLD and SLD drug resistance conferring mutations were associated with bacterial FLD drug resistant phenotypes. The results are showed in **Table III-10**. The frequencies of gene mutations including the most frequent mutations in each gene were compared to two drug resistant categories based on the number of FLD drug resistances (mono-, double-, tripe- and quadruple-drug resistance) and the MDR versus non-MDR phenotypes.

Table III-10. Frequencies of mutations according to FLD drug resistance patterns

Drug resistance	Gene/frequent mutation	Drug resistance patterns							
		Categories by number of FLD drug resistance					Categories by MDR and non-MDR		
		Mono-resistance	Double-resistance	Triple-resistance	Quadruple-resistance	<i>p-value</i>	MDR	Non-MDR	<i>p-value</i>
INH	<i>katG</i>	24.5	70.1	65.2	89.5	< 0.001	76.1	37.1	< 0.001
	<i>katG</i> Ser315Thr	13.3	53.7	54.3	76.3	< 0.001	60.6	26.4	< 0.001
	<i>inhA</i>	17.3	20.9	23.9	7.9	0.204	15.6	20.0	0.367
	<i>inhA</i> C-15T	15.3	19.4	23.9	7.9	0.206	15.6	17.9	0.635
	<i>katG</i> and <i>inhA</i>	37.8	88.1	87.0	94.7	< 0.001	89.9	52.9	< 0.001
RIF	<i>rpoB</i>	21.4	50.7	91.3	97.4	< 0.001	94.5	22.1	< 0.001
	<i>rpoB</i> RRDR	20.4	47.8	87.0	97.4	< 0.001	92.7	20.0	< 0.001
	<i>rpoB</i> Leu531Leu	7.1	25.4	34.8	73.7	< 0.001	56.0	5.0	< 0.001
STM	<i>rpsL</i>	22.4	46.3	60.9	65.8	< 0.001	47.7	38.6	0.148
	<i>rpsL</i> Lys43Arg	15.3	28.4	41.3	44.7	< 0.001	31.2	25.7	0.341
	<i>rpsL</i> Lys88Arg	6.1	11.9	19.6	21.1	0.038	15.6	10.0	0.186
	<i>rrs-F1</i>	3.1	7.5	10.9	26.3	0.001	13.8	5.7	0.029
	<i>rrs-F1</i> A514C	2.0	3.0	8.7	21.1	0.001	11.0	2.9	0.008
	<i>rpsL</i> and <i>rrs-F1</i>	25.5	53.7	71.7	92.1	< 0.001	61.5	44.3	0.006
EMB	<i>embB</i>	4.1	11.9	34.8	81.6	< 0.001	45.0	7.1	< 0.001
	<i>embB</i> Met306Val	1.0	1.5	13.0	31.6	< 0.001	14.7	2.9	< 0.001
	<i>embB</i> Met306Ile	1.0	4.5	6.5	10.5	0.08	9.2	0.7	< 0.001

Regarding the INH resistance, the frequencies of *katG* mutations was significantly higher than the frequencies of *inhA* mutations ($p < 0.01$) (**Table III-10**). Compared to mono-drug resistant isolates, the frequencies of *katG* and *katG315* mutations were significantly higher in double-drug, triple-drug and quadruple-drug resistant isolates ($p < 0.01$), while frequencies of *inhA* and *inhA* C-15T mutations were not different according to the drug resistant patterns ($p > 0.05$). Similarly, the frequencies of *rpoB* and *rpoB* Leu531Leu mutations, conferring resistance to RIF, and the frequencies of *rpsL* and *rrs-F1* gene, including *rpsL* Lys43Arg, *rpsL* Lys88Arg and *rrs-F1* A514C, conferring resistance to STM, were also associated with poly- and multi-drug resistance (double-drug, triple-drug and quadruple-drug resistance) compared to mono drug resistance ($p < 0.01$). Globally, the frequencies of *embB* mutations conferring EMB resistance were significantly associated with different drug resistant patterns ($p < 0.001$). Among *embB* mutations, only *embB* Met306Val mutation was found significantly higher in double-drug, triple-drug and quadruple-drug resistance ($p < 0.001$). In contrast, *embB* Met306Ile mutation was not significantly different among the resistant patterns ($p = 0.08$). Furthermore, except for *inhA* C-15T substitution, *katG* Ser315Thr, *rpoB* Leu531Leu, *rpsL* Lys43Arg, *rpsL* Lys88Arg, *rrs-F1* A514C and *embB* Met306Val were significantly associated with triple and quadruple drug resistance compared to mono and double drug resistant patterns.

Regarding the comparison of MDR and non-MDR isolates, we found that *katG* mutation (76.1%), *KatG* Ser315Thr (60.6%), *rpoB* mutation (94.5%), *rpoB* Leu531Leu (56.0%), *rrs-F1* mutation (13.8%), *rrs-F1* A514C (11.0%), *embB* mutation (45.0%), *embB* Met306Val (14.7%) and *embB* Met306Ile (9.2%) were more frequent among MDR isolates ($p < 0.05$). Mutations in *inhA* gene including C-15T mutation and *rpsL* gene including Lys43Arg and Lys88Arg mutations were detected in similar proportion in MDR and non-MDR isolates.

The frequencies of *pncA* mutations were the highest in quadruple-resistant isolates (71.1%), followed by triple-resistant (30.4%), double-resistant (9.0%) and mono-resistant isolates (2.0%) (**Table III-11**). The crude odds ratio (COR) calculation showed that triple-resistant and quadruple-resistant isolates were associated with *pncA* mutations. The COR comparing triple-resistant and quadruple-resistant isolates with mono-resistant isolates was 20.9 (95% CI 4.5 to 97.4; $p < 0.001$) and 117.8 (95% CI 24.6 to 564.0; $p < 0.001$), respectively. The association with *pncA* mutations was stronger in MDR isolates (COR, 14.6; 95% CI 5.9-35.9; $p < 0.001$).

Table III-11. Frequencies of *pncA* mutations according to FLD drug resistance patterns

FLD drug resistant patterns	<i>pncA</i> gene conferring PZA resistance		Frequency of mutations	Odds ratio (95% CI)	<i>p</i> -value
	With mutation	Without mutation			
Mono resistance	2	96	2.0	1	
Double resistance	6	61	9.0	4.7 (0.9-24.1)	0.06
Triple resistance	14	32	30.4	20.9 (4.5-97.4)	< 0.001
Quadruple resistance	27	11	71.1	117.8 (24.6-564.0)	< 0.001
Non-MDR	6	134	4.3	1	
MDR	43	66	39.5	14.6 (5.9-35.9)	< 0.001

The frequencies of SLD resistance-associated mutations (*gyrA* and *rrs-F2*) were strongly associated with triple and quadruple-FLD resistances, with crude OR of 5.7 (95% CI 1.7 to 19.7; $p = 0.006$) and 6.3 (95% CI 1.8 to 22.3; $p = 0.005$), respectively, and were significantly different between non-MDR and MDR isolates with crude OR of 5.7 (95% CI 2.1 to 15.8; $p < 0.001$) (**Table III-12**).

Table III-12. Frequencies of SLD-resistant mutations according to FLD drug resistance patterns

FLD drug resistant patterns	<i>gyrA</i> and <i>rrs-F2</i> genes conferring resistance to SLD		Frequency of mutations	Odds ratio (95% CI)	<i>p</i> -value
	With mutation	Without mutation			
Mono resistance	4	94	4.1	1	
Double resistance	3	64	4.5	1.1 (0.2-5.1)	0.901
Triple resistance	9	37	19.6	5.7 (1.7-19.7)	0.006
Quadruple resistance	8	30	21.1	6.3 (1.8-22.3)	0.005
Non-MDR	5	135	3.6	1	
MDR	19	90	17.4	5.7 (2.1-15.8)	< 0.001

2.6 Link between *M. tuberculosis* genotypes and drug resistance

2.6.1 Association between genotypes and drug resistant profiles

To test whether the genetic background could influence the drug resistance in *M.tb*, we categorized all isolates into three main groups: Beijing (n=141 isolates), EAI (n=85 isolates) and "Other" (n=23 isolates). "Other" regroups isolates belonging to minor families including T (n=4 isolates), H (n=3 isolates), Manu-ancestor (n=3 isolates), Zero-copy (n=2 isolates), Cameroon (n=2 isolates), Unknown (n=6 isolates) and not defined families (n=3 isolates). The crude OR and 95% confidence interval (CI) and *p*-value were calculated to test the association between the genotypes and the drug resistance patterns. The results are showed in **Table III-13**. Compared to EAI isolates, Beijing and "Other" isolates were associated with any phenotypic resistance to RIF and EMB. Beijing was also associated with any resistance to STM. There is no association between genotypes and any phenotypic resistance to INH. In terms of number of FLD resistances, there is no association between genotypes and double drug resistance and EAI isolates were associated with mono drug resistance. Interestingly Beijing isolates were associated with multiple drug resistance (triple drug resistance) and this association was stronger for quadruple drug resistance (Crude OR, 7.39; 95% CI, 2.18 to 25.04; $p = 0.001$). "Other" isolates were also associated with quadruple drug resistance with similar OR. Both Beijing and "Other" isolates were associated with multidrug resistance.

Among the isolates harboring mutations in *gyrA/B* and *rrs-F2* genes tested by phenotypic DST for SLD, 23 among 34 isolates including 18 MDR and 5 non-MDR exhibited resistance to at least one SLD. Among them, 4.4% (1/5 isolates), 82.6% (19/26 isolates) and 13.0% (3/3 isolates) were EAI, Beijing and "Other" isolates, respectively. Beijing isolates were associated with SLD resistance with crude OR of 10.86 (95% CI, 1.03 to 114.58; $p = 0.025$) when comparing to EAI isolates.

Table III-13. Association between M.tb genotypes and FLD drug resistance profiles

Drug resistance pattern	<i>M. tuberculosis</i> genotypes						Beijing versus EAI		Other versus EAI	
	Beijing		EAI		Other		Odds ratio (95% CI)	<i>p</i> -value	Odds ratio (95% CI)	<i>p</i> -value
	N° of resistant isolates	%	N° of resistant isolates	%	N° of resistant isolates	%				
INH	109	77.3	64	75.3	19	82.6	1.12 (0.59-2.10)	0.730	1.56 (0.48-5.10)	0.463
RIF	82	58.2	32	37.7	13	56.5	2.30 (1.33-3.99)	0.003	2.15 (0.85-5.48)	0.108
STM	105	75.5	39	45.4	10	43.5	3.44 (1.94-6.09)	< 0.001	0.91 (0.36-2.29)	0.837
EMB	36	25.5	7	8.2	6	26.1	3.82 (1.62-9.04)	0.002	3.93 (1.17-13.19)	0.027
Mono resistance	44	31.2	44	51.8	10	43.5	0.42 (0.24-0.76)	0.002	0.72 (0.28-1.81)	0.482
Double resistance	33	23.4	28	32.9	6	26.1	0.62 (0.34-1.13)	0.190	0.72 (0.26-2.02)	0.531
Triple resistance	34	24.1	10	11.8	2	8.7	2.38 (1.10-5.12)	0.026	0.71 (0.15-3.51)	0.679
Quadruple resistance	30	21.3	3	3.5	5	21.7	7.39 (2.18-25.04)	0.001	7.59 (1.66-34.69)	0.009
MDR	73	51.8	24	28.2	2	52.2	2.73 (1.53-4.86)	0.001	2.77 (1.08-7.13)	0.034

2.6.2. Association of genotypes with drug resistance-conferring mutations

To test whether the genetic background could influence the evolutionary pathway to anti-TB drugs resistance, we compared mutation frequencies as well as predominant mutations in *katG*, *inhA*, *rpoB*, *rpsL*, *rrs-F1*, *embB*, *pncA*, *gyrA*, *gyrB* and *rrs-F2* within the main M.tb families (Beijing and EAI). The Beijing family was found to be associated with *katG*, *rpoB*, *rpsL*, *embB*, *pncA* and *gyrA* mutations (**Table III-14**). In contrast, *inhA* mutations were associated with EAI family and *rrs* mutation including *rrs-F1* and *rrs-F2* were not associated with any family. However, the mutation in *rrs-F2* gene conferring resistance to SLID were only observed in three Beijing isolates.

A comparison performed with the predominant mutations in each gene revealed similar results. Beijing family was associated with *katG* Ser315Thr, *rpoB* Ser531Leu, *rpsL* Lys43Arg, *rpsL* Lys88Arg and *gyrA* Asp94Gly, as well as *rrs-F1* A514C, while *inhA* C-15T, *embB* Met306Val and *embB* Met306Ile were not linked to any genotype. Interestingly, we also observed interactions between drug resistance mutations, M.tb genotypes and highly drug resistance (MDR, triple DR, QDR, pre-XDR and XDR). Double mutants *rpoB* Ser531Leu/*katG* Ser315Thr were significantly associated with Beijing family (85.7% versus 59.1%; COR 4.15; 95% CI 1.35 to 17.00; $p = 0.004$) and only found in highly resistant isolates ($p < 0.001$). Double mutants *rpsL* Lys43Arg/*katG* Ser315Thr were associated with Beijing family (90.9% versus 55.5%; COR 8.02; 95% CI 2.71 to 31.91; $p < 0.001$) as well as with highly resistant isolates (68.2% versus 40.0%; COR 3.21; 95% CI 1.54 to 6.95; $p < 0.001$). Double mutants *rpsL* Lys43Arg/*rpoB* Ser531Leu were only found in Beijing family ($p < 0.001$) and in highly resistant isolates ($p < 0.001$).

