



HAL
open science

Genetic determinants and evolution of drug resistance in Mycobacterium tuberculosis in Vietnam : toward new diagnostic tools

Quang Huy Nguyen

► To cite this version:

Quang Huy Nguyen. Genetic determinants and evolution of drug resistance in Mycobacterium tuberculosis in Vietnam : toward new diagnostic tools. Human health and pathology. Université Montpellier, 2016. English. NNT : 2016MONTT081 . tel-01560449

HAL Id: tel-01560449

<https://theses.hal.science/tel-01560449>

Submitted on 11 Jul 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



THÈSE

Pour obtenir le grade de
Docteur

Délivré par l'**Université de Montpellier**

Préparée au sein de l'école doctorale
Sciences Chimiques et Biologiques pour la Santé

Et de l'unité de recherche
**UMR MIVEGEC (224 IRD-5290 CNRS
Université de Montpellier)**

Spécialité : **Biologie santé**

Présentée par **NGUYEN Quang Huy**

**Genetic Determinants and evolution of
drug resistance in *Mycobacterium
tuberculosis* in Vietnam: towards new
diagnostic tools**

Soutenue le 20/12/2016 devant le jury composé de

Dr. Maria-Laura Boschioli, HDR, Agence Nationale de Sécurité Sanitaire (ANSES)	Rapporteur
Dr. Rogier Van Doorn, L'Unité de Recherche Clinique de l'Université d'Oxford (OUCRU)	Rapporteur
Pr. Michel Lebrun, Université des Sciences et des Technologies de Hanoi (USTH)	Président
Dr. Le Quang Hoa, Polytechnique de Hanoi (HUST)	Examineur
Dr. Nguyen Thi Van Anh, National Institute of Hygiene and Epidemiology (NIHE)	Co-directeur de thèse
Dr. Anne-Laure Bañuls, UMR MIVEGEC, Montpellier (IRD)	Co-directeur de thèse



SUMMARY

Tuberculosis (TB) is one of the deadliest infectious diseases worldwide, mainly caused by *Mycobacterium tuberculosis*. Multidrug resistant (MDR) and extensively drug resistant (XDR) TB are currently main challenges for TB control. In high MDR-TB burden countries like Vietnam, one of the main factors of drug resistant strain spread is the insufficient capacity of drug resistance detection. Besides, still little is known in these countries about the resistance to second line and pyrazinamide drugs (key drugs in the MDR-TB treatment) and the genetic determinants linked to these resistances. In this context, this work aimed to acquire knowledge on drug resistance in Vietnam and to understand how *M. tuberculosis* evolved from sensitive to highly drug resistance form by molecular analysis.

260 clinical isolates collected in Vietnam between 2005 and 2009 were included. Various techniques and analyses were used: drug susceptibility testing (development of a test with a reduced turn-around time), spoligotyping and 24-MIRU-VNTR typing and gene sequencing. The data were analyzed by statistical and phylogenetic analyses. First, this work was focused on quadruple-first line drug (isoniazid, rifampicin, streptomycin and ethambutol) resistance and pyrazinamide resistance in *M. tuberculosis* clinical isolates. Out of 91 quadruple-FLD resistant isolates, 21 (23.1%) isolates were pre-XDR and 7 (7.7%) ones were XDR by DNA sequencing. The molecular analysis revealed also 91/205 (44.4%) drug resistant isolates carrying highly confident pyrazinamide resistance-associated mutations, particularly in MDR and quadruple resistant isolates and in Beijing family.

Second, the genetic and phylogenetic analyses showed high diversity of mutation patterns within each family and each MIRU-VNTR cluster suggesting various evolutionary trajectories towards first and second-line drug resistance. The predominance of specific mutations and combinations of mutations associated with high level of resistance and low fitness cost suggests a cumulative effect of mutations and a role for epistasis in multiple-drug resistance acquisition. In addition, 19/123 (15.4%) MDR isolates carried fitness-compensatory mutations associated with rifampicin resistant mutations. These processes may drive the evolution of drug resistance in this sample and lead to a successful spread of highly drug resistant

strains. It is worth noting that Beijing family was specifically linked to drug resistances, low fitness cost mutations and to compensatory mutations.

In conclusion, this work provides knowledge on the resistance to the first and second-line anti-TB drugs in clinical *M. tuberculosis* samples collected in Vietnam between 2005 and 2009. These data predict an evolution towards a more problematic situation in terms of drug resistance. First, because the Beijing family, which is currently invading Vietnam. Second, the 69.1% (85/123) of MDR isolates carrying highly confident pyrazinamide resistance-associated mutations in our sample challenge the efficacy and the use of this drug in MDR-TB treatment. Third, the high diversity of drug-resistance mutation patterns with some prevailing mutations suggest an evolution of *M. tuberculosis* towards a higher potential of drug resistance because of a probable cumulative effect of drug resistant mutations and epistatic interactions. Since the samples under study were collected between 2005 and 2009, the next step is to test our hypotheses on a recent sampling. Finally, this study together with published data allowed making, for the first time, an inventory of the drug resistance associated mutations in *M. tuberculosis* isolates from Vietnam. This database will be used for the development of a DNAChip for the fast detection of drug resistance in Vietnam.

RÉSUMÉ

La tuberculose (TB), provoquée par *Mycobacterium tuberculosis*, est une des trois maladies prioritaires dans le monde. Les TB multi-résistantes (MDR) et ultra-résistantes (XDR-TB) représentent des obstacles majeurs pour la lutte antituberculeuse. Dans les pays à MDR-TB élevée, comme le Vietnam, la détection insuffisante de la résistance aux antibiotiques est un des facteurs principaux qui favorisent la transmission des souches résistantes. De plus, dans ces pays, encore très peu de choses sont connues sur la résistance à la pyrazinamide et aux antibiotiques de seconde ligne (antibiotiques essentiels dans le traitement de la MDR-TB) et sur les déterminants génétiques liés à ces résistances. Dans ce contexte, ce travail vise donc à acquérir des connaissances sur la résistance aux antibiotiques au Vietnam et à étudier comment *M. tuberculosis* évolue de l'état sensible à l'état ultra-résistant.

260 isolats cliniques collectés au Vietnam entre 2005 et 2009 ont été inclus. Diverses techniques et analyses ont été utilisées: tests de sensibilité aux médicaments (développement d'un test à temps réduit), spoligotypage et MIRU-VNTR (24 loci) et séquençage de gènes. Les données ont été analysées par des analyses statistiques et phylogénétiques.

Ce travail s'est d'abord focalisé sur la caractérisation d'isolats hautement résistants et sur la résistance à la pyrazinamide. Une forte proportion d'isolats quadruple résistants aux antibiotiques de première ligne a été identifiée comme pré-XDR et XDR et en majorité appartenant à la famille Beijing. L'analyse moléculaire a également révélé une forte proportion d'isolats, en particulier MDR, quadruple résistants et de la famille Beijing, portant des mutations associées à la résistance à la pyrazinamide.

L'analyse génétique et phylogénétique globale a ensuite montré une grande diversité de profils de mutations dans chaque famille et chaque cluster MIRU-VNTR. Ces données suggèrent que *M. tuberculosis* peut suivre des chemins évolutifs variés pour devenir ultra-résistant. La prédominance de mutations et de combinaisons de mutations associées à un haut niveau de résistance et à un faible coût en termes de fitness suggère un effet cumulatif des mutations et un rôle de l'épistasie dans l'acquisition de la résistance multiple. De plus, une fréquence élevée de mutations

compensatoires associées à la résistance à la rifampicine a été détectée chez les isolats très résistants. Ces processus semblent donc influencer fortement l'évolution de la résistance dans notre échantillon. Il est à noter que les mutations liées à des niveaux de résistance élevée et à de faibles coûts en termes de fitness, ainsi que les mutations compensatoires étaient plus particulièrement associées à la famille Beijing.

En conclusion, ce travail fournit des connaissances uniques sur la résistance aux antibiotiques chez *M. tuberculosis* au Vietnam entre 2005 et 2009. En particulier, ces données prédisent une évolution de la résistance vers une situation de plus en plus préoccupante. Premièrement, la famille Beijing, en cours d'invasion au Vietnam, apparaît associée à de hauts niveaux de résistance, de faibles coûts en termes de fitness et aux mutations compensatoires. Deuxièmement, le risque élevé de résistance à la pyrazinamide remet en question son efficacité et son utilisation dans les traitements contre la MDR et la XDR-TB. Troisièmement, les données suggèrent une évolution de *M. tuberculosis* vers un potentiel de résistance plus élevé par effet cumulatif des mutations associées à la résistance et l'existence de phénomènes d'épistasie. Comme les échantillons étudiés dans ce travail ont été collectés entre 2005 et 2009, l'étape suivante est de valider nos hypothèses sur des données actualisées.

Enfin, ce travail, avec les données déjà publiées, a permis d'établir, pour la première fois, un inventaire des mutations associées à la résistance aux antibiotiques chez *M. tuberculosis* au Vietnam. Cette base de données sera utilisée pour le développement d'une puce à ADN pour la détection rapide de la résistance aux antibiotiques au Vietnam.

Acknowledgments

I would like to express my gratitude to all the people who made this thesis possible.

Foremost I would like to sincerely thank Dr. Anne-Laure Bañuls (MIVEGEC, Institute of Research for Development (IRD), Montpellier) and Dr. Nguyen Thi Van Anh (Laboratory of Tuberculosis, National Institute of Hygiene and Epidemiology (NIHE), Hanoi) who provided me with brilliant supervision and wise mentorship. For teaching me the value of rigorous scientific inquiry, while guiding me through an exploration of the field, I am deeply grateful. I am very happy to be your student.

My sincere thanks go to Dr. François Chevenet and Dr. Eric Elguero (MIVEGEC, IRD, Montpellier) for advising me on the aspects of my project which deal with evolution. I would like to thank Lucie Contamin (DRISA, NIHE, Hanoi) for her experimental assistance. I would further like to thank Tran Thi Thanh Hoa and Vu Thi Thuong (Laboratory of Tuberculosis, NIHE, Hanoi), Dr. Cécile Cassan and Dr. Mallorie Hide (MIVEGEC, IRD, Montpellier) for making themselves available to give technical support.

In addition, I would like to thank all members of GeneSys Team (MIVEGEC, IRD, Montpellier) and of Laboratory of Tuberculosis (NIHE, Hanoi) for sharing their helpful experiences. You all contributed to a welcoming atmosphere in both laboratories. I have learnt something from each of the staff and students at IRD and NIHE. Most of all I thank them for the laughter we shared and for the friendships that we have developed.