Table III-14. Distribution of the most frequent mutations associated with FLD and SLD resistance according to the M.tb families

Drug resistance	Gene/frequent mutation	<i>M. tuberculosis</i> Genotypes								
		Beijing			EAI			Beijing versus EAI		
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	COR	95% CI	<i>p</i> -value
INH	<i>katG</i>	86	55	60.9	31	54	36.5	2.72	1.51-4.95	< 0.001
	<i>katG</i> Ser315Thr	64	77	45.4	23	62	27.1	2.24	1.21-4.21	0.006
	<i>inhA</i>	19	122	13.5	24	61	28.2	0.39	0.19-0.82	0.006
	<i>inhA</i> C-15T	19	122	13.5	21	64	24.7	0.47	0.22-1.01	0.032
	<i>katG</i> and <i>inhA</i>	101	40	71.6	51	34	60.0	1.68	0.92-3.08	0.071
RIF	<i>rpoB</i>	85	56	60.3	35	50	41.2	2.17	1.21-3.89	0.005
	<i>rpoB</i> RRDR	82	59	58.2	33	52	38.8	2.19	1.22-3.95	0.005
	<i>rpoB</i> Ser531Leu	45	96	31.9	15	70	17.7	2.19	1.09-4.56	0.019
STM	<i>rpsL</i>	91	50	64.5	14	71	16.5	9.23	4.55-19.39	< 0.001
	<i>rpsL</i> Lys43Arg	63	78	44.7	7	78	8.2	9.00	3.77-24.55	< 0.001
	<i>rpsL</i> Lys88Arg	27	114	19.2	3	82	3.5	6.47	1.88-34.23	< 0.001
	<i>rrs-F1</i>	12	129	8.5	8	77	9.4	0.89	0.32-2.65	0.817
	<i>rrs-F1</i> A514C	12	129	8.5	1	84	1.2	7.81	1.11-333.87	0.022
	<i>rpsL</i> and <i>rrs-F1</i>	103	38	73.1	22	63	25.9	7.76	4.04-15.04	< 0.001
EMB	<i>embB</i>	39	102	27.7	12	73	14.1	2.33	1.10-5.21	0.018
	<i>embB</i> Met306Val	16	125	11.4	3	82	3.5	3.49	0.95-19.23	0.040
	<i>embB</i> Met306Ile	5	136	3.6	3	82	3.5	1.00	0.19-6.63	0.995
PZA	<i>pncA</i>	34	107	24.1	10	75	11.8	2.38	1.07-5.73	0.023
FQ	<i>gyrA</i>	19	122	13.5	1	84	1.2	13.08	1.98-549.80	0.002
	<i>gyrA</i> Asp94Gly	13	128	9.2	0	85	0.0	-	-	0.001
SLID	<i>rrs-F2</i>	3	138	2.1	0	85	0.0	-	-	0.293
SLD	<i>gyrA</i> and <i>rrs-F2</i>	20	121	14.2	1	84	1.2	13.88	2.12-582.08	< 0.001

2.7 Compensatory mutations

2.7.1 Mutations in *ahpC* gene of INH-resistant isolates

The sequencing of *ahpC* gene was successfully performed for 176/192 (91.7%) INH-resistant isolates. Among them, only 2 (1.1%) INH-resistant isolates carried mutation (C-51T) in *ahpC* gene; L191F mutation and 570 ins ACTC (**Table III-15**). Both isolates were MDR and belonged to EAI5 family, suggesting that this mutation may act as compensatory mutation for INH-resistant mutants.

Table III-15. Compensatory mutations in *ahpC*, *rpoA* and *rpoC* genes and their distribution according to *M.tb* families

Gene	Nucleotide position	Nucleotide change	Codon position	Amino acid change	<i>M. tuberculosis</i> families			
					Beijing	EAI	other	Total
<i>ahpC</i>	571	CTC-TTC	191	Leu-Phe	0	1	0	1
	570	ins ACTC	-	-	0	1	0	1
<i>rpoA</i>	379	ACG-GCG	127	Thr-Ala	0	1	0	1
	544	CGG-TGG	182	Arg-Trp	0	1	0	1
<i>rpoC</i>	1448	GTG-GGG	483	Val-Gly	3	0	0	3
	1448	GTG-GCG	483	Val-Ala	1	0	0	1
	1452	TGG-TGC	484	Trp-Cys	0	0	1	1
	1681	TCC-CCC	561	Ser-Pro	0	1	0	1
	1802	CCG-CTG	601	Pro-Leu	4	1	1	6

2.7.2 Mutations in *rpoA* and *rpoC* gene of RIF-resistant isolates

Due to a technical issue, sequencing of *rpoA* and *rpoC* genes were successfully obtained for respectively 76 (59.8%) and 41 (32.3%) of the 127 RIF-resistant isolates and only 7 of them had sequence results for both genes. Mutations in *rpoA* gene were found in two (2.6%) among the 76 RIF-resistant isolates; one isolate was poly-resistant and the other one was MDR quadruple resistant. Both of the isolates belonged to EAI family; one isolate carried *rpoB* Val146Phe mutation and the other one carried *rpoB* His526Leu mutation.

Twelve (29.3%) out of the 41 RIF-resistant isolates revealed five different mutations in *rpoC* gene (**Table III-15**). The most common mutations were detected at codons 601 (n=6) and 448 (n=4). Of these 12 isolates, 11 had *rpoB* mutations, mainly at codon 531 (Ser-Leu, n=6) followed by H526Y, D516V3, S531L + F584S, T480I + S531L and L511R + D516Y one each. Except one isolate which was resistant to only RIF and Ofx, all the 11 isolates carrying compensatory mutations in *rpoC* gene were MDR, in which 5 were quadruple drug resistant and 2 were pre-XDR_{FQ}.

Overall, compensatory mutations in either *rpoA* or *rpoC* were detected in 14/110 (12.7%) of RIF-resistant isolates. Among these mutants, 12 were MDR or pre-XDR. The compensatory mutations were mainly found in isolates carrying mutations in the RRDR of *rpoB* gene (12/14) and involved

mainly codon 531 (n=8, 80%; 6 single mutations and 2 double mutations). The majority of *rpoA* and *rpoC* mutants was Beijing (n=8), followed by EAI (n=4) and “Other” class (n=2).

2.8 Analysis of combined spoligotyping and MIRU-VNTR data and drug resistance

The phylogenetic tree based on spoligotyping and MIRU-VNTR data clearly showed the two main clades corresponding to the two main M.tb lineages including lineage 1 (mainly EAI family) and lineage 2 (Beijing family) (**Figure III-1**). Beijing isolates were well distinguished from other families; however, two Zero-copy isolates were found among EAI family. Twenty-four clusters of 55 isolates were observed. Mapping drug resistance-associated mutations and resistance patterns on phylogenetic tree, the result revealed that globally various resistance patterns and mutations patterns conferring resistance to FLD and SLD were distributed on the tree without any specific structuring. Moreover, the combinations of mutations were diverse within the majority of clusters (19/24 clusters). These data suggest various evolutionary events towards FLD and SLD resistances. It is worth noting that five clusters (C8, C11, C13, C18 and C30) shared the same mutation profiles. Except for C11 and C18 isolates, isolates in the three other clusters, including one MDR cluster (C8) and two INH-R clusters (C13 and C30), had the same drug resistance pattern and were isolated in similar time period and from patients living in the same province (see **Appendix 4**). This might suggest a recent transmission of primary resistant isolates.

Regarding overall drug resistance profiles, we observed that highly resistance isolates, particularly MDR isolates (COR 2.13; 95% CI 1.10 to 4.16; $p = 0.014$) and isolates resistant to three or four drugs (COR 2.80; 95% CI 1.43 to 5.47; $p < 0.001$) were associated with clustering. In terms of individual mutations, *katG* Ser315Thr and *gyrA* Asp94Gly were associated with clustering with crude OR of 2.43 (95% CI 1.25 to 4.74; $p = 0.004$; 58.5% versus 36.7%) and 4.10 (95% CI 1.16 to 14.38; $p < 0.001$; 13.2% versus 3.6%) respectively, while mutation *rpoB* Ser531Leu, *rpsL* Lys43Arg, *rpsL* Lys88Arg, *rrs* A514C and *embB* Met306Val/Ile did not show any association. Interestingly, *inhA* C-15T were associated with low clustering (5.7% versus 19.9%; COR 0.024; 95% CI 0.05 to 0.82; $p = 0.014$).

3. Discussion

3.1 Genetic determinants of FLD and SLD drug resistance

In this study, we reported the genetic determinants of resistance to FLD and SLD and their ability to predict phenotypic resistance. As expected, the most common mutations detected in genes involving resistance to INH, RIF, STM and EMB are *katG* Ser315Thr (53.6%) and *inhA* C-15T (21.4%), *rpoB* Ser531Leu (55.1%), *rpsL* Lys43Arg (45.5%) and *embB* Met306Val (36.7%), respectively. In one INH-resistant isolate, *katG* could not be amplified after repetitions using two pairs of primers targeting two different regions on the gene. The result suggests partial or complete deletion of *katG* gene, as reported by Ramaswamy et al ⁽¹⁵⁵⁾. New mutations such as *katG* (Gly99Ala, Gly125Val, Gly268Asp, Gly277Ala, Trp351Cys, Thr394Met, Ala467Ser and Phe720Ser), *rpsL* (Val23Ala and Val37Leu), *rpoB* (Thr563Ile and Met736Thr) and *embB* (Val309Phe, Asn318Asp, Phe330Ser and Leu561Arg) were observed in our sample. Further study is needed to explore their role and involvement in resistance mechanisms. Nevertheless, respectively 14.6%, 6.3%, 19.5%, 26.5% of isolates resistant to INH, RIF, STM and EMB did not present any mutation in studied genes, suggesting the involvement of other mechanisms (other resistance genes or drug efflux pumps) ^(128, 147).

The sensitivity and specificity for detecting phenotypic resistance from DNA sequencing data were calculated and the results showed that from 73.5 to 92.9% of isolates with FLD resistance were detected by sequencing of studied genes; with the highest rate for RIF resistance and the lowest rate for EMB resistance. The result is consistent with many previous studies supporting the usefulness of molecular tools for rapid detection of RIF-resistance and MDR ^(210, 417).

To detect resistance to SLD, the genes involved in drug resistance were sequenced. Among the 109 MDR, 15 (13.8%) were resistant to either Ofx (13; 11.9%) or at least one SLID (2; 1.8%) corresponding to pre-XDR profile, and three (2.8%) MDR isolates were resistant to Ofx and at least one SLID simultaneously corresponding to XDR isolates. Five (3.6%) Ofx-resistance were also found in 140 non-MDR isolates. All 21 FQ-resistant isolates had mutations in the QRDR region of the *gyrA* gene. Mutations at codon 94 were the most frequent (66.7%), followed by codon 90 (23.8%), representing 90.5% of Ofx-resistance. These findings are in accordance with previous reports, in which 60.0%-90% of FQ-resistant isolates had mutations at codons 88-94 ^(201, 418, 419). These mutations have also been reported to cause cross resistance among FQ compounds (Ofx, Lfx and Mfx) ⁽¹⁸⁴⁾. Unlike the *gyrA* gene, mutations in the *gyrB* gene were less commonly associated with resistance to FQ in *M.tb* ⁽²⁰¹⁾. In our study, all mutations in the *gyrB* gene were located outside the QRDR region and most of them were observed in FQ-susceptible isolates. The Ala644Asp mutation in the *gyrB* was found in 1/20 FQ-resistant isolates but was observed together with the Asp94Gly mutation in *gyrA*, known to be FQ determinant. Overall, all the 7 isolates with mutations outside the QRDR region of *gyrA/B* were susceptible to FQ, suggesting no impact on FQ resistance. However, one FQ-susceptible isolate harbored a double mutation in the QRDR region of the *gyrA* gene: mutation Asp94Gly, known to be FQ determinant, and mutation His87Gln, reported in one study but not associated with FQ-

resistance⁽⁴²⁰⁾. This double *gyrA* mutation has never been reported elsewhere before. The presence of a double *gyrA* mutation in a FQ-susceptible isolate might suggest: i) hetero-resistance with a lack of detection of the resistant population by the pDST method; ii) deactivation of FQ resistance caused by the Asp94Gly mutation due to the presence of mutation His87Gln or iii) other mechanisms.

Resistance to SLID was found in 4.6% (5/109) of MDR isolates, three times less than the rate of FQ resistance. This can be explained by the fact that in Cambodia, injectable agents are not usually used to treat common infections compared to FQ. Mutation A1401G in the *rrs*-F2 gene is reported as the most prominent mutation causing cross-resistance among SLID.^{22,47,48} In our study, cross-resistance was detected in 4/5 SLID-resistant isolates and 3 of them carried the A1401G mutation, in agreement with previous reports. One isolate (DR_001), carrying a new mutation named C1489T, also exhibited cross-resistance to Am and Km. Another isolate (DR_002) resistant only to Km did not reveal any mutation in the *rrs* gene, suggesting the contribution of other mechanisms. It is worth noting that two isolates with mutations (G1177A and 1204 ins A) located outside the 1400-1500 region of the *rrs* gene were susceptible to all the 3 SLID tested, suggesting no impact on SLID resistance.

Briefly, among the 34 isolates harboring mutations in genes associated with SLD resistance (ie *rrs*-F2, *gyrA* and *gyrB*), seven were SLD susceptible by pDST. Among these seven SLD susceptible isolates, six isolates carried mutations not conferring resistance and one isolate carried the Asp94Gly mutation linked to FQ resistance in association with other mutations (see above). Even if the sampling is small, these results suggest that molecular detection of SLD resistance is beneficial in our study in complement to pDST and is time saving.

Globally, more than 75% and 90% of the pre-XDR and XDR isolates, respectively, have been reported with phenotypic resistance to PZA⁽⁴²¹⁾. In our study, mutations conferring PZA resistance were observed in all XDR isolates and 83.3% of pre-XDR isolates. These results are important and worrying since PZA is an essential antibiotic for TB treatment because of its specific bactericidal action against semi-dormant bacilli (non-growing persister bacteria) located in acidic environments (TB lesions). Since PZA resistance is not routinely tested in Cambodia, the drug is systematically added in the treatment regimen for drug-resistant TB patients when there is no clinical contraindication for its use. Our findings underline that the resistance to PZA should be assessed among MDR-TB before using this drug in pre-XDR and XDR-TB treatment regimens.

3.2 Fitness cost of drug resistance associated mutations and compensatory mutations

As the drug targets are generally involved in important biological functions, the acquisition of drug resistance caused by mutations impairing those function might lead to fitness cost and low transmission rate⁽²⁶⁶⁾. Nevertheless, in *M.tb*, the drug resistant mutations associated with low fitness cost, high level of drug resistance, and successful transmission in the community were reported^(266,422,423). These mutations included *katG* Ser315Thr, *rpoB* Ser531Leu, *rpsL* Lys43Arg and *gyrA* Asp94Gly conferring resistance to INH, RIF, STM and FQ, respectively^(266,424-426). In our study, these five mutations were the most common mutations responsible of resistance to respective drugs and associated with

Beijing family. A statistical significance in the occurrence of these mutations in triple or quadruple drug resistant isolates were also observed. Beside *katG* Ser315Thr and *gyrA* Asp94Gly were associated with clustering. These data confirmed the previous findings that *katG* Ser315Thr, *rpoB* Ser531Leu, *rpsL* Lys43Arg and *gyrA* Asp94Gly might incur low fitness cost and that the isolates with these mutations, especially *katG* Ser315Thr and *gyrA* Asp94Gly, are capable of accumulating mutations causing resistance to multiple drugs. Consequently, multiple resistant isolates with low fitness cost mutations emerge and spread successfully in the population.

Based on the available data, the proportion of INH-resistant isolates with compensatory mutation in *ahpC* gene was low (1.1%; 2/176). The two isolates with compensatory mutations in *ahpC* gene harboring mutation *inhA* C-15T were MDR. The low number of isolates with compensatory mutations might be due to a positive selection of mutation with low fitness costs as showed by the high prevalence of low biological cost mutations *katG* Ser315Thr gene (74.4%) in our sample. These results are in concordance with previous reports^(422, 423). The mutation in *rpoA* (2.6%; 2/76) and *rpoC* (29.3%; 12/41) genes in RIF-resistant isolates were observed more frequently, in total 14 out of the 110 isolates (12.7%). Among them, 10 (71.4%) were MDR or pre-XDR. Compared to *rpoA*; isolates with mutation in *rpoC* belonged in higher proportion to Beijing family and carried mutations in *rpoB* codon 531. This observation was also reported in previous studies, assuming that the interactions (epistasis, see below) between a fitness-compensatory mutation in *rpoC* mutation and *rpoB* Ser531Leu mutation induce a positive selection of the mutation *rpoB* Ser531Leu in RIF-resistant clinical isolates and incur a low fitness cost^(266, 426, 427). However, the data in our study did not allow statistical analysis.