I gratefully acknowledge the NIHE, IRD, CNRS, USTH and French Embassy in Hanoi for their support. I would like to sincerely thank the Vietnam International Education Development, Ministry of Education and Training, Vietnamese Government for financial assistance (the project 911 scholarship), allowing me to study in France. This study was also supported by grants of the following projects: the PHC Lotus, the GDRI ID-BIO, the KC.10.15/06-10, the JEAI MySA, the LMI DRISA, and an ADB BIO3 USTH project. I would like to thank the Vietnam National Tuberculosis Programme, National Lung Hospital, Pham Ngoc Thach Hospital and Hue Central Hospital for *M. tuberculosis* sampling.

My deepest gratitude is reserved for my family who always support me throughout my studies. Their patience, encouragement and exhilaration provided me enormous energy. I am indebted to my little family, my wife and my daughter, for their love and understanding.

Last, but not least, I would like to thank all my friends particularly the Association of Vietnamese Student in Montpellier (AEVM) for being special friends in this little lovely city.

Thank you,

TABLE OF CONTENTS

Chapter 1. INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW	14
1.1 Introduction and Objectives	14
1.2 Literature review	17
1.2.1 Global tuberculosis burden and risk factors.....	17
1.2.2 Detection of <i>M. tuberculosis</i>	19
1.2.3 Anti-TB drugs	21
1.2.4 TB Treatment	24
1.2.5 BCG vaccine, new vaccines and perspectives	26
1.2.6 General characteristics, lineage classification, evolution and distribution of <i>Mycobacterium tuberculosis</i>	28
1.2.7 Mechanisms of drug resistance in <i>Mycobacterium tuberculosis</i>	34
1.2.8 Evolution of drug resistance in <i>M. tuberculosis</i>	50
1.2.9 The tuberculosis situation in Vietnam	67
Chapter 2. MATERIALS AND METHODS	71
2.1 Study population	71
2.2 Ethics approval of research	71
2.3 Bacterial culture	71
2.4 Drug susceptibility testing (DST)	72
2.4.1 FLD-susceptibility testing.....	72
2.4.2 SLD-susceptibility testing.....	73
2.5 DNA preparation	73
2.6 Molecular Genotyping.....	73
2.6.1 Spoligotyping typing.....	73
2.6.2 24-locus MIRU-VNTR typing.....	74
2.7 Detection of drug resistance-associated mutations by DNA sequencing.....	76
2.8 Detection of compensatory mutations in RIF-resistant and INH-resistant isolates by DNA sequencing	78
2.9 Phylogenic analysis	78
2.10 PhyloType analysis	78
Chapter 3. RESULTS AND DISCUSSIONS	79
3.1 Reduced turn-around time for <i>Mycobacterium tuberculosis</i> drug susceptibility testing with a proportional agar microplate assay.....	81

3.2	Quadruple-first line drug resistance in <i>Mycobacterium tuberculosis</i> in Vietnam: What can we learn from genes?	82
3.3	Molecular analysis of pyrazinamide resistance in <i>Mycobacterium tuberculosis</i> in Vietnam highlights the high rate of pyrazinamide resistance-associated mutations in clinical isolates	83
3.4	Evolution of drug resistance in <i>Mycobacterium tuberculosis</i> in Vietnam	84
3.4.1	Objectives	84
3.4.2	Results.....	84
3.4.3	Discussion	110
Chapter 4. GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES		115
4.1	General discussion.....	115
4.1.1	PZA and SLD resistance, and drug-resistant TB control.....	115
4.1.2	Mutations in drug resistance-associated genes	116
4.1.3	Evolution of drug resistance in <i>M. tuberculosis</i>	116
4.1.4	Towards new diagnostic tool for drug resistance detection.....	117
4.2	Conclusion.....	118
4.3	Perspectives	120