3.3 Epistasis

Despite the high variability of mutation patterns, our data showed significant associations between double mutants *rpoB* Ser531Leu/*katG* Ser315Thr, *rpsL* Lys43Arg/*katG* Ser315Thr and *rpsL* K43Arg/*rpoB* Ser531Leu with Beijing family and highly resistant isolates (MDR, triple resistance, QDR, pre-XDR and XDR). This is in agreement with previous reports showing these specific combinations of mutations offer a selective advantage in some M.tb populations such as Beijing family. These strains with a selective advantage have the ability to acquire multiple drug resistance and to become highly resistant with a minimized or absent fitness cost. These data strongly suggest the existence of epistasis interactions between drug resistance mutations as experimentally demonstrated^(266, 428).

3.4 Genetic background and drug resistance

Beijing family, predominant in Eastern Asia and often associated with drug resistance⁽⁴²⁹⁻⁴³²⁾, hypervirulence, younger ages⁽⁴³¹⁾ and outbreak^(433, 434) has been spread worldwide in recent decade^(429, 435, 436). Consistent, with previous reports, in our study, Beijing family were also likely to be drug resistant, especially MDR (COR, 2.73; 95% CI, 1.53 to 4.86; $p = 0.001$), quadruple resistant (COR, 7.39; 95% CI, 2.18 to 25.04; $p = 0.001$) and SLD drug resistance including pre-XDR and XDR (COR 10.86; 95% CI 1.03 to 114.58; $p = 0.025$). The analyses of gene sequences and genotypes also

revealed that the distribution of specific mutations depends on M.tb family. As showed in **Table III-13**, compared to EAI, Beijing family was associated with high level of drug resistance and low fitness cost mutations such as *katG* Ser315Thr, *rpoB* Ser531Leu, *rpsL* Lys43Arg and *gyrA* Asp94Gly ($p < 0.05$), consistent with other reports ^(266, 429-432). The interactions between the genetic background and the drug resistant mutations was also explained by epistatic processes ⁽²⁶⁶⁾.

3.5 Various evolutionary pathways towards highly drug-resistance

The genetic determinants of resistance to FLD and SLD and the evolution of resistance were investigated. Genetic and phylogenetic analysis demonstrated a diversity of drug resistant patterns from mono-resistance to XDR and an important diversity of mutation patterns in each M.tb family and each cluster. These results suggest various evolutionary trajectories towards resistance to FLD and SLD. The Beijing family was also associated with MDR and XDR and low fitness cost mutations in resistant genes such as *katG* Ser315Thr, *rpoB* Leu531Leu and *gyrA* Asp94Gly. The existence of compensatory mutations in some resistant isolates highlights that the M.tb population is evolving towards increasingly resistant strains with a high transmission potential. Our data suggest a cumulative effect of mutations, a role of epistasis in the acquisition of multiple resistance and the spread of highly resistant and transmissible isolates in the population.

Result 4: Zooanthroponosis: Human to animal transmission of drug resistant *Mycobacterium tuberculosis* in a rescue center

Poster presented in the 50th Union World Conference on Lung Health, 30 October to 2 November 2019, Hyderabad, India (Appendix 3)

Running title: Zoonosis in reverse: *Mycobacterium tuberculosis* in a population of captive sun bears

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Abstract

Background: Between 2009 and 2017, tuberculosis, caused by *Mycobacterium tuberculosis* (M.tb), was confirmed in 19 sun bears (*Helarctos malayanus*) at a wildlife rescue centre in Cambodia. All cases either died or underwent euthanasia and M.tb was confirmed on post-mortem tissue culture. One staff member developed TB in 2011 and was fully recovered after completed TB treatment. This work aims to illustrate the molecular features of M.tb isolates from both the sun bears and the in-contact human.

Methods: 43-spacer spoligotyping and 24-locus MIRU-VNTR methods were used to genotype already cultured and confirmed M.tb isolates from each case. Historical temporal and spatial records were used to model possible transmission paths.

Results: Spoligotyping revealed two patterns. Sixty percent of isolates belonged to the East African-Indian (EAI) family whereas 40% belonged to the Beijing family. All the isolates belonging to the Beijing lineage were resistant to isoniazid and streptomycin, while the EAI isolates were pan-sensible. MIRU-VNTR typing distinguished three patterns. The first cluster contained all Beijing spoligotype isolates, including the human case. The second cluster contained 11 out of 12 isolates belonging to the EAI spoligotype, with the final isolate having one locus different. Contact tracing indicated transmission pathways for each infection, with the human case providing a link between two temporally separated bear cases in the Beijing spoligotype pathway.

Conclusions: Genotyping revealed two lineages of M.tb and confirmed involvement of a human case, with timing and exposure indicating likely transmission from bear to human and back to

bear. This illustrates TB susceptibility of a wildlife species placed in contact with humans in a high TB prevalence country, captivity creating necessary conditions for pathogen exposure and disease development. There are serious human health implications when drug-resistant zoonotic disease emerges in a previously unreported species, as well as important conservation impacts when the species is endangered.

1. Background

Two bear species, the Malayan sun bear (*Helarctos malayanus*) and the Asiatic black bear (*Ursus thibetanus*), are found in mainland Southeast Asia, with both considered vulnerable to extinction⁽⁴³⁷⁾. The two bear species are seriously threatened in Cambodia by habitat destruction and the illegal wildlife trade. If intercepted from illegal holding and trade, these two bear species are found in relatively high numbers in rescue centres. Furthermore, they are frequently kept in zoos, circuses and, unfortunately, in private homes and “bile farms” where their bile is extracted for medicinal uses. Poaching, illegal captivity, and rescue centres all bring bears into close and prolonged contact with humans, amplifying the potential for pathogen transfer. Cambodia is among high TB burden countries (1). Bidirectional transmission of M.tb is known to occur between humans and elephants^(438, 439) and the spill-over of M.tb to wildlife is recognized in certain species and settings, including captive sloth bears in India⁽⁴⁴⁰⁾. There are no published reports of TB occurring in either bear species. However, between 2009 and 2017, TB, caused by *Mycobacterium tuberculosis* (M.tb), was confirmed in 19 sun bears (*Helarctos malayanus*) at a wildlife rescue centre in Cambodia, where the bears are managed by bear conservation and welfare NGO Free the Bears. All cases either died or underwent euthanasia and M.tb was confirmed on post-mortem tissue culture. One staff member developed tuberculosis in 2011 and was fully recovered after completed TB treatment. This work aims to illustrate the molecular features of M.tb isolates from both the sun bears and the in-contact human and determine if transmission of TB can occur between humans and bears.

2. Results

2.1 Drug susceptibility to first line drugs

DST for FLD including INH, STM, RIF and EMB was performed on all 20 M.tb isolates including 19 from the sun bears and 1 from human case. Of them, 8 M.tb isolates including the human isolate were resistant to INH and STM while the others were susceptible to all the 4 drugs tested (**Table III-16**). The sequencing for FLD and SLD drug resistant genes were performed for all isolates and only isolates resistant to INH and STM harboring *inhA* C-15T and *rpsL* Lys88Arg mutation conferring low level resistance to INH and resistance to STM, respectively. The mutation conferring resistance to SLD were not observed in all isolates. Table III-16. DST, mutation patterns, spoligotype and MIRU-VNTR results of the 20 M.tb isolates

Table III 16. DST, mutation patterns, spoligotype and MIRU-VNTR results of the 20 M.tb isolates

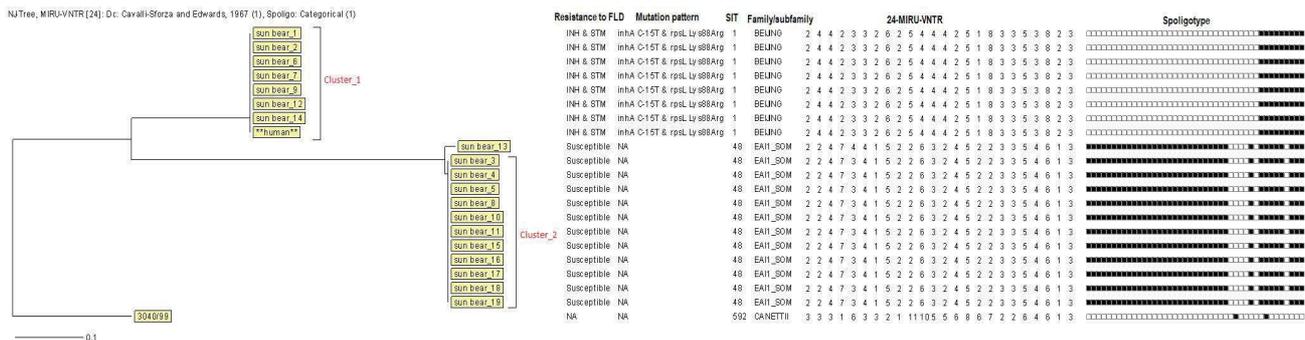
N	ID	Diagnosis date	Resistance to FLD	Drug resistant mutations	Spoligo Octal	SIT	Family/subfamily	24-MIRU-VNTR	Cluster
1	sun bear_1	Dec-09	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
2	sun bear_2	Jan-11	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
3	sun bear_3	Feb-12	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
4	sun bear_4	Mar-14	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
5	sun bear_5	Jul-15	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
6	sun bear_6	Apr-16	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
7	sun bear_7	Jun-16	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
8	sun bear_8	Aug-16	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
9	sun bear_9	Aug-16	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
10	sun bear_10	Nov-16	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
11	sun bear_11	Nov-16	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
12	sun bear_12	Feb-17	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
13	sun bear_13	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224744152263245223354613	Cluster_2.1
14	sun bear_14	Mar-17	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1
15	sun bear_15	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
16	sun bear_16	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
17	sun bear_17	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
18	sun bear_18	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
19	sun bear_19	Mar-17	Susceptible	NA	77777777413731	48	EAI1-SOM	224734152263245223354613	Cluster_2
20	**human**	Aug-11	INH & STM	<i>inhA</i> C-15T & <i>rpsL</i> Lys88Arg	00000000003771	1	Beijing	244233262544425183353823	Cluster_1

FLD, first line anti-TB drug; SIT, spoligo-international type; INH, Isoniazid; STM, Streptomycin; NA, not available.

2.2 Analysis of genotyping results

Spoligotyping revealed two spoligotype patterns shared by 20 M.tb isolates (**Table III-16**). Sixty percent of isolates belonged to the EAI (EAI1_SOM, SIT48) family whereas 40% belonged to the Beijing family (Beijing, SIT1), including the human isolate. All the isolates belonging to Beijing lineage were resistant to INH and STM, while the isolates belonging to EAI were pan-sensible.

The 24 loci MIRU-VNTR typing allowed distinguishing 3 patterns, including 1 unique pattern and patterns shared by 19 isolates (ranging from 8 to 11 isolates per pattern). The dendrogram generated by Neighbor-Joining (NJ) algorithm based on Spoligotyping and MIRU-VNTR data allowed to determine phylogenetic relationships between the 20 M.tb isolates (**Figure III-2**). The NJ tree was also used to determine the clusters defined as group of isolates having the same MIRU-VNTR and spoligotype profile and two clusters of 19 isolates were observed (8 to 11 isolates per cluster; clustering rate was 85%). The first cluster contained the 8 Beijing isolates (Beijing cluster), of which 1 isolate was obtained from a staff member in contact with bears. All the Beijing clustered isolates also shared the same drug resistant profiles and mutation patterns. The second cluster contained 11 out of 12 M.tb isolates that belonged to the EAI family (EAI cluster). One EAI isolate that did not belong to the cluster had only one MIRU loci (MIRU 40) different from that of clustered isolates.



From left to right: i) NJ tree built using Cavalli-Sforza and Edwards distance matrix calculated from the combination of MIRU-VNTR and spoligotyping data for the 20 isolates and *M. canettii* from MIRU-VNTR Plus reference strain used as outgroup; ii) cluster identification and; iii) Resistant profiles to first line anti-TB drugs (FLD: INH, isoniazid; STM, streptomycin); iv) Mutation patterns for genes involving resistance to first and second line drugs; v) Spoligo-international type (SIT) number; vi) spoligotype family and subfamily; vii) Number of repetitions of each of the 24 MIRU VNTR loci; and viii) 43-spacer spoligotypes (black spots represent the presence and white spot represent the absence of 1–43 spacers).

Figure III-2. Neighbor-joining tree based on the MIRU-VNTR and spoligotyping data.

Records of sun bear arrivals, social group compositions, and translocations within the rescue centre were collated for the previous 11 years to derive contact data. The information was combined with genotyping data to model possible transmission pathways. An apparent index case followed by bear-to-bear contact potentially accounted for entry and spread within the EAI cluster (cluster_2). However, the entry and transmission route of the Beijing cluster was not so apparent, with less clear temporal and spatial contact dimensions and the human case appearing to bridge a bear-to-bear transmission chain. Records of arrivals and TB disease confirmed for the sun bears and staff member of the Beijing cluster were showed in **Figure III-3**.

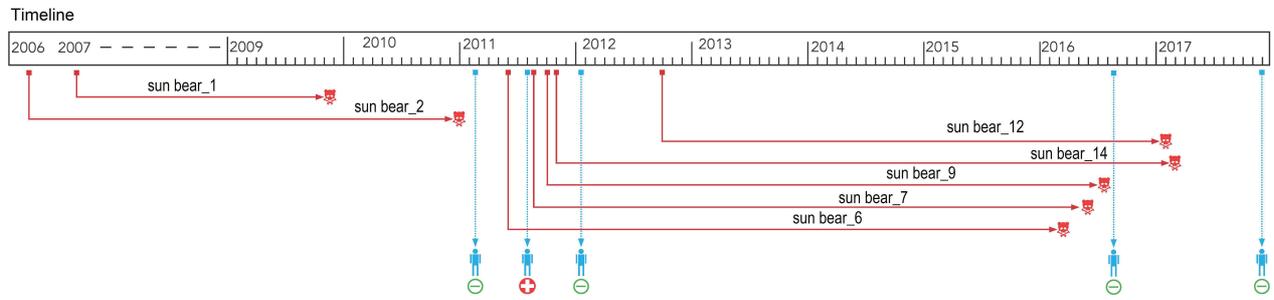


Figure III-3. Timeline of arrival, contact and TB disease confirmed among bear and human in the Beijing cluster.

Genetic profiles of the 20 M.tb isolates were compared with those obtained from human isolates (**Result 1**). The results suggested the M.tb isolates from the bears were genetically closed to M.tb isolates from human (**Figure III-4**).

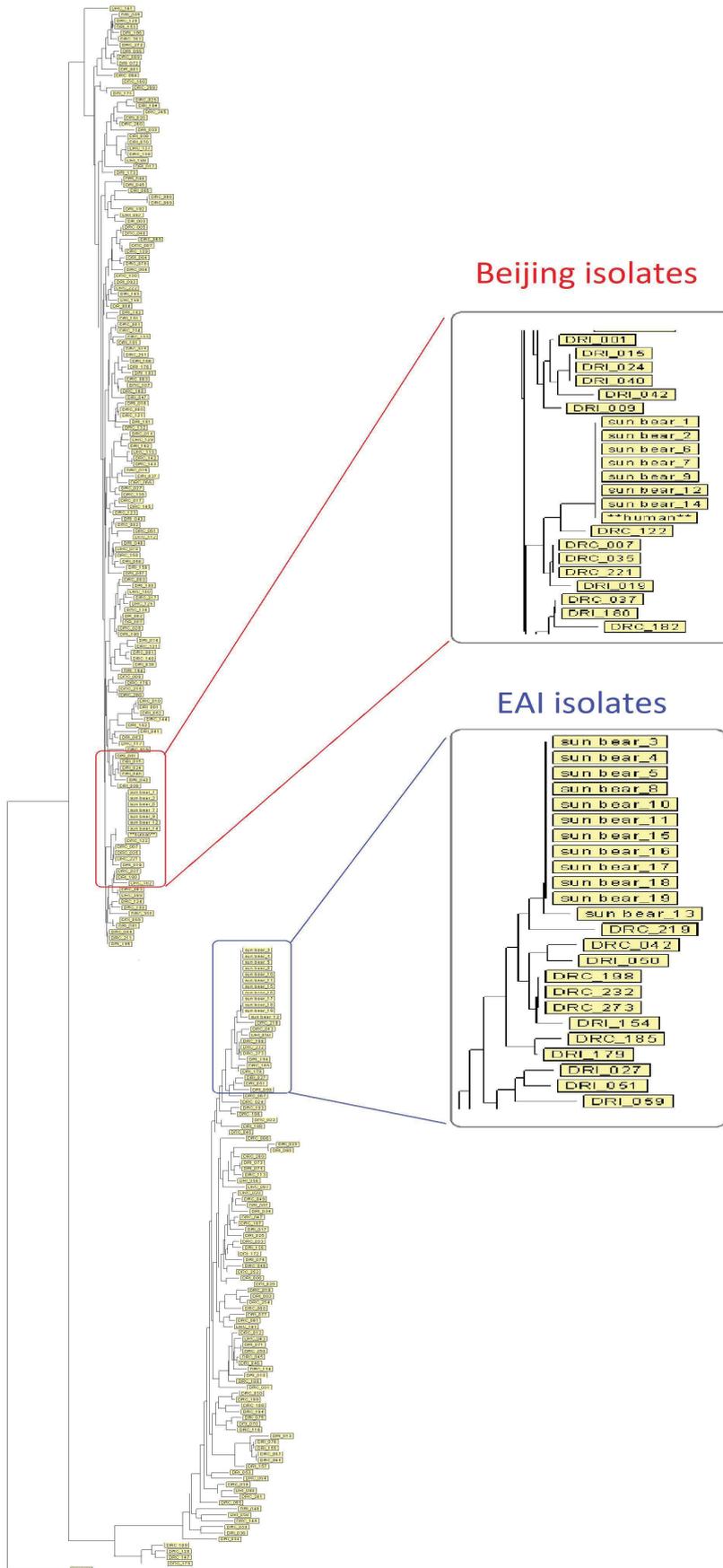


Figure III-4. Neighbor-joining tree based on the MIRU-VNTR and spoligotyping data of EAI and Beijing isolates from sampling 1 (Human M.tb isolates) and sampling 2 (Bear isolates).

3. Discussion and conclusion

TB was confirmed in 19 sun bears and one staff member at a Cambodian bear rescue centre that houses bears confiscated from the illegal wildlife trade. In all cases TB was caused by *M.tb*, the same mycobacteria that causes TB in humans. This is the first-time TB has been confirmed in this species. In the current absence of viable and safe treatment options, all cases resulted in death, either from the disease, or by euthanasia. The bear species is classified as vulnerable to extinction, and infectious diseases impact their conservation by affecting future release, translocation and breeding initiatives.

Genotyping results confirmed two families of *M.tb* in TB cases in this bear population; EAI (60%) and Beijing (40%). These two lineages are reported to be the most prevalent in Cambodia and in the South-East Asian region (432, 441). Two clusters were described with the same spoligotype and MIRU patterns, suggestive of two chains of transmission of *M.tb* isolates among the bears and between a human and bears inside the rescue center. The 24 MIRU-VNTR loci pattern of one isolate that did not belong to any cluster was very close to the EAI cluster isolates with only one loci with one repetitive unit different. The result highly suggests that the strain has evolved from the other strain in EAI cluster by gaining one more repetitive unit on MIRU 40 loci.

The genotyping also revealed involvement of a human case in the transmission chain, with timing and exposure indicating transmission from bear to human and back to bear. Contact tracing indicated a clear transmission pathway for each infection due to the Beijing cluster, with the human case providing a link between two bear cases. This illustrates TB susceptibility of a wildlife species placed in prolonged contact with humans in a high TB prevalence country. Captivity created the necessary conditions for pathogen transmission and disease development. There are serious human health implications when zoonotic disease emerges in a previously unreported species, as well as potential conservation impacts when the species is endangered. Beyond the TB human-bear transmission, anti-TB drug resistance complicated the management and control of TB in the rescue center.

Further studies including surveillance of TB disease in the rescue center among the bear species and also other in-contact species and full genome sequencing are needed for early detection of disease, rapid management of the case, prevent further spread and better understanding of how the disease progresses within individual bears. WGS will allow a better understanding of how the infection entered, and then spread, which in turn will inform management strategies to prevent recurrence, and ultimately impact the health and welfare of the remaining bears, as well as human safety.

Chapter IV: GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

1. General discussion

In this study, several aspects were investigated including the genetic diversity of the *M.tb* isolates population circulating in Cambodia over a six years period and their link with FLD resistance and the socio-demographic data; epidemiological patterns including the estimation of recent transmission of drug and multidrug resistance isolates (MDR, QDR, pre-XDR, XDR) and the transmission between humans and bears; phenotypic and genotypic characteristics as well as the prevalence of highly resistant isolates including pre-XDR and XDR; and the type and frequency of mutations associated with resistance to FLD and SLD; the genetic processes responsible for drug resistance and the evolutionary pathways toward highly resistant isolates. The major findings obtained in this study and their potential implications and applications in terms of public health are discussed below.

1.1 Diversity of *M. tuberculosis* population circulating in Cambodia

The present study gives the first outline of the genetic diversity and the structure of the *M.tb* population among patients suspected of having drug resistant TB in Cambodia over a period of 6 years. Our results showed that Beijing (47.4%) and EAI (42.2%) are the two most predominant families among our sample. The other families like T, Haarlem, LAM, Cameroon, Manu_ancestor and Unknown were in low proportion. For the first time, we report the presence of Zero_copy family (SIT 405; n=3; 0.8%) characterized by the absence of IS6110 insertion elements ⁽⁴⁴²⁾.

Beijing family, predominant in Eastern Asia and often associated with drug resistance ⁽⁴²⁹⁻⁴³²⁾, hypervirulence ^(429, 443), younger ages ⁽⁴³¹⁾ and outbreak ^(433, 434) has been spread worldwide in recent decades ^(429, 435, 436). In accordance to these reports, the proportion of Beijing family in our study was on the rise and now become the most predominant family. In addition, the Beijing family was associated with drug resistant, especially MDR and QDR, and with clustering which suggests involvement in recent transmission. The increase of Beijing circulation in the country can have a major impact on the TB epidemiology due to its high transmission ability as well as its association with drug resistance. A failure in measures to prevent and control TB infection might result into a more worrying TB situation in the future with the emergence of a highly resistance isolates or larger outbreak clusters. None of the demographic and clinical characteristics was significantly associated with Beijing genotype, in contrast to previous report by Nguyen et al, in which Beijing was associated with younger age, likely to be attributable to recent transmission ⁽⁴³¹⁾. In our sample, the global lack of association can be due

to a sampling bias since our study included isolates from presumed MDR-TB patients recruited by the NTP. The sampling is not thus representative of the M.tb population circulating in Cambodia and the clustering rate could be underestimated.

1.2 SLD drug resistance and its impact on TB control

Although XDR TB cases have been reported worldwide, Cambodia, with nearly 35 000 cases of TB and 150 MDR-TB cases annually, reported the first XDR-TB case to the WHO in 2016 ⁽³⁶⁾. Due to the absence of surveillance programs and systematic screening for SLD drug resistance among MDR patients, the burden of pre-XDR and XDR-TB has been under reported in Cambodia and is still unknown. The present study reports the first description of SLD drug resistance in M.tb in Cambodia using a combination of molecular screening for mutations in the *gyrA/B* gene conferring FQ resistance and the *rrs-F2* gene conferring SLID resistance. The molecular resistant patterns were then checked by pDST. Overall, only 25 of the 37 M.tb isolates harboring mutations in the *gyrA/B* and/or *rrs-F2* genes were resistant to FQ or SLID by pDST. The proportion of pre-XDR and XDR cases among MDR isolates in our study were 13.6% and 2.5%, respectively. These proportions remain lower than the global estimation of 6.2% for XDR-TB and 20.8% for FQ-resistance among RR/MDR-TB ⁽²⁾ but higher than the two previous reports in Cambodia ^(385, 444). It should be noted that, for the first time in Cambodia, one XDR isolate (DRI_151), named XDR+ in our study, was resistant to the 4 SLD and 5 FLD tested reflecting the circulation of highly resistant strains in Cambodia.

Among the isolates resistant to SLD in our study, the resistance to FQ, 14.4% ($n=17$) in the MDR group and 3.9% in non-MDR group ($n=6$), was more common than the resistance to SLID. This result was expected since FQ is one of the most commonly antibiotics used in non-TB infections in Cambodia and the drug is accessible without prescription at the counter of pharmacies ⁽⁴⁴⁵⁾. High incidence of FQ-resistance has also been reported in many parts of the world, particularly in countries with high TB burden ⁽⁴⁴⁶⁾. FQ is used in both standardized and shorter MDR-TB regimens and is considered as an essential part of the MDR-TB regimen ⁽³⁷⁾. Considering the high incidence of FQ-R described in our study, resistance to FQ should be determined right after a diagnostic of MDR-TB. Resistance to SLID was found in 4.2% (5/118) of MDR isolates, three times less than the rate of FQ resistance. This can be explained by the fact that these injectable agents are not usually used to treat common infections compared to FQ.

Overall, 12 out of the 37 isolates harboring mutations in genes associated with SLD resistance (ie *rrs-F2*, *gyrA* and *gyrB*), were SLD susceptible by pDST. Among these 12 SLD susceptible isolates, 11 isolates carried mutations not conferring resistance. Even if the sampling is small, these results suggest that molecular detection of SLD resistance is beneficial in our study in complement to pDST and is time saving.

In our study, mutations conferring PZA resistance were observed in all XDR isolates and 70% of pre-XDR isolates, in consistency with previous reports (e.g. Günther *et al*, 2015 ⁽⁴²¹⁾). Since PZA

resistance is not routinely tested in Cambodia, the drug is systematically added in the treatment regimen for drug-resistant TB patients when there is no clinical contraindication for its use. Our findings underline that the resistance to PZA should be assessed in MDR strains before using this drug in pre-XDR and XDR-TB treatment regimens.

The genetic analysis revealed that the dominant family was Beijing, representing 81.3% of pre-XDR and 100% of XDR isolates, in agreement with previous reports ^(418, 419, 444, 447). The phylogenetic analysis showed the presence of one cluster of two isolates belonging to the Beijing family (DR_002-XDR-2016 and DR_007-pre-XDR-2012); with another additional isolate (DRC_003-pre-XDR-2015), they form a clonal complex. This finding suggests that resistant isolates propagate in Cambodia and evolve from pre-XDR to XDR. This underlines the necessity of a more efficiency control strategy in terms of diagnostic and treatment to stop the spread of highly resistant strains such as the XDR+ strain (DRI_151). More worrying, the patient with the highly resistant XDR+ Beijing isolate (DRI_151) refused the treatment. This XDR+ strain was isolated in 2017 from a smear-positive sputum sample from a patient who lived in Phnom Penh capital city, suggesting a high risk of transmission in the population and a possible evolution towards increased drug resistance ⁽²⁶⁶⁾.

1.3 Genetic determinants of drug resistance

In this study, we reported the diversity of mutations associated with FLD and SLD drug resistance. The most common mutations were *katG* Ser315Thr, *inhA* C-15T, *rpoB* Ser531Leu, *rpsL* Lys43Arg and *embB* Met306Val, in agreement with previous reports ^(419, 448). The partial or complete *katG* gene deletion was observed in one INH-resistant isolate, as reported by Ramaswamy et al ⁽¹⁵⁵⁾. New mutation patterns in *katG*, *rpsL*, *rpoB* and *embB* were observed in our sample, suggesting the need for further study to illustrate their role and involvement in drug resistance. The sensitivity for detecting phenotypic resistance from DNA sequencing was estimated between 73.5 to 92.9% for FLD resistance. The result is consistent with previous studies supporting the usefulness of molecular tools for rapid detection of RIF-resistance and MDR ^(210, 417).

Compared to other studied genes, the mutation patterns in *pncA* gene are highly dispersed throughout the gene. The majority of them were previously associated with PZA resistance or confirmed by pDST in our study. The FQ-resistance in our sample were only caused by mutations in the QRDR region of the *gyrA* gene, with the most predominant mutations observed in codon 94 as in previous reports ^(201, 418, 419). This substitution has also been reported to cause cross resistance among FQ compounds (Ofx, Lfx and Mfx) ⁽¹⁸⁴⁾. Mutations found outside the QRDR region of *gyrA/B* were found in isolates susceptible to FQ, suggesting no impact on FQ resistance. Mutation a1401g in the *rrs-F2* gene was reported as the most prominent mutation causing cross-resistance among SLID ^(185, 447, 449) and it is the most frequent mutation found in our sample.

Since we used a limited set of genes in our study, for 14.6%, 6.3%, 19.5%, 26.5% of isolates resistant to INH, RIF, STM and EMB, respectively, we did not find any mutation in the studied genes,

suggesting the involvement of other mechanisms (other resistance genes or drug efflux pumps)^(128,147). It is likely that we underestimated the proportion of FQ- and SLID-resistant isolates for which the resistance mechanisms involved other resistant genes, such as *eis* promoter, *whiB*, *tlyA* or genes encoding mycobacterial efflux pumps^(184, 450).

1.4 Evolution of drug resistance in *M. tuberculosis*

In this study, we have highlighted intrinsic factors that could influence the evolution of drug resistance in *M.tb*, using genotyping and drug resistant mutation data. Genetic and phylogenetic analyses demonstrated a diversity of drug resistant patterns from mono-resistance to XDR and an important diversity of mutation patterns in each *M.tb* family and each cluster. These results suggest various evolutionary trajectories towards highly drug resistance including MDR, QDR, pre-XDR and XDR in the Cambodia. The intrinsic factors include the genetic background of *M.tb*, the fitness cost of mutations, the epistatic interactions between drug resistance mutations and between drug resistance mutations and compensatory mutations, as reported by Nguyen et al⁽²⁶⁶⁾. In our study, the Beijing family was associated with MDR and XDR and low fitness cost mutations in resistant genes such as *katG* Ser315Thr, *rpoB* Leu531Leu, *rpsL* Lys44Arg and *gyrA* Asp94Gly. The existence of compensatory mutations in some resistant isolates highlights that the *M.tb* population is evolving towards increasingly resistant strains with a restored fitness. These data predict an evolution of resistance towards a more problematic situation in the future since Beijing family seems on the rise in Cambodia. These results underline the imperative need to improve the diagnostic capacity of highly resistant TB cases in order to give to the patients the most appropriate treatment and prevent the spread and emergence of a more and more resistant isolates.