List of Tables

Table 1.1. The five groups of anti-TB drugs recommended by WHO for treating drug-sensitive and drug-resistant TB (including MDR and XDR).....	22
Table 1.2. The major drug efflux pumps in <i>M. tuberculosis</i> and affected drugs	35
Table 1.3. Description of the modes of action of anti-TB drugs as well as the genes involved in drug resistance, the mutation rates and the mutation frequencies in clinical drug-resistant <i>Mycobacterium tuberculosis</i> isolates.....	37
Table 2.1. Primers used for DNA amplification and sequencing of genes involved in anti-TB drug resistance and compensatory mutation in <i>M. tuberculosis</i>	77
Table 3.1. Distribution of the 260 isolates according to the FLD-resistance patterns and the <i>M. tuberculosis</i> families	85
Table 3.2. FLD and SLD susceptibility testing profiles of the 125 isolates according to the <i>M. tuberculosis</i> families.....	86
Table 3.3. Number of isolates with and without genetic mutations according to drugs and sensitivity and specificity data between phenotypic and genotypic data	87
Table 3.4. The INH resistance-associated mutations found in <i>katG</i> and <i>inhA</i> genes and <i>inhA</i> promoter of the 170 INH-resistant isolates and their distribution according to the <i>Mycobacterium tuberculosis</i> families	89
Table 3.5. Mutations detected in the <i>rpoB</i> gene of the 126 RIF-resistant isolates and their distribution according to <i>M. tuberculosis</i> families	90
Table 3.6. Mutations found in the <i>embB</i> gene of the 100 EMB-resistant isolates and their distribution according to the <i>M. tuberculosis</i> families	92
Table 3.7. Mutations found in the <i>rpsL</i> and <i>rrs</i> genes of the 163 STR-resistant and their distribution according to the <i>M. tuberculosis</i> families	93
Table 3.8. Distribution of SLD resistant mutations among drug resistance patterns and mutation frequency.....	94
Table 3.9. Mutations detected in <i>gyrA</i> and <i>gyrB</i> genes and <i>rrs</i> -F2 fragment and distribution within the <i>M. tuberculosis</i> families	95
Table 3.10. SLD resistances, pre-XDR and XDR identified by genetic mutations	96
Table 3.11. Frequencies of specific mutations according to FLD drug resistance patterns (mono, double, triple and quadruple resistance, MDR and non-MDR).....	97
Table 3.12. Frequencies of SLD-resistant mutations according to FLD-resistant patterns (sensitive, mono, double, triple and quadruple resistance and Odds Ratio estimation).....	98
Table 3.13. Frequency of mutations according to <i>M. tuberculosis</i> families	100
Table 3.14. Distribution of the most frequent mutations associated with FLD resistance according to the <i>M. tuberculosis</i> families	101
Table 3.15. Compensatory mutations found in <i>rpoA</i> and <i>rpoC</i> genes in RIF-resistant isolates and distribution according to the <i>M. tuberculosis</i> families.....	103
Table 3.16. Phylotypes linked to specific drug resistance-associated mutations. Each phylotype is characterized by the number of isolates, the drug resistance phenotype and the <i>Mycobacterium tuberculosis</i> family.....	107

List of figures

Figure 1.1. Global picture of MDR- and XDR-TB in 2014. Percentage of new TB cases with MDR-TB (A) and countries with XDR-TB (B) (WHO, 2015).....	18
Figure 1.2. Global TB drug pipeline in 2016.....	24
Figure 1.3. Global TB vaccine pipeline in 2014.....	27
Figure 1.4. Visualization of <i>M. tuberculosis</i> colonies on Lowenstein-Jensen medium (A) and <i>M. tuberculosis</i> visualization using the Ziehl–Neelsen stain (B).....	28
Figure 1.5. <i>Mycobacterium tuberculosis</i> scanning electron micrograph.....	29
Figure 1.6. Evolutionary relationships between selected mycobacteria and members of the <i>Mycobacterium tuberculosis</i> complex.....	30
Figure 1.7. Out-of-Africa migration and Neolithic expansion of human MTBC.....	31
Figure 1.8. Phylogeny of the MTBC and distribution.....	32
Figure 2.1. Schema of spoligotyping technique.....	74
Figure 2.2. Schema of the different molecular markers used for <i>M. tuberculosis</i> genotyping of which MIRU-VNTR typing.....	75
Figure 3.1. Phylogenetic tree based on MIRU-VNTR data obtained from the 260 <i>Mycobacterium tuberculosis</i> isolates collected in Vietnam.....	105

Appendix

List of Tables

Table A 1. Critical concentrations for first-line and second-line drug susceptibility testing (Source from WHO 2012).....	143
Table A 2. Phenotypes, spoligotypes and MIRU-VNTR patterns of the clinical 260 <i>Mycobacterium tuberculosis</i> isolates under study.....	144
Table A 3. Mutation patterns of the 260 clinical <i>Mycobacterium tuberculosis</i> isolates in Vietnam.....	156

List of Abbreviations

Abbreviations	Full words
General	
DR	Drug resistance
DS	Drug sensitive
DST	Drug susceptibility testing
FLD	First-line drug
FQs	Fluoroquinolones
HBCs	High tuberculosis burden countries
HIV	Human immunodeficiency virus
MIC	Minimum inhibitory concentration
MDR-TB	Multidrug-resistant tuberculosis
TB	Tuberculosis
RR-TB	Rifampicin-resistant tuberculosis
XDR-TB	Extensively drug-resistant tuberculosis
SLD	Second-line drug
SLID	Second-line injectable drugs
WHO	World Health Organization
Molecular typing and drug resistance region	
DRS	Direct repeat sequence
ERDR	Ethambutol resistance-determining region

LSPs	Large Sequence Polymorphisms
MIRU-VNTR	Mycobacterial interspersed repetitive units- variable number of tandem repeats
IS	Insertion sequence
QRDR	Quinolone resistance- determining region
RRDR	Rifampicin resistance-determining region
RDs	Regions of difference
SNPs	Single Nucleotide Polymorphisms

Anti-tuberculosis drugs

Amikacin	AMK or Amk in the regimens
Bedaquiline	BDQ or Bdq in the regimens
Capreomycin	CAP or Cap in the regimens
Clofazimine	CFZ or Cfz in the regimens
Cycloserine	CS or Cs in the regimens
Delamanid	DLM or Dlm in the regimens
Ethambutol	EMB or E in the regimens
Ethionamide	ETO or Eto in the regimens
Gatifloxacin	GAT or Gfx in the regimens
High-dose isoniazid	H ^h in the regimens
Isoniazid	INH or H in the regimens
Kanamycin	KAN or Kan in the regimens
Levofloxacin	LVX or Lfx in the regimens
Linezolid	LZD or Lzd in the regimens