1.5 Zooanthroponosis: Human to animal transmission of drug resistant *M. tuberculosis*

An outbreak of presumed human-origin TB in bears at a rescue centre in Cambodia has been identified in our study. Two families of *M.tb* were involved in TB cases in this bear-human population; EAI (60%) and Beijing (40%). These two lineages are reported to be the most prevalent in Cambodia and in the South-East Asian region^(432, 441). With the exception of one isolate, the isolates formed two clusters; an EAI cluster and a Beijing cluster including the human case. The non-cluster isolate (only one different allele) was identified as EAI, suggesting an evolution of the population in the center. The cluster analysis confirmed the involvement of the human case in the transmission chain. The timing and exposure indicated probable transmission from bear to human and back to bear. Nevertheless, the close genetic relationships of the strains with the human TB strains indicate that the source of bear infection is likely a human. All the Beijing isolates were resistant to INH and

STM whereas the bears were not treated by antibiotics indicating a primary resistance. These data illustrate TB susceptibility of a wildlife species placed in prolonged contact with humans in a high TB prevalence country, captivity creating the necessary conditions for pathogen exposure and disease development. This result suggests serious human health implications when zoonotic disease merges in an unreported species that can play the role of reservoir. In addition, the TB susceptibility of these bears has a conservation impact since these species are endangered. It is worth noting that the human-animal TB transmission was previously demonstrated involving bears or other animals. As examples, a bidirectional transmission of *M.tb* between humans and elephants has been reported^(438, 439) as well as a spill-over of *M.tb* to wildlife in certain species and settings⁽⁴⁴⁰⁾, including captive sloth bears in India. Our data and these previous reports show the necessity to carefully studied these potential epidemiological routes in order, first, to avoid the persistence of unknown reservoirs of TB which can be a risk for human health and second to endanger susceptible species that are in danger of extinction. Beyond the TB human-bear transmission, anti-TB drug resistance complicates the management and control of TB in the rescue center. These results suggest an urgent need for further investigation in order to describe and address the diagnostic and management challenges associated with the TB outbreak to protect humans and bears in the rescue center. Whole genome sequencing of all the bear and human staff samples could help trace how the infection has spread. Furthermore, isolates from different infected organs of individual bear are available; WGS of those isolates can help to determine how the infection spread within the bear body. This information is essential for a better understanding of physiopathology of TB in bear body which can help to address the diagnosis and management issue.

2 Conclusion and perspectives

In conclusion, this work provides knowledge on the genetic diversity, population structure of *M.tb* and resistance to FLD and SLD in our sample collected from MDR-TB presumptive patients between 2012 and 2017. These data predict an evolution of resistance to a more problematic situation in the future. First, the Beijing family is associated with MDR and XDR as well as mutations associated with high level of resistance and low fitness cost and recent transmission. Second, the proportion of XDR and pre-XDR isolates remains low but seems to increase over time. The identification of the XDR+ isolate in a patient who refused treatment underlines the risk of transmission of highly resistant strains in the population. The detection of mutations associated with PZA resistance in MDR, pre-XDR and XDR isolates challenges the efficacy and the use of this antibiotic in the treatment of MDR-TB. PZA should not be used in the treatment regimen for pre-XDR TB and XDR-TB unless the resistance has been ruled out. DST using phenotypic or molecular methods for PZA should be performed for all MDR-TB patients in order to avoid a treatment failure and the risk of transmission of highly resistant strains. The genotypic results confirmed that Beijing strains circulate in the population and can

evolve into highly resistant forms such as pre-XDR or XDR isolates. These results strongly indicate the need for rapid interventions in terms of diagnostic and treatment to prevent the spread of Beijing isolates in the population and the emergence of more resistant strains.

This work also illustrates the susceptibility of the captive sun bear (*Helarctos malayanus*) to human TB. The susceptibility of this endangered species to human TB and in particular to resistant TB poses problems in terms of conservation of the species but also of public health. While this study provides essential information, it is important to note that this is a retrospective study on a relatively small number of genes and loci. A genetic study based on whole genome sequencing and on an extended sample of the M.tb population in Cambodia will complete this study on the evolution of drug resistance and the risk of M.tb transmission from humans to animals and from animals to humans.

Appendix 1. Article 1: Isolation of Nontuberculous Mycobacteria in Southeast Asian and African Human Immunodeficiency Virus-infected Children With Suspected Tuberculosis

Clinical Infectious Diseases

BRIEF REPORT



Isolation of Nontuberculous Mycobacteria in Southeast Asian and African Human Immunodeficiency Virus-infected Children With Suspected Tuberculosis

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We enrolled 427 human immunodeficiency virus-infected children (median age, 7.3 years), 59.2% severely immunodeficient, with suspected tuberculosis in Southeast Asian and African settings. Nontuberculous mycobacteria were isolated in 46 children (10.8%); 45.7% of isolates were *Mycobacterium avium* complex. Southeast Asian origin, age 5–9 years, and severe immunodeficiency were independently associated with nontuberculous mycobacteria isolation.

Clinical Trials Registration. NCT01331811.

Keywords. HIV; children; nontuberculous mycobacteria; tuberculosis.

Nontuberculous mycobacteria (NTM) are saprophytic organisms present in the environment [1]. NTM are not systematically

pathogenic but can cause various diseases in humans including respiratory disease, lymphadenitis, skin and soft tissue infection, and disseminated disease occurring mostly in immunodeficient hosts [2].

The diagnosis of NTM pulmonary disease is challenging in settings of high tuberculosis (TB) burden as NTM can also be detected by smear microscopy, and clinical and radiological features may mimic TB. However, unlike *Mycobacterium tuberculosis* (MTB), for which any positive culture is interpreted as TB disease, the major challenge with diagnosis and management of NTM is to differentiate environmental contamination, or colonization, from disease [3]. Criteria to help differentiate colonization from respiratory disease have been proposed by the American Thoracic Society (ATS) for adults, but these are not validated in children [2].

Human immunodeficiency virus (HIV)-infected adults and children are particularly at risk of NTM-related diseases, including respiratory and disseminated infections due to the *Mycobacterium avium* complex (MAC) [3]. NTM infections, MAC in particular, are globally occurring in adults and children with profound immunodeficiency and CD4 count usually <50 cells/μL. Data regarding epidemiology and clinical significance of NTM isolation in HIV-infected children from high-TB-burden and resource-limited countries remain scarce [1, 4, 5].

We assessed the rate of NTM isolation in HIV-infected children with a clinical suspicion of TB in a prospective cohort followed in 4 countries with high and very high TB burden in Southeast Asia and Africa, as well as NTM species distribution and factors associated with NTM isolation in this cohort.

METHODS

The French National Agency for Research on AIDS and Viral Hepatitis (ANRS) 12229 Pediatric Asian African Network for Tuberculosis and HIV Research (PAANTHER) 01 study (ClinicalTrials.gov identifier NCT01331811) was a TB diagnostic cohort study that enrolled HIV-infected children aged ≤13 years with suspected intrathoracic TB from 8 hospitals in Burkina Faso, Cambodia, Cameroon, and Vietnam, from April 2010 to May 2014. Relevant ethics committees, institutional review boards, and health authorities in each country approved the study. We included in this analysis children who had at least one interpretable culture result on any microbiological sample.

Inclusion procedures and study design were described elsewhere [6]. According to his/her age, each child had 5 or 6 systematic bacteriological samples, including self-expectorated sputum or gastric aspirate, nasopharyngeal aspirate, string test, and stools collected. All samples were tested with smear microscopy, Xpert MTB/RIF assay (Xpert, Cepheid, Sunnyvale, California, USA), mycobacterial culture on Lowenstein-Jensen medium, and automated liquid culture (Bactec MGIT, Becton

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Dickinson, Maryland, USA), except in Burkina Faso where culture was only performed on solid media. MTB complex was identified by biochemical method, immunochromatographic tests, or commercial multiplex line-probe assays. Speciation of NTM was performed using the GenoType Mycobacterium CM/AS line probe assay (Hain Lifescience, Nehren, Germany) in Cambodia and Cameroon, and using an in-house 16S ribosomal RNA sequencing in Vietnam. NTM treatment was initiated on a case-by-case basis based on lack of response to prior TB treatment, species of pathogen identified, and immune status. Children were followed for 6 months after enrollment.

We used χ^2 or Fisher exact tests, and Student or Wilcoxon rank-sum tests to compare characteristics between patients with and without NTM isolated. Factors associated with NTM isolation overall were evaluated using logistic regression models. We entered in multivariate models previously described associated characteristics and variables associated with the outcome in univariate analysis at a threshold of $P < .2$. We obtained final models using manual stepwise backward selection. We performed analyses using Stata software, version 13.1 (StataCorp, College Station, Texas, USA). All tests were two-sided and a P value $< .05$ was considered statistically significant.

RESULTS

Of 438 HIV-infected children enrolled in the study, 5 died and 5 withdrew before sample collection, and one had a positive culture without identification. Finally, we included 427 children in this analysis (Supplementary Figure 1), including 245 (59.2%) with severe immunodeficiency (Supplementary Table 1).

NTM were isolated in 46 (10.8%) children, including 5 (1.2%) with both NTM and MTB. Additionally, 50 (11.7%) children had bacteriologically confirmed MTB alone. NTM were isolated in 16.7% of Southeast Asian children and 2.7% of African children.

NTM were isolated from 102 specimens. Among these, species identification was successful for 85 (83.3%) specimens obtained from 41 of 46 (89.1%) children. Overall, 11 different NTM species were identified (Table 1). The most frequently isolated NTM species were MAC in 21 (45.7%) children, *Mycobacterium fortuitum*, *Mycobacterium simiae*, and *Mycobacterium scrofulaceum*. The same NTM species was identified in at least 2 separate specimens in 18 (39.1%) children. MAC was isolated almost exclusively in Southeast Asian children (20 of 21 children).

A similar proportion of children with and without NTM isolated initiated anti-TB treatment. Additionally, 12 (26.1%) patients with NTM isolated received specific NTM treatment, initiated at a median time of 6.0 (interquartile range, 2.2–9.3) weeks following inclusion. After 6 months of follow-up, 7 (15.2%) children with NTM had died and 39 were still followed up. Mortality did not significantly differ between children with NTM isolated (7/46 [15.2%]) and the overall cohort (50/381 [13.1%]; $P = .69$).

In multivariate analysis, factors independently associated with NTM isolation were Southeast Asian origin (odds ratio [OR], 7.0; 95% confidence interval [CI], 2.7–18.3), age 5–9 years (OR, 7.7; 95% CI, 1.7–33.9), and severe immunodeficiency (OR, 2.6; 95% CI, 1.2–5.4) (Supplementary Table 2). In Southeast Asian children, age 5–9 years (OR, 7.2; 95% CI, 1.5–35.0), absolute CD4 count < 50 cells/ μ L (OR, 2.6; 95% CI, 1.2–5.7), indeterminate Quantiferon (OR, 11.4; 95% CI, 1.2–108.2), and presence of a BCG scar (OR, 3.0; 95% CI, 1.2–7.2) were independently associated with NTM isolation.

Complementary clinical and biological data on NTM isolation, factors associated with NTM, and comparison of characteristics of children with NTM and those with confirmed TB and NTM positivity rate according to the specimen type are presented in Supplementary Tables 3–5.

DISCUSSION

In this multicenter study, NTM was isolated in 10.8% of HIV-infected children with suspected TB. Patients with NTM isolates were mostly Southeast Asian children with severe immunodeficiency. MAC was the most frequently identified NTM. To our knowledge, this is the first prospective study specifically investigating the frequency of NTM isolation and associated factors in HIV-infected children with suspected TB in both Africa and Southeast Asia.

The observed higher NTM isolation rate in Southeast Asian as compared to African children may be explained by differential virulence of NTM strains, variable exposure to NTM, and, possibly, genetic factors influencing host susceptibility [4]. High NTM isolation rates were previously reported in Vietnam, Thailand, and Cambodia in HIV-infected individuals > 7 years old assessed for pulmonary TB [5, 7]. Higher age associated with NTM isolation in our study is consistent with known bacteriological confirmation challenges in young children, and possibly longer environmental exposure in older children. The rate of NTM isolation in African settings is in line with most previous studies, which reported rates ranging from 2.1% up to 26.3% [8–10]. We identified 11 different NTM species, most of them previously described as pathogenic in HIV-infected patients, except for *Mycobacterium interjectum* and *Mycobacterium cosmeticum* [1, 9]. Among them, MAC, *Mycobacterium kansasii*, and *M. fortuitum* are well-known to be associated with respiratory disease in children [11]. In our study, the most frequently observed species were MAC, mostly in Southeast Asian sites, followed by *M. fortuitum*. Interestingly, *M. simiae*, previously associated with disseminated disease and severe outcome in HIV-infected individuals, was identified in 5 Asian children, confirming the circulation of this rarely observed species in this continent [5].

Isolating NTM in respiratory samples of symptomatic individuals is not sufficient to confirm NTM respiratory disease.

Table 1. Nontuberculous Species Identified

Mycobacterial Species	All Children With NTM Identified ^a (n = 46)		Severe Immunodeficiency (n = 33)		Absence of Severe Immunodeficiency (n = 13)	
	No.	(%)	No.	(%)	No.	(%)
1 NTM species identified	28	(60.9)	18	(39.1)	10	(21.7)
MAC	13	(28.3)	10	(21.7)	3	(6.5)
<i>M. fortuitum</i>	7	(15.2)	3	(6.5)	4	(8.7)
<i>M. simiae</i>	2	(4.3)	2	(4.3)		
<i>M. kansasii</i>	1	(2.2)	1	(2.2)	1	
<i>M. scrofulaceum</i>	2	(4.3)	1	(2.2)	1	(2.2)
<i>M. lentiflavum</i>	1	(2.2)	1	(2.2)		
<i>M. goodii</i>	1	(2.2)			1	(2.2)
<i>M. interjectum</i>	1	(2.2)			1	(2.2)
2 NTM species identified	11	(23.9)	9	(19.6)	2	(4.3)
MAC + nontypeable NTM ^b	3	(6.5)	3	(6.5)	2 ^c	
MAC + <i>M. simiae</i>	2	(4.3)	2	(4.3)		
MAC + <i>M. scrofulaceum</i>	1	(2.2)	1 ^d	(2.2)	1	
MAC + unidentified NTM ^e	1	(2.2)	1	(2.2)		
<i>M. fortuitum</i> + <i>M. interjectum</i>	1	(2.2)			1	(2.2)
<i>M. goodii</i> + nontypeable NTM	1	(2.2)			1	(2.2)
<i>M. scrofulaceum</i> + <i>M. flavescens</i>	1	(2.2)	1	(2.2)		
<i>M. simiae</i> + <i>M. cosmeticum</i>	1	(2.2)	1	(2.2)		
NTM species + MTB	5	(10.9)	4	(8.7)	1	(2.2)
MAC + nontypeable NTM + MTB	1	(2.2)	1	(2.2)		
Nontypeable NTM + MTB	2	(4.3)	2	(4.3)		
<i>M. fortuitum</i> + MTB	1	(2.2)	1	(2.2)	1	
Unidentified NTM + MTB	1	(2.2)			1	(2.2)
NTM species unidentified	2	(4.3)	2	(4.3)	0	(0.0)
Total	46	(100.0)	33	(69.6)	17	(28.3)

Abbreviations: MAC, *Mycobacterium avium* complex; MTB, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria.