Moxifloxacin	MXF or Mfx in the regimens
Prothionamide	PTO or Pto in the regimens
Pyrazinamide	PZA or Z in the regimens
Rifampicin	RIF or R in the regimens
Ofloxacin	OFX or Ofx in the regimens
<i>p</i> -aminosalicylic acid	PAS or Pas in the regimens
Terizidone	TRD or Trd in the regimens
Streptomycin	STR or S in the regimens

***M. tuberculosis* lineages**

CAS	Central Asian Strain
EAI	East-African-Indian
H	Haarlem
MTBC	<i>Mycobacterium tuberculosis</i> complex
LAM	Latin-American-Mediterranean

Chapter 1. INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1.1 Introduction and Objectives

Tuberculosis (TB), one of the deadliest infectious diseases in the world, caused by *Mycobacterium tuberculosis*, is a major problem of public health in Vietnam. Although many successes in treatment and TB management of drug-resistance were achieved between 1990 and 2015, Vietnam ranks 13th in the list of the highest TB and 14th in the list of multidrug resistant (MDR-TB, resistant TB at least to isoniazid (INH) and rifampin (RIF)) burden countries in the world (1). Vietnam, as many developing or emerging countries, is facing an increase of MDR-TB (from 2.7% in 2005 to 4.0% in 2011) and the emergence of highly drug resistant strains such extensively drug resistant TB (XDR-TB, MDR TB resistant to any fluoroquinolone (FQ) and at least to one second-line injectable drug (SLID)) (1). However, accessibility to drug susceptibility testing (DST) is still limited in these countries leading to high spread of drug resistant strains. Several studies deal with MDR-TB in Vietnam and thus with RIF and INH resistance, but still little is known about the resistance to the other FLDs (streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA)) and second line drugs (SLDs = FQs and SLIDs). However, it is worth noting that PZA is a key drug in current and future treatment regimens for sensitive TB, MDR-TB, XDR-TB and TB relapses and SLDs in the MDR-TB treatment (2-4). On a genetic point of view, five papers demonstrated a link between Beijing family, one of the most transmissible and drug resistant *M. tuberculosis* families, with MDR-TB in Vietnam and two of them investigated the drug resistant mutations associated with FQ and STR resistance (5-9). Thus, the data are still limited and do not allow to have a detailed view of drug resistance, to estimate the risk of XDR, to limit the risk of using and introducing ineffective TB treatment regimens and to help the implementation of new diagnostic tools and efficient control strategies.

In this context, this work aimed to acquire knowledge on drug resistance within clinical samples of *M. tuberculosis* collected in Vietnam and to understand

how this bacteria evolved from sensitive to highly drug-resistant form by molecular analysis. Finally, the data allowed to discuss about the risk of drug resistance increase over time and to build a database of mutations associated with drug resistance in order to develop a diagnostic kit specifically adapted to Vietnam based on the microarray technology.

This study is of high interest because we will acquire global knowledge on FLD and SLD resistance within a collection of clinical *M. tuberculosis* from Vietnam belonging to different families. It is obvious that knowledge on drug resistant patterns, their frequencies and distribution in *M. tuberculosis* population and the insights into the contributing evolutionary processes are essential to determine the best TB treatment, to implement efficient control strategies and to help the development of diagnostic tools.

The specific objectives were as follows:

1. To develop a method with a reduced turn-around time and cheaper cost for *M. tuberculosis* drug susceptibility testing.
2. To determine the FLDs and SLDs drug resistant patterns by drug susceptibility testing in a clinical *M. tuberculosis* sample collected between 2005 and 2009 and to identify the genetic determinants linked to FLD and SLD drug resistance by the sequencing of 11 main genes known to be associated with these resistances including the following genes or promoters: *rpoB*, *katG* and *inhA* and *inhA* promoter, *rpsL*, *rrs*, *embB*, *pncA* and *pncA* promoter, and *gyrA* and *gyrB*. The objective here was to assign a genetic drug resistance pattern to each isolate. The compensatory mutations linked to RIF and INH resistance were also investigated in the genes, *ahpC* and its promoter, *rpoA* and *rpoC*, in order to investigate the potential risk of fitness increase within highly drug resistant isolates.
3. To characterize the *M. tuberculosis* isolates by spoligotyping and 24-loci MIRU-VNTR typing in order to identify the families and determine the genotypic diversity.

4. To acquire knowledge on drug resistance in Vietnam. The objective here was to determine the link between FLD and SLD drug resistance patterns and the *M. tuberculosis* genetic background.
5. To determine what we can learn from the genes and to better understand how *M. tuberculosis* evolves from drug sensitive to MDR, pre-XDR and XDR forms. The objective here is to explore the processes that drive the evolution of drug resistance.
6. To investigate the sensitivity and the specificity of the molecular analysis for the drug resistance detection. This work will help build a database of drug resistance-associated mutations for the further implementation of a specific diagnostic kit based on microarray technology.

1.2 Literature review

1.2.1 Global tuberculosis burden and risk factors

Over tens of thousands of year, human has been infected with an ancient pathogen named *Mycobacterium tuberculosis*, which is the main causative agent of TB also historically called “White plague” or “Consumption”(10, 11).