^aAll NTM patients: including NTM + MTB patients.

^bNontypeable NTM: after molecular biology assays, NTM remained nontypeable.

^c≥2 isolates for MAC.

^d≥2 isolates for MAC and ≥2 isolates for *M. scrofulaceum*.

^eUnidentified NTM: identification not performed.

ATS criteria for NTM diagnosis and treatment decision in adults have not been validated for children, especially HIV-infected children with severe immunodeficiency, and there is currently no internationally accepted definition for NTM respiratory disease in children [2]. In children with severe immunodeficiency, NTM isolation was more frequent, MAC being isolated in more than half of cases, and frequently confirmed on another sample, supporting the hypothesis of NTM-related opportunistic infection in children from our study. However, unlike bacteriologically confirmed TB, which was associated with a 5-fold increase in mortality in ART-naive children in our study despite access to anti-TB treatment, NTM or MAC isolation was not associated with a higher risk of death in children with severe immunodeficiency [12]. In

children without severe immunodeficiency, NTM isolation was less frequent, MAC were rare, and only one child had the same species confirmed on a second sample, which is more in favor of environmental exposure and colonization.

Our study had limitations. First, blood cultures were not performed and we may thus have missed NTM disseminated diseases. Second, respiratory samples were not repeated along time, probably contributing to lower confirmation of NTM disease on a second sample. Third, children were managed heterogeneously across sites in the absence of specific recommendations on NTM treatment.

In this international cohort, the rate of NTM isolation in HIV-infected children with suspected TB was high, particularly in Southeast Asian settings. The high proportion of MAC isolated

on repeated samples in children with severe immunodeficiency raises the question of genuine respiratory NTM opportunistic infections, even if we did not show a difference in mortality between children with NTM isolated and other children. Further research should be conducted in settings of high TB and NTM burden to assess clinical significance of NTM isolation in HIV-infected children and develop guidelines on NTM pulmonary disease diagnosis and treatment for this population.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Appendix 2. Article 2: Evaluation of Loopamp Assay for the Diagnosis of Pulmonary Tuberculosis in Cambodia

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Research Article

Evaluation of Loopamp Assay for the Diagnosis of Pulmonary Tuberculosis in Cambodia

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The Loopamp™ MTBC kit (TB-LAMP) is recommended by WHO for *Mycobacterium tuberculosis* complex detection in low-income countries with a still low drug-resistant tuberculosis (TB) rate. This study is aimed at testing its feasibility in Cambodia on sputa collected from presumptive tuberculosis patients. 499 samples were tested at a smear microscopy center and 200 at a central-level mycobacteriology laboratory. Using mycobacterial cultures as reference, TB-LAMP results were compared with those of LED fluorescent microscopy (LED-FM) and Xpert® MTB/RIF. At the microscopy center, TB-LAMP sensitivity was higher than that of LED-FM (81.5% [95% CI, 74.5-87.6] versus 69.4% [95% CI, 62.2-76.6]), but lower than that of the Xpert assay (95.5% [95% CI 92.3-98.8]). At the central-level laboratory, TB-LAMP sensitivity (92.8% [95% CI, 87.6-97.9]) was comparable to that of Xpert (90.7% [95% CI, 85.0-96.5]) using stored sample. No significant difference in terms of specificity between TB-LAMP and Xpert assays was observed in both study sites. In conclusion, our data demonstrate that TB-LAMP could be implemented at microscopy centers in Cambodia to detect TB patients. In addition, TB-LAMP can be a better choice to replace smear microscopy for rapid TB diagnosis of new presumptive TB patients, in settings with relative low prevalence of drug-resistant TB and difficulties to implement Xpert assay.

1. Introduction

Despite the availability of highly efficacious treatments for decades, tuberculosis remains a major public health issue worldwide. In 2017, WHO reported that 10.0 million people developed TB, mainly in developing countries, and that approximately 3.6 million (36%) were not diagnosed or notified to the national authorities [1]. The most widely used method for TB diagnosis is sputum smear microscopy. This test is simple, rapid, and inexpensive but has a relatively low sensitivity, particularly in patients with extrapulmonary TB, children, and in people with HIV [2]. Hence, the development of new diagnostic tools suitable for use in

low- and middle-income countries is one of the top priorities for TB control.

Among the technologies developed and implemented over the past decade, nucleic acid amplification technologies hold the greatest promise of substantial gains in turnaround-time (compared with culture) and in sensitivity and specificity (compared with smear microscopy) [2–4]. Loop-mediated isothermal amplification (LAMP) has several advantages: no need of sophisticated instrumentation, DNA amplification of partially processed or unprocessed samples, and visual readout of the amplified products [5]. Therefore, in 2016, WHO recommended using the Loopamp™ MTBC assay (TB-LAMP) for the detection of *Mycobacterium*

tuberculosis complex (MTBC) in sputum as a replacement of smear microscopy and as a follow-on test after negative smear microscopy [6]. Despite these conditional recommendations, further data are required to establish TB-LAMP usefulness in the TB diagnostic pathway in developing countries. Therefore, we assessed the feasibility and usefulness of the commercial TB-LAMP assay for the early detection and management of patients with TB at smear microscopy centers in Cambodia. Specifically, we wanted to determine the sensitivity and specificity of TB-LAMP (compared with culture as the gold standard) for the analysis of sputum specimens collected from patients with suspected pulmonary TB and tested at a smear microscopy center. We also compared TB-LAMP sensitivity and specificity with those of the Xpert® MTB/RIF (Xpert) assay and smear microscopy.

2. Materials and Methods

2.1. Study Design. We conducted two cross-sectional studies between 2013 and 2015: one at the central-level mycobacteriology laboratory using a panel of stored specimens and one in a smear microscopy center using sputum specimens prospectively collected from people undergoing TB testing in the framework of the National TB Control Program (NTP). Both study sites are located in Phnom Penh, the capital city of Cambodia. Before starting the study, the operators of TB-LAMP assay received a one-week training course administered by Eiken staffs and passed a proficiency test.

The study was approved by the National Ethics Committee for Health Research, Phnom Penh, Cambodia. Patients' informed consent was obtained.

2.2. Smear Microscopy Center Study. Patient enrolment was conducted at the Out-Patient Department (OPD) of TB Referral Hospital of the National Center for TB and Leprosy Control (CENAT), Phnom Penh, Cambodia (see Figure 1). Individuals aged 18 years and over with symptoms suggestive of pulmonary TB, as defined by the NTP (typically including a cough that persists for longer than two weeks and one other TB symptom, such as fever, night sweats, or recent weight loss), were eligible for the study. Patients who had been receiving any antituberculosis treatment 30 days prior to the enrolment date were excluded. Eligible patients were informed about the study by a clinician, and those who volunteered to participate were asked to sign a written informed consent and to complete a questionnaire.

Sputum samples were collected according to the NTP protocol for TB diagnosis. Each participant provided three sputum samples: a spot sputum at the time of recruitment in OPD of CENAT, a morning sputum the following day at home, and at least 1 hour later, a last spot sputum specimen when returning to OPD. It was explained to all participants in local language how to produce a good-quality sputum specimen of at least 2 ml. The spot specimen was collected under supervision. For the morning specimen, the participants received sterile, leak proof, screw-cap, and labeled sputum containers and they were asked to collect the sputum at home in the morning and to bring the specimen to CENAT the next day.

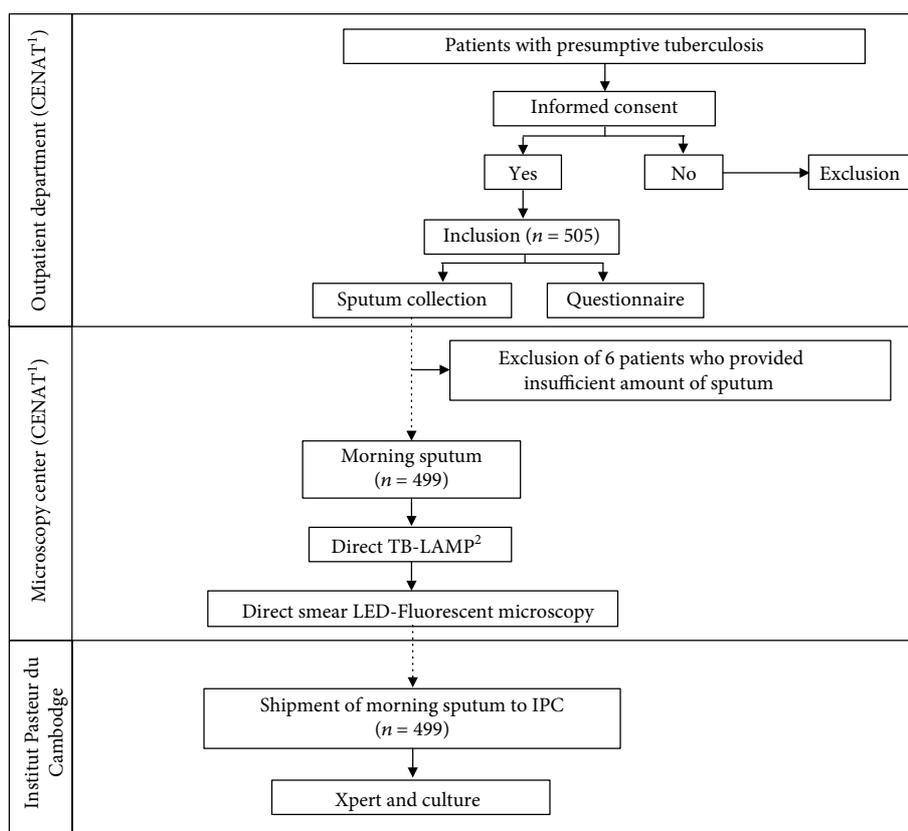
Following NTP protocol for TB diagnosis, all three sputum specimens were sent to CENAT Microscopy Center for light-emitting diode fluorescence microscopy (LED-FM). In the framework of our study, the morning sputum specimens were tested using the TB-LAMP assay by applying the same local biosafety conditions as for the preparation of microscopy. The remaining morning sputum samples were then stored at 2-8°C until shipment twice per week to the IPC Mycobacteriology Laboratory for Xpert MTB/RIF and mycobacterial culture.

2.2.1. Central-Level Laboratory Evaluation. A panel of sputum samples with known smear and mycobacterial culture results was selected from the biobank of the Institut Pasteur du Cambodge (IPC) and used for the retrospective laboratory evaluation of the diagnostic performance of TB-LAMP at IPC Mycobacteriology Laboratory, which is categorized as a central-level laboratory. Two hundred sputum specimens (including 83 smear- and culture-positive, 17 smear-negative and culture-positive, and 100 smear- and culture-negative samples) that were collected between 2010 and 2012 and had been frozen at -80°C for 5 months to 2 years were included in this evaluation. Approximately 1 ml of each specimen was kept in a cryotube without the addition of chemical preservatives and without decontamination procedure prior to storage. On the specimens, TB-LAMP and Xpert MTB/RIF were performed by applying the same local biosafety conditions as for the preparation of specimens for mycobacterial culture.

2.3. Laboratory Procedures

2.3.1. Loopamp™ MTBC Detection Kit, Eiken, Tokyo (TB-LAMP). The TB-LAMP test was performed following the manufacturer's recommendations. DNA was extracted from the sputum samples using the Loopamp™ PURE DNA Extraction Kit. A 60 µL aliquot of purulent sputum portion was mixed with the DNA extraction solution in a heating tube and incubated at 90°C in a LF-160 incubator for 5 minutes to allow bacterial cell lysis and DNA release in the solution. The DNA solution was purified through porous absorbent powder in an adsorbent tube. A 30 µL aliquot of purified DNA was dispensed in the LAMP reaction tube containing the lyophilised LAMP reagents (*Bst* DNA polymerase, dNTPs, calcein, and MTBC-specific primers targeting DNA gyrase subunit B and insertion sequence 6110). High specificity was reported by Yuki regarding the combination of primers included in the kit [7]. The dried LAMP reagents (stored in the cap of the reaction tube) were reconstituted by mixing by inversion with the DNA solution. The reaction tube was incubated at 67.0°C for 40 minutes to allow DNA amplification by the LAMP reaction. Amplified products were visually detected on the basis of their fluorescence under ultraviolet light.

2.3.2. Light-Emitting Diode Fluorescence Microscopy (LED-FM). The sputa were examined microscopically for the presence of acid-fast bacilli (AFB) by fluorescent microscopy. Direct smears were prepared for staining by Auramine O, counterstained with potassium permanganate. The slide



¹National Center for Tuberculosis and Leprosy Control

²Loop-mediated isothermal amplification

FIGURE 1: Study flowchart in smear microscopy center.

was examined on the same day of staining at 400x magnification using an LED-based fluorescent microscope to detect yellow fluorescent rods on a dark background. The smear result was provided using a semiquantitative scale [8].

2.3.3. Culture and Species Identification. At the central laboratory, 2 mL of all sputum samples underwent NALC-NaOH decontamination (1% final NaOH concentration), followed by neutralization with phosphate buffer (PB) and centrifugation [9]. Pellets were resuspended in 0.7 mL of PB and inoculated on a Löwenstein-Jensen (LJ) slant and in MGIT liquid culture tubes (Bactec MGIT; BD Microbiology Systems, Cockeysville, MD). The inoculated LJ slants were incubated at 37°C and monitored weekly for 8 weeks. Isolates from positive cultures were examined by smear microscopy using Kinyoun staining and the AccuProbe MTBC culture identification test (Gen-Probe, San Diego, CA) to identify MTBC.

2.3.4. Xpert MTB/RIF (Xpert). The Xpert assay was performed according to the manufacturer’s instructions using 1 mL of untreated sputum sample or 0.5 mL of sputum pellets after a NALC-NaOH digestion method, as described above.

2.3.5. Data Analysis. Using a combination of solid and liquid mycobacterial cultures as reference standard, patients were classified as being “TB-positive” when they had at least one sputum culture-positive for MTBC on solid or liquid MGIT media and as “TB-negative” in the case of negative culture or culture-positive for mycobacteria other than tuberculosis (MOTT). To assess the performance of TB-LAMP, LED-FM, and Xpert, their sensitivity was calculated as the proportion of positive tests among “TB” patients and their specificity as the proportion of negative tests among “non-TB” patients. Each value was calculated with the 95% confidence interval (95% CI). TB-LAMP and Xpert sensitivity values were also stratified according to the smear results.

TB-LAMP performance at the smear microscopy center was compared with that of the LED-FM and Xpert assays by measuring the difference in sensitivity and specificity between the tests, using a method appropriate for matched (within-subject) designs [10]. The TB-LAMP assay was considered noninferior to Xpert if the upper limit of the 95% CI of the difference for sensitivity and specificity was <5%. TB-LAMP was considered superior to smear if the lower limit of the 95% CI of the difference for sensitivity and specificity was >0. Statistical analyses were done using SAS version 9.4 (SAS Institute, Cary, NC).

3. Results and Discussion

3.1. Smear Microscopy Center Study. Between February and June 2014, 505 eligible patients with suspected TB were enrolled in the study. As six patients were excluded due to insufficient sputum volume, the study population included 499 patients (among whom 280 men). Their median age was 52 years (range 19 to 92). HIV status was unknown for most patients (471/499, 94.4%). In the 28 patients with known HIV results, three (10.7%) were HIV positive. The clinical and demographic characteristics of the study population are shown in Table 1.

Among the sputum samples of the 499 patients, 180 (36.1%) had positive mycobacterial culture on solid and/or liquid media. Most positive isolates were identified as MTBC (155/180), 23 as MOTT, and two as mixed cultures of MTBC and MOTT. Finally, 157 samples (31.5%) were classified as “TB-positive” and 342 as “TB-negative”.

The LED-FM analysis showed that 115 of the 499 samples (23%) were positive for acid-fast bacilli (AFB). Of 115 smear-positive samples, 6 (5.2%) were MOTT culture-positive. MTBC DNA was detected in 137 (27.5%) and 161 (32.3%) samples by the TB-LAMP and Xpert tests, respectively. The overall sensitivity and specificity of the LED-FM, TB-LAMP, and Xpert were calculated with 95% CI and are shown in Table 2. Using a combination of liquid and solid cultures (MGIT and LJ media) as the reference standard, the sensitivity values of TB-LAMP, Xpert, and LED-FM were 81.5% (74.5-87.6), 95.5% (92.3-98.8), and 69.4% (62.2-76.6), respectively. The sensitivity of TB-LAMP was 94.5% (90.2-98.8) in smear- and culture-positive and 52.1% (38.0-66.2) in smear-negative and culture-positive TB patients. The specificity values of TB-LAMP, Xpert, and LED-FM were 97.4% (95.7-99.1), 96.8% (94.9-98.7), and 98.2% (96.8-99.6), respectively.

Of 499 Xpert testing, 357 (71.5%) were performed on untreated sputum samples and 142 (28.5%) were performed on sputum pellets after the NALC-NaOH digestion method. The comparison of the sensitivity and specificity of Xpert performed on untreated sputum samples (96.6% [91.6-99.1] and 96.6% [93.5-98.5]) and Xpert performed on concentrated sputum (92.1% [78.6-98.3]) and 97.1% [91.8-99.4]) did not highlight any significant difference between methods.

During the study, 67 series (runs) of TB-LAMP (mean number of samples tested per series: 7.6) were performed by only one operator. For each series, photos of reaction tubes exposed to ultraviolet light were captured (Figure 2). All the TB-LAMP results were read by two different individuals, first by the TB-LAMP operator and later by the laboratory supervisor using photos captured by the operator. For all the results, there was no discrepancy between the two readers. One series, which included eight samples, was needed to be repeated because of one invalid result for the positive control. All 8 samples had clear positive or negative results after repetition of TB-LAMP assay on the same sputum. No instance of contamination of the negative control or indetermination of fluorescent read-out was observed.

TABLE 1: Demographic and clinical features of the study population and culture-confirmed tuberculosis cases in smear microscopy center study.

Characteristics	Study population N (%)	Culture-confirmed TB cases N (%)
Total	499	157
Age (year)	18-24	36 (7.2)
	25-34	83 (16.6)
	35-44	67 (13.4)
	45-54	94 (18.8)
	55-64	98 (19.6)
	≥ 65	121 (24.2)
Sex	Total	499
	Men	280 (56.1)
	Women	219 (43.9)
Symptoms	Total	499
	Fever	497 (99.6)
	Cough	487 (97.6)
	Chest discomfort	463 (92.8)
	Weight loss	453 (90.8)
	Breathlessness	98 (19.6)
Hemoptysis	63 (12.6)	
Abnormal chest X-ray	421/426 (98.8)	141/141 (100)
Previous treatment for TB disease	129/482 (26.8)	18/154 (11.7)
TB contact	74/496 (14.8)	13/156 (8.3)

3.2. Comparison of TB-LAMP, Xpert, and LED-FM at the Smear Microscopy Center. The difference in sensitivity and specificity of TB-LAMP with that of the LED-FM and Xpert assays was calculated, and the results are shown in Table 3. The upper limit of the 95% CI for the difference in sensitivity between TB-LAMP and Xpert was 19.7%, which is above the noninferiority threshold (5%). Hence, compared with the Xpert assay sensitivity, TB-LAMP was inferior. The lower limit of the 95% CI for the difference in sensitivity between TB-LAMP and LED-FM was 5.4%, which is above the superiority threshold (0%). Hence, TB-LAMP was superior to LED-FM concerning sensitivity.

The upper limit of the 95% CI for the difference in specificity between TB-LAMP and Xpert was 0.8%, which is below the noninferiority threshold (5%). Hence, TB-LAMP was noninferior to Xpert concerning specificity. The lower limit of the 95% CI for the difference in specificity between TB-LAMP and LED-FM was -1.2%, which is below the superiority threshold (0%). Hence, TB-LAMP was not superior to LED-FM with respect to specificity.

3.3. Central-Level Laboratory Evaluation. In this evaluation, error results with Xpert were obtained for four samples

TABLE 2: Sensitivity and specificity of LED-fluorescent microscopy, TB-LAMP, and Xpert MTB/RIF.

Test	Culture result		Sensitivity among culture-positive MTB			Specificity % (95% CI)
	MTB-positive (n = 157) positive/negative	MTB-negative (n = 342) positive/negative	Smear-positive % (95% CI)	Smear-negative % (95% CI)	Overall % (95% CI)	
Smear microscopy center						
Microscopy	109/48	6/336	—	—	69.4 (62.2-76.6)	98.2 (96.8-99.6)
TB-LAMP	128/29	9/333	94.5 (90.2-98.8)	52.1 (38.0-66.2)	81.5 (74.5-87.6)	97.4 (95.7-99.1)
Xpert	150/7	11/331	99.1 (93.6-99.9)	87.5 (74.2-94.4)	95.5 (92.3-98.8)	96.8 (94.9-98.7)
Central laboratory						
TB-LAMP	90/7	4/95	100	56.3 (29.8-79.6)	92.8 (87.6-97.9)	96.0 (92.1-99.8)
Xpert	88/9	3/96	100	43.8 (20.4-70.2)	90.7 (85.0-96.5)	97.0 (93.6-1.0)

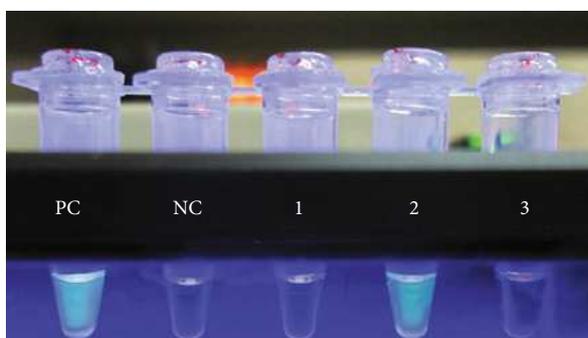


FIGURE 2: Visual detection of LAMP products under UV light. The combination of turbidity and bright green fluorescence observed in tube 2 and positive control (PC) indicated positive reactions, while a blue color without turbidity indicated a negative reaction in tubes 1, 3, and negative control (NC).

TABLE 3: Matched comparison between TB-LAMP performed at the smear microscopy center and the Xpert and LED-fluorescent microscopy.

Comparison	Difference (%)	Lower 95% confidence limit (%)	Upper 95% confidence limit (%)
Sensitivity			
TB-LAMP versus Xpert	14.0	8.3	19.7
TB-LAMP versus microscopy	12.1	5.4	18.8
Specificity			
TB-LAMP versus Xpert	-0.6	-2.0	0.8
TB-LAMP versus microscopy	0.9	-1.2	2.9

which were excluded from the analysis, giving an indeterminate rate of 2%. Hence, only data for 196 sputum specimens (including 81 smear- and culture-positive, 16 smear-negative and culture-positive, and 99 smear- and culture-negative

samples) were used to estimate TB-LAMP and Xpert sensitivity and specificity with 95% CI at the central laboratory. The results are presented in Table 2. The comparison of the sensitivity and specificity of TB-LAMP (92.8% [87.6-97.9] and 96.0% [92.1-99.8]) and Xpert (90.7% [85.0-96.5] and 97.0% [93.6-1.0]) in this setting did not highlight any significant difference between tests.

4. Discussion

Our findings indicate that the sensitivity of the Loopamp™ assay for TB diagnosis (TB-LAMP) is superior to that of sputum smear microscopy using the LED-fluorescent method, but inferior to that of the Xpert MTB/RIF test at the smear microscopy center. Conversely, its specificity is not superior to that of smear microscopy and is noninferior to that of the Xpert assay. However, no significant difference in sensitivity and specificity was found for TB-LAMP compared to Xpert in central-level laboratory evaluation.

In microscopy center study, TB-LAMP overall sensitivity (81.5% [95% CI, 74.5-87.6]) and specificity (97.4% [95% CI, 95.7-99.1]) were similar to the TB-LAMP pooled sensitivity (80.3% [95% CI, 70.3-87.5]) and specificity (97.7% [95% CI, 96.1-98.7]) reported by WHO [6]. TB-LAMP inferiority concerning sensitivity compared with Xpert is probably explained by the lower volume of sputum used in the test (60 μL for TB-LAMP versus 1000 μL for Xpert) [11]. The superiority of TB-LAMP compared with smear microscopy is highlighted in our study by the finding that approximately 50% of smear-negative samples were TB-LAMP-positive, as previously observed [3, 12–15]. In addition to the sensitivity, TB-LAMP could distinguish fairly well between MOTT and MTB; hence, it avoids inappropriate anti-TB treatment for patient with NTM infection, which is observed around 10.8% and 5.6% among patients with presumptive TB and all smear-positive cases, respectively [16]. Our results support the WHO recommendation for using the TB-LAMP test instead of smear microscopy for rapid detection of new TB patients or for follow-on testing. The inferior sensitivity

(with similar specificity) of TB-LAMP compared with Xpert suggests that the latter test should be preferred. However, considering the ease of use and the low cost compared to Xpert, the TB-LAMP might be a better choice for rapid TB diagnosis of new presumptive TB patients in low- and middle-income countries (LMICs) with relative low prevalence of drug-resistant TB.

The sensitivity of TB-LAMP was reported ranging from 48% (29%-68%) in Vietnam to 100% (94%-100%) in India [6]. Among patients with bacteria-positive pulmonary TB, our data showed high sensitivity for TB-LAMP (81.5% [95% CI, 74.5-87.6]). In two previous meta-analysis, the positivity rates of Xpert and smear microscopy were relatively high as well since the values varied from 58% to 100% and from 32% to 83%, respectively [17, 18]. Several aspects can explain these results. First, as LED-FM was performed at the CENAT microscopy center which is the reference microscopy center, the high sensitivity of smear microscopy was expected. Second, a higher positivity rate has been previously reported for the early morning sputum compared to spot sputum samples using smear microscopy and Xpert [19, 20]. Third, the selected patients had a high probability to be at an advanced state of TB since more than 80% presented a combination of symptoms such as fever, cough, chest discomfort, and weight loss and 90% had abnormal chest X-ray [21, 22]. Last, the low HIV prevalence in Cambodia can explain the high sensitivity of microscopy as well as of TB-LAMP and Xpert compared to other countries with a high HIV prevalence [1]. Besides, we cannot exclude the low performance of culture examination due to the complexity of the method [2]. However, to monitor the performance of the culture procedures, the quality indicators such as the contamination rate, artificial sputum to control cross-contamination; the systematic internal quality control; and the proficiency testing were implemented. The results of these indicators and tests were within the range defined by the European Centre for Disease Prevention and Control (ECDC). The cold chain was ensured during the transportation of the specimens until they reached culture laboratory [23].

Our result from central-level laboratory evaluation showed that TB-LAMP sensitivity and specificity were equal to those of Xpert assay. The similar performance of TB-LAMP and Xpert assays was also reported by Pham et al. in a multicenter study conducted in reference laboratories in Peru, Brazil, South Africa, and Vietnam [24]. The high sensitivity of TB-LAMP comparable to Xpert in our central-level laboratory evaluation was obtained using frozen sputum samples. The freeze and thaw processes of the samples may improve the sensitivity of TB-LAMP assay. Even if the TB-LAMP operators were well trained to take the most purulent portion of sputum for the test, due to the very low volume of sputum required (60 μ L), the performance of direct TB-LAMP might be affected by the viscosity of the sputum during sample splitting. It is worth noting that compared to fresh sputa, the thawed frozen sputa were relatively less viscous and easier to homogenize by a simple vortexing. Our results showed that the TB detection rate of the direct TB-LAMP was dependent on sputum quality and viscosity, as mentioned by Nguyen et al. [11]. It is noteworthy that the perfor-

mance of TB-LAMP assay can reach those of Xpert when the quality and homogeneity of the sputum sample are ensured. These findings might also suggest a simple and cost-effective alternative method for TB diagnosis or for retrospective confirmation using frozen sputum sampled when the transport or processing of fresh samples is delayed, such as samples collected at remote settings in low-resource countries or used for epidemiological studies.

One limitation of the study is that follow-up for participants with TB-LAMP-positive and/or Xpert MTB/RIF-positive and bacteriologically negative results was not carried out. Mycobacterial culture is the current best available method for bacteriologically confirmed active TB patients; however, culture is not 100% sensitive due to the harmful effect of the decontamination method on mycobacteria [2]. The use of an imperfect reference standard method will have a more deleterious effect on assay with a higher specificity [24].

Another limitation is that the smear microscopy center where the study was carried out is the reference microscopy center in the country. In this reference microscopy center, the routine sputum smears are prepared in a biosafety cabinet because of the high number of sputum samples per day compared to other microscopy centers in the country. Therefore, the DNA extraction of samples for TB-LAMP assay was also performed in a biosafety cabinet. This may explain the relative low number of false positive results and the low rate of contaminations for the smear microscopy and TB-LAMP assays. However, the technician who performed TB-LAMP assay at the microscopy center has no experience on a molecular method or other laboratory method than microscopy and only received one-week training to perform the test. Further studies in microscopy centers at peripheral levels are recommended to confirm the expected benefits.

In conclusion, our findings suggest that the TB-LAMP assay can be implemented in smear microscopy centers in Cambodia and can rapidly and accurately detect patients with TB using sputum samples, with a sensitivity of 81.5% and a specificity of 97.4%. In addition, TB-LAMP sensitivity is superior to that of LED-fluorescent microscopy and requires similar infrastructure as smear microscopy. Therefore, TB-LAMP assay can be a better choice to replace smear microscopy for rapid TB diagnosis of new presumptive TB patients, in settings with a relatively low prevalence of drug-resistant TB and difficulties to implement Xpert assay.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix 3: Zoonosis in reverse: *Mycobacterium tuberculosis* in a population of captive sun bears (Poster number: PS -17-684-01)

Zoonosis in reverse: *Mycobacterium tuberculosis* in a population of captive sun bears

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Introduction

The illegal wildlife trade in Cambodia sees bears kept in prolonged close contact with humans.