Despite the undeniable progresses that has been made in global TB control, with a decrease of TB prevalence and mortality rate since 1990, TB is still one of the most devastating human infectious diseases, with HIV and malaria (1). It is a major public health problem worldwide and 2-3 billion people have been infected with *M. tuberculosis* of whom 5-15% will develop TB during their lifetime (1). According to the WHO global TB report 2016, an estimated 10.4 million people developed TB. South-East Asia and Western Pacific regions account for 58% of the estimated TB cases (1). TB killed 1.8 million people in 2015, among which 0.4 million were HIV-positive patients (1). Several parameters can explain this epidemiological situation, such as the social and health conditions, the association with HIV/AIDS (1.2 million of patients are co-infected by MTB/HIV), the movement of populations and the existence of multidrug-resistant strains (1).

Regarding the last point, the emergence of MDR and XDR-TB is one of the main challenges threatening the success of global TB control program (**Figure 1.1**) (1). Globally, 480 000 (5%) TB cases were estimated to be MDR-TB in 2015 (1). Drug resistance surveillance data showed that the proportion of MDR-TB was 3.3% among new TB cases and 20% among previously treated cases (1). Besides, XDR-TB has been reported in 117 countries by the end of 2015 (**Figure 1.1**) (1). During this year, an estimated 9.5% of people with MDR-TB developed XDR-TB. In some regions of the world, this proportion was over than 15% such as in Latvia (19%), Lithuania (25%) and Belarus (29%) (1).

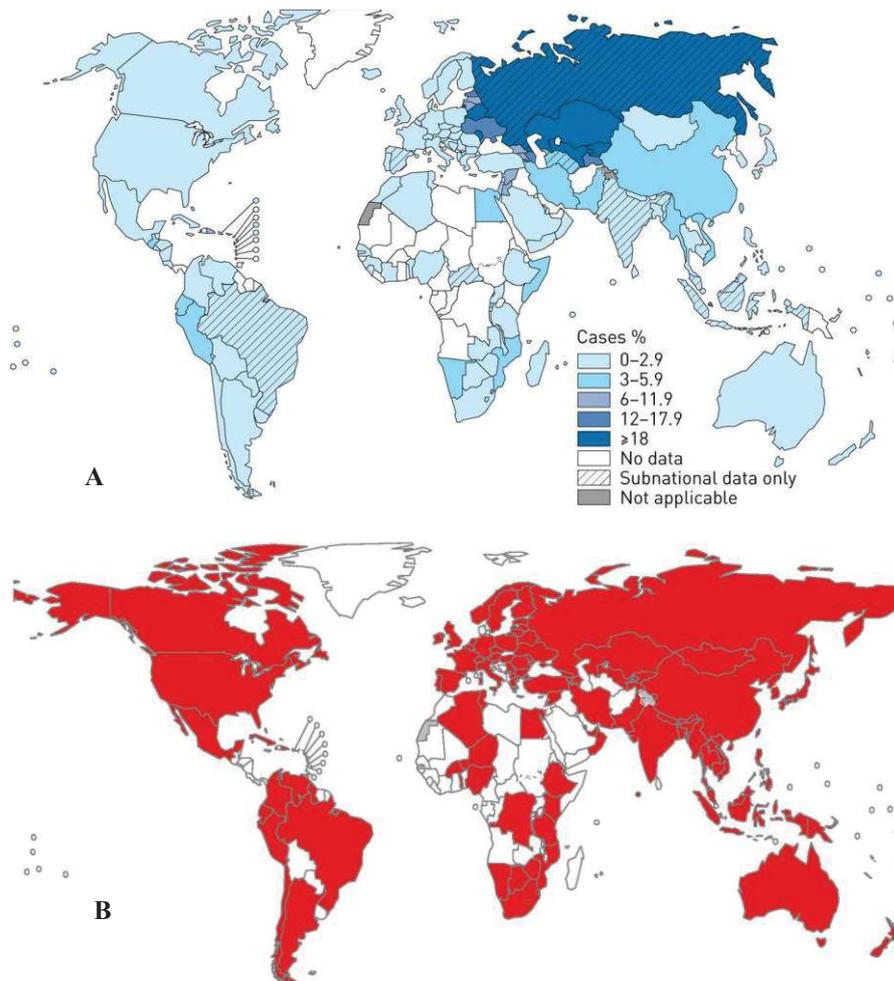


Figure 1.1. Global picture of MDR- and XDR-TB in 2014. Percentage of new TB cases with MDR-TB (A) and countries with XDR-TB (B) (WHO, 2015).

Overall, the treatment success rate was of 83% for new TB cases in 2014 (1). The treatment of MDR and XDR-TB is much more difficult, complex and costly with higher rates of treatment failure and mortality. Globally, only 50% of MDR-TB and 26% of XDR-TB patients are successfully treated (1). The economic situation of the countries may also impact the success of TB control programs. It is estimated that about \$ 8 billion per year is required for a full response to TB epidemic in these countries, of which about 20% is for detection and treatment of MDR-TB (1, 12). Additionally about \$ 2 billion per year is required for research and development for new TB diagnostics, drugs and vaccines. In brief, the global economic burden of TB is approximately \$ 12 billion annually (1).