Between 2009 and 2017 at one wildlife rescue centre 19 sun bears (*Helarctos malayanus*) were diagnosed with tuberculosis (TB) caused by *M. tuberculosis*.

One staff member developed TB in 2011 and was treated and made a full recovery.

Drug susceptibility testing (DST) patterns suggested the involvement of at least two types of *M. tuberculosis* strain; drug sensitive and drug resistant (resistance to isoniazid (INH) and streptomycin (SM)).

Genotyping was undertaken and combined with contact information to investigate possible sources and transmission patterns.

Methods

43 - spacer spoligotyping and 24 - loci MURU-VNTR methods were used to genetically characterise cultured isolates of *M. tuberculosis* from each case.

Records of sun bear arrivals, social group compositions, and translocations within the rescue centre were collated for the previous 21 years to derive contact data.

The information was combined with genotyping data to model possible transmission pathways.

Results

Spoligotyping revealed two patterns, consistent with the two DST patterns and representing Beijing and East African-Indian (EAI) lineages.

MIRU-VNTR typing distinguished three patterns; one containing all the Beijing spoligotype isolates, one containing 11 out of 12 EAI isolates, and one with only one locus different to the EAI group.



Acknowledgements

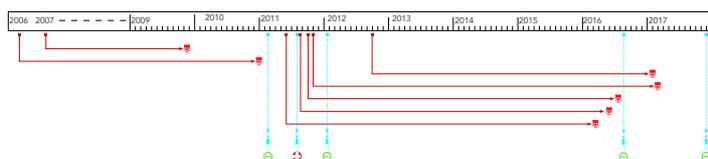
- Veterinary staff and colleagues, Cambodian Wildlife Hospital
- Dr Jonathan Cracknell, Cracknell Wildlife and Veterinary Services
- Staff at the Mycobacteriology Laboratory, Institut Pasteur du Cambodge

Results cont.

CASE	DIAGNOSIS DATE	RMP	SM	EMB	SPOLOGOTYPE OCTAL	SPOLOGOTYPE	MIRU-VNTR CODE	GENOTYPE CLUSTER
sun bear_1	Dec 2009	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_2	Jan 2011	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
human	Aug 2011	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_6	Apr 2016	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_7	Jun 2016	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_9	Aug 2016	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_12	Feb 2017	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_14	Mar 2017	S	R	S	00000000003771	Beijing (SIT1)	244233262544425183353823	cluster_1
sun bear_3	Feb 2012	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_4	Mar 2014	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_5	Jul 2015	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_8	Aug 2016	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_10	Nov 2016	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_11	Nov 2016	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_13	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2_1
sun bear_15	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_16	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_17	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_18	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2
sun bear_19	Mar 2017	S	S	S	77777777413731	EAI1-SOM (SIT48)	224734152263245223354613	cluster_2

A clearly apparent index case and bear-to-bear contact accounted for entry and spread within the EAI1 spoligotype cluster (not shown).

The human case appeared to bridge a bear-to-bear transmission chain for the Beijing spoligotype cluster.



Conclusions

Genotyping revealed two lineages and confirmed involvement of the human case.

While the original source is likely to be human, these data combined with contact information suggest transmission occurred from sun bear to human and back to sun bear within the rescue centre.

Full genome sequencing is needed to clarify this apparent zoonosis and reverse zoonosis.

This work illustrates TB susceptibility in a wildlife species placed in contact with humans, with captivity creating conditions necessary for pathogen exposure and transmission.

There are human health implications when drug resistant zoonotic disease emerges in a previously unreported species.

There are also conservation impacts when the species, such as the sun bear, is vulnerable to extinction.



Appendix 4: Demographic information, drug resistance, mutation patterns, spoligotype and MIRU-VNTR patterns of the clustered isolates

Isolate ID	Sex	Year of isolation	Age	Residence	Address code	FLD Resistance	SLDs Resistance	Phenotypes	Mutation patterns	SIT SpolDB4	Family	24-loci MIRU-VNTR	Cluster
DRI_076	M	2012	24	Provincial	KH-03	S	-	Mono-R	WT	459	EAI1-SOM	223634252273245223354613	C1
DRI_155	M	2017	65	Capital	KH-12	H	-	Mono-R	katG315Thr	459	EAI1-SOM	223634252273245223354613	C1
DRC_198	F	2017	65	Provincial	KH-08	S	-	Mono-R	WT	48	EAI1-SOM	224734252263245223354613	C3
DRC_232	M	2012	62	Provincial	KH-01	HS	-	Poly-R	katG315Thr & rrs878A	48	EAI1-SOM	224734252263245223354613	C3
DRC_273	M	2012	43	Provincial	KH-02	H	-	Mono-R	WT	48	EAI1-SOM	224734252263245223354613	C3
DRC_137	F	2013	76	Capital	KH-12	HRS	Ofx	Pre-XDR	katG315Thr & rpoB531Leu & rps-L43Arg & pncA-11G & gyrA94Gly & gyrB644Asp	1	Beijing	234233342144425183343733	C4
DRC_138	M	2013	27	Provincial	KH-09	HRS	Sensitive	MDR	katG315Thr & rpoB516Val & rps-L43Arg & gyrA87Gln & gyrA94Gly & gyrB644Asp	1	Beijing	234233342144425183343733	C4
DRI_070	F	2012	23	Provincial	KH-03	HS	Sensitive	Poly-R	katG315Thr & rpsL43Arg & gyr-B644Asp	1	Beijing	234233342144425183343733	C4
DRC_010	M	2015	40	Provincial	KH-07	HRS	Am, Km	Pre-XDR	katG315Thr & rpoB526Arg & rps-L43Arg & rrs1401G	1	Beijing	244213352544426171352823	C5
DRI_061	M	2013	16	Provincial	KH-03	H	-	Mono-R	WT	1	Beijing	244213352544426171352823	C5
DRC_007	M	2015	52	Capital	KH-12	HRS	-	MDR	katG315Thr & rpsL43Arg	1	Beijing	244233252644425173353823	C6
DRC_035	M	2015	54	Provincial	KH-14	HS	-	Poly-R	katG315Thr & rpsL43Arg	1	Beijing	244233252644425173353823	C6
DRC_221	M	2012	48	Capital	KH-12	HRS	-	MDR	katG315Thr & rpoB516Val & rps-L43Arg	1	Beijing	244233252644425173353823	C6
DRC_120	F	2014	32	Provincial	KH-07	E	-	Mono-R	WT	1	Beijing	244233342744425173253743	C7
DRI_153	NA	2016	NA	Capital	KH-12	H	-	Mono-R	katG315Thr	1	Beijing	244233342744425173253743	C7
DRI_015	M	2014	26	Provincial	KH-03	HRSE	-	MDR	katG315Thr & rpoB531Leu & rpsL43Arg & embB354Ala & pncA-138Gly	1	Beijing	244233352644425165353823	C8

DRI_024	M	2013	15	Provincial	KH-03	HRSE	-	MDR	katG315Thr & rpoB531Leu & rpsL43Arg & embB354Ala & pncA-138Gly	1	Beijing	244233352644425165353823	C8
DRI_040	M	2013	27	Provincial	KH-03	HRSE	-	MDR	katG315Thr & rpoB531Leu & rpsL43Arg & embB354Ala & pncA-138Gly	1	Beijing	244233352644425165353823	C8
DRC_057	M	2014	52	Capital	KH-12	HE	-	Poly-R	WT	459	EAI1-SOM	223634252273245223344613	C10
DRC_064	M	2014	17	Provincial	KH-08	R	-	Mono-R	WT	459	EAI1-SOM	223634252273245223344613	C10
DR_002	F	2016	23	Capital	KH-12	HRS	Ofx, Km	XDR	inhA-15T & inhA94Ala & rpoB511Pro & rpoB526Gln & rpsL88Arg & pncA-171Glu & gyrA90Val	1	Beijing	224232352544425173353823	C11
DR_007	M	2012	67	Provincial	KH-03	HRS	Ofx	Pre-XDR	inhA-15T & inhA94Ala & rpoB511Pro & rpoB526Gln & rpsL88Arg & pncA-171Glu & gyrA90Val	1	Beijing	224232352544425173353823	C11
DRC_250	M	2012	36	Capital	KH-12	H	-	Mono-R	katG315Thr	48	EAI1-SOM	224534362263245223344613	C13
DRI_071	F	2012	68	Capital	KH-12	H	-	Mono-R	katG315Thr	48	EAI1-SOM	224534362263245223344613	C13
DRI_073	F	2012	44	Provincial	KH-03	HR	-	MDR	katG315Thr & rpoB531Leu & embB306Ile & pncA48 del T	236	EAI5	224734252273245223347513	C15
DRI_074	M	2012	44	Provincial	KH-03	HR	-	MDR	katG315Thr & rpoB531Leu & pncA48 del T	236	EAI5	224734252273245223347513	C15
DRC_005	F	2015	26	Capital	KH-12	HR	-	MDR	katG277Ala & rpoB526Tyr	621	Beijing	234233342544425173353823	C16
DRC_040	M	2015	41	Capital	KH-12	R	Ofx	Mono-R_FQ	rpoB526Tyr & gyrA94Gly	621	Beijing	234233342544425173353823	C16
DRC_028	F	2015	49	Provincial	KH-14	S	-	Mono-R	WT	1	Beijing	244232352544425173353823	C17
DRI_190	M	2017	71	Provincial	KH-14	HRS	-	MDR	rpoB531Leu & embB306Leu	1	Beijing	244232352544425173353823	C17
DRC_142	F	2013	71	Provincial	KH-18	HRS	-	MDR	katG315Gly & rpoB531Leu & rps-L43Arg & embB306Val	1	Beijing	244233242744425153333823	C18
DRC_143	M	2013	31	Provincial	KH-08	HRS	-	MDR	katG315Gly & rpoB531Leu & rps-L43Arg & embB306Val	1	Beijing	244233242744425153333823	C18
DRC_070	M	2014	29	Capital	KH-12	HRSE	-	MDR	katG315Thr & rpoB531Trp & rps-L43Arg & embB306Val	1	Beijing	244233352544425173343823	C20
DRC_150	M	2013	50	Provincial	KH-14	S	Ofx	Mono-R_FQ	rpsL43Arg & gyrA94Gly	1	Beijing	244233352544425173343823	C20
DRC_063	M	2014	42	Capital	KH-12	HRSE	-	MDR	katG315Thr & rpoB516Val & rps-L43Arg & embB306Val	1	Beijing	244233352644425163343823	C22
DRC_088	M	2014	17	Capital	KH-12	H	-	Mono-R	inhA-15T	1	Beijing	244233352644425163343823	C22

DRC_037	M	2015	45	Capital	KH-12	HRS	-	MDR	katG315Thr & rpoB516Val & rps-L43Arg	1	Beijing	244233352644425163353823	C23
DRI_180	F	2017	29	Provincial	KH-05	HRSE	-	MDR	katG315Thr & rpoB531Leu & rpsL43Arg & embB354Ala & pncA-138Gly	1	Beijing	244233352644425163353823	C23
DRC_044	F	2015	31	Provincial	KH-21	R	-	Mono-R	rpoB531Leu	1	Beijing	244233352644425173353823	C24
DRC_211	M	2012	47	Capital	KH-12	HRSE	-	MDR	katG315Thr & rpoB531Leu & rps-L43Arg	1	Beijing	244233352644425173353823	C24
DRI_185	M	2017	30	Provincial	KH-14	HRS	-	MDR	katG315Thr & rpoB526Arg & rps-L43Arg	1	Beijing	244233352644425173353823	C24
DRC_001	M	2015	59	Capital	KH-12	S	-	Mono-R	rpsL88Arg	1	Beijing	244233382244425173353823	C25
DRC_234	M	2012	26	Provincial	KH-03	HRSE	-	MDR	katG360 del CGGCGC & rpoB531Leu & rpsL88Arg & embB497Lys & pncA64Stop	1	Beijing	244233382244425173353823	C25
DRC_015	F	2015	40	Capital	KH-12	HRS	-	MDR	katG315Thr & rpoB516Val & rrs514C & pncA136Gly	1	Beijing	244233382344425133353823	C26
DRC_251	M	2012	44	Capital	KH-12	HRSE	Ofx	Pre-XDR	katG315Thr & rpoB531Leu & rrs514C & embB306Val & pncA8Glu & gyrA94Gly	1	Beijing	244233382344425133353823	C26
DRC_080	M	2014	28	Provincial	KH-08	HRS	-	MDR	katG315Thr & rpoB531Leu & rps-L88Arg & pncA35Pro	1	Beijing	244233382644425173355823	C27
DRC_121	M	2014	46	Capital	KH-12	HRSE	Ofx	Pre-XDR	katG315Thr & rpoB531Leu & rps-L88Arg & pncA35Pro & gyrA94Gly	1	Beijing	244233382644425173355823	C27
DRC_195	F	2016	51	Capital	KH-12	HRE	Ofx	Pre-XDR	katG315Thr & rpoB511Arg & rpoB-516Tyr & embB306Leu & pncA7Gly & gyrA94Gly	523	Manu_ancestor	244237242854235173341813	C28
DRC_210	F	2012	27	Capital	KH-12	HRSE	-	MDR	katG315Thr & rpoB531Leu & embB306Leu & pncA-11G	523	Manu_ancestor	244237242854235173341813	C28
DRC_215	M	2012	44	Capital	KH-12	HRSE	-	MDR	katG315Thr & katG614Thr & rpoB-526Asn & rpoB757Ala & rpsL43Arg & embB406Cys	1	Beijing	244333352544425173353823	C29
DRC_280	F	2012	18	Provincial	KH-08	HRS	-	MDR	katG315Thr & katG614Thr & rpoB-526Thr & rpsL43Arg & embB406Cys	1	Beijing	244333352544425173353823	C29
DRI_044	F	2013	56	Provincial	KH-03	H	-	Mono-R	katG155Cys	1	Beijing	254233352734425173353843	C30
DRI_045	M	2013	43	Provincial	KH-03	H	-	Mono-R	katG155Cys	1	Beijing	254233352734425173353843	C30

Appendix 5: Postgraduate student theses supervised during this PhD

CHHAY Sokpanhana, Diploma of Specialized Doctor of Pharmacy in Medical Biology (Diplôme d'Études Spécialisées de Biologie médicale. « Genetic diversity of drug-resistant *Mycobacterium tuberculosis* isolates circulating in Cambodia between 2013 and 2015 ». Defensed on December 21, 2018 at University of Health Sciences, Phnom Penh, Cambodia.

SROEUN Maline, Diploma of Specialized Doctor of Pharmacy in Medical Biology (Diplôme d'Études Spécialisées de Biologie médicale. « Etude des mutations des gènes liées à la résistance chez les *Mycobacterium tuberculosis* aux antituberculeux au Cambodge ». University of Health Sciences, Phnom Penh, Cambodia. Upcoming defense.

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