The detection rate of TB patients remains low (57% of the pulmonary cases reported were bacteriologically confirmed), which may seriously underestimate the

burden of the disease and favor the spread of the disease in the community. One of the main factors contributing to the high rate of DR-TB is the treatment non-adherence of TB patients (13, 14). The treatment duration is long (usually six months or longer) and is a combination of multiple drugs that often have side effects, therefore patients can be refractory to the treatment. Furthermore, patients often experience rapid improvement of symptoms, and then stop the treatment leading to TB relapses and DR. In 1990, WHO recommended the DOTS (Directly Observed Treatment, Short-course) strategy for TB control. However, the lack of full supervision during the intensive phase of treatment, of follow up, drug supply and management and of infrastructure largely contributes to the development of DR (15, 16). In addition, the low quality of drugs can be also one of the reasons of relapses and DR (16, 17). The major concerns of the MDR-TB treatment are the duration (20 - 30 months), the use of SLDs, which are very expensive and the complex management (1, 4). Factors such as poverty, migration, traveling, HIV infection, homelessness, being in prison, substance abuse, weak immune system, alcohol and smoking were also linked to DR transmission (14-17). Finally, in the regions with high prevalence of virulent *M. tuberculosis* strains such as Beijing or LAM genotypes, the risk of TB and MDR-TB infection is also higher (7, 18, 19).

1.2.2 Detection of *M. tuberculosis*

The most common method for diagnosing *M. tuberculosis* worldwide remains the conventional Ziehl–Neelsen (ZN) method, that has been developed more than 100 years ago (1). Bacteria are observed in sputum samples examined under a light microscope. This method is a low-cost and frontline tool for TB diagnosis. However, this method cannot distinguish between drug-susceptible and drug-resistant *M. tuberculosis* or between different species of mycobacteria such as non-tuberculous mycobacteria. Moreover, this method has variable sensitivity (20 - 80%) than can be very low in HIV/TB co-infected patients (1, 12, 20). Currently, WHO recommends the use of fluorescent light-emitting diode (LED) microscopy as alternative technique because it is more sensitivity (10%) than ZN method (20). Nevertheless, the use of LED microscopy is limited because of its high cost. Therefore only 7% of TB centers worldwide used this technology in 2014.

The culture-based method is the gold standard for detection of *M. tuberculosis*. The detection rate often increases of 30-50% compared to microscopy (20). In addition, culture provides the bacterial material for drug susceptibility testing (DST). However, this method is more complex and expensive than microscopy, time consuming (4-8 weeks by solid culture) and strict biosafety measures (20-22). Besides, liquid culture media is a more sensitive and faster culture system but does not permit to identify contamination with other bacteria (20, 22). Regarding the DST, the reliability also varies with the anti-TB drugs. DST is more accurate in detecting susceptibility to INH, RIF, FQs and SLIDs, but results are less reliable and reproducible for ethambutol (EMB), streptomycin (STR), pyrazinamide (PZA) and for drugs of groups 4 and 5 (20, 22, 23).

The development of genetic methods showed considerable advantages in *M. tuberculosis* and DR detection. The two main molecular tools endorsed by WHO in 2008 and 2010 are Line Probe Assay (LPA) and Xpert MTB/RIF, respectively (24, 25). LPA allows rapid detection of *M. tuberculosis* and RIF resistance alone (INNO-LiPA®Rif.TB assay, Innogenetics, Ghent, Belgium) or in combination with INH (GenoType® MTBDR assay, Hain Lifescience, Nehren, Germany) within 24 hours (20). LPA is suitable for both AFB smear-positive sputum specimens and *M. tuberculosis* isolates grown by conventional culture method. This test shows high sensitivity for detection of RIF resistance (over 94%), but is less sensitive for the detection of INH resistance (approximately 85%) (20, 26). Therefore, it may underestimate the number of MDR cases (20). The Xpert MTB/RIF assay allows to identify *M. tuberculosis* complex and the RIF resistance directly from sputum specimens in less than two hours (20, 24). The assays had similar sensitivity, specificity and accuracy as culture on solid media and this tool has been recommended by WHO as initial diagnostic test for persons with a risk of MDR-TB and HIV. However, the tests for detection of RIF resistance alone cannot accurately predict RIF resistance and MDR-TB since about 10% of RIF resistant isolates were sensitive to INH (20). Besides, despite the overall high sensitivity (99%), Xpert MTB/RIF sensitivity was 60 - 88% compared with liquid culture and false results have been reported (27, 28). Recently, a new version called Xpert Ultra is able to detect *M. tuberculosis* with a limit of detection of 10 – 100 CFU/ml sputum (27). This new version has already shown higher sensitivity and specificity for detection of *M.*

tuberculosis and RIF-resistant samples compared to the previous Xpert MTB/RIF and is expected to be launched in 2017.

For the rapid identification of XDR-TB, the Genotype MTBDRs/® test has been developed in 2009 with the capacity to detect resistance to FQs and SLIDs (25). However, the moderate sensitivity (69%) for XDR-TB detection leads to an underestimation of XDR-TB cases (WHO 2016). The accuracy of MTBDRs/ by indirect testing for the detection of FQ resistance in patients with RIF-resistant or MDR-TB was 86% of sensitivity and 99% of specificity (27). For detection of SLID resistance by indirect testing, this test showed 77% of sensitivity and 99% of specificity (27). Therefore, the results obtained by MTBDRs/® may be used as initial test for detection of SLD resistance but cannot be used to properly guide the choice of SLIDs for the MDR-TB treatment (20, 22, 27).

Overall, the TB case detection remains low. In 2014, only 6 million (63%) out of the 9.6 million estimated TB cases by WHO were reported (1). Furthermore, if the 6 million notified TB cases were tested for DR, an estimated 300 000 people would have been found to have MDR-TB (globally 5% MDR). However, only 12% of new TB cases and 58% of previously treated TB cases were examined for drug susceptibility (1). Therefore, only 123 000 MDR-TB cases were notified globally, represented 41% of the estimated 300 000 MDR-TB cases in the world in 2014. As a result, many TB patients with undetected MDR were not potentially correctly treated leading to treatment failure and an increased risk of MDR transmission in the community. Moreover, among the 110 000 notified MDR-TB patients enrolled on MDR-TB treatment according to WHO standards, only 24% received DST for both FQs and SLIDs (1). This suggests that many XDR-TB cases were never diagnosed. These data show the depth of the challenge for the management of MDR-TB and emergence of XDR-TB worldwide.

1.2.3 Anti-TB drugs

More than 20 anti-TB drugs are currently available for the treatment of TB (**Table 1.1**) (WHO 2016). These drugs have traditionally been divided into FLDs and SLDs, and have been classified into 5 different groups.

The FLDs (group 1, **Table 1.1**) generally have the greatest activity against TB bacilli and they are the core elements of the TB control programs in the world. These drugs have been combined to form the backbone of the directly observed therapy (DOT) program launched by WHO since 1995 (29). The SLDs (groups 2 – 4, **Table 1.1**) are mainly used for treating MDR and XDR-TB cases (4). The drugs in group 5 are the third-line drugs including anti-TB drugs with limited data on their efficacy and/or on their long-term safety. They are mainly used for treating XDR-TB (4), but some drugs were designed for new treatment regimens of sensitive-drug cases, such as LZD and CFZ (30-33). The mode of action of the main FLDs and SLDs and the mechanisms of DR are described in the part 1.2.7 (mechanisms of drug resistance in *M. tuberculosis*) of this chapter (see below).

Table 1.1. The five groups of anti-TB drugs recommended by WHO for treating drug-sensitive and drug-resistant TB (including MDR and XDR)

Group	Description	Drug (year of discovery)	TB treatment
1	<i>First-line oral anti-TB drugs</i>	Isoniazid/INH (1952)	DS-TB
		Rifampicin/RIF (1966)	DS-TB
		Ethambutol/EMB (1961)	DS-TB, DR-TB (add-on agents)
		Pyrazinamide/PZA (1952)	DS-TB, DR-TB (add-on agents)
		Rifabutin/RBT (1975)	DS-TB
		Rifapentine/RPT (1975)	DS-TB
2	<i>Injectable anti-TB drugs</i>	Streptomycin/STR (1943)	DS-TB
		Kanamycin/KAN (1957)	DR-TB
		Amikacin/AMK (1957)	DR-TB
		Capreomycin/CAP (1960)	DR-TB
3	<i>Fluoroquinolones (FQs)</i>	Levofloxacin/LFX (1987)	DR-TB
		Moxifloxacin/MFX (1996)	DR-TB
		Gatifloxacin/GFX (1998)	DR-TB
		Ofloxacin/OFX (1982)	DR-TB
4	<i>Oral bacteriostatic second-line anti-TB drugs</i>	Ethionamide/ETO (1956)	DR-TB
		Prothionamide/PTO (1956)	DR-TB
		Cycloserine/CS (1954)	DR-TB

	Terizidone/TZD (1956)	DR-TB
	p-aminosalicylic acid/PAS (1946)	DR-TB
	p-aminosalicylate sodium/ASS (1946)	DR-TB
5	<i>Anti-TB drugs with limited data on efficacy and/or long-term safety in the treatment of drug-resistant TB</i>	Bedaquiline/BDQ (2006)
		DR-TB (add-on agents), clinical trial
		Delamanid/DLM (2006)
		DR-TB (add-on agents), clinical trial
		Linezolid/LZD (1996)
		DR-TB
	<i>(This group includes new anti-TB agents)</i>	Clofazimine/CFZ (1950s)
		DR-TB
		Amoxicillin/Clavulanate/AMX-CLV
		DR-TB (add-on agents)
	Imipenem/Cilastatin/LPM	
	DR-TB (add-on agents)	
	Meropenem (1976)/MPM	
	DR-TB (add-on agents)	
	High-dose isoniazid/H ^h	
	DR-TB (add-on agents)	
	Thioacetazone/T	
	DR-TB (add-on agents)	
	Clarithromycin/CLA	
	DR-TB (add-on agents)	

Adapted from WHO 2014 (Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis) and WHO 2016 (WHO treatment guidelines for drug-resistant tuberculosis: 2016 update). Add-on agents: PZA and EMB are added in the regimens if drug-sensitive strains are confirmed; drugs in group 5 are added in the regimen if the full regimen cannot contain drugs from the groups 2, 3 and 4. DS=drug sensitive; DR=drug resistant.

New anti-TB drugs are now in the pipeline (**Figure 1.2**) and combinations of drugs that include new compounds are being tested in clinical trials (3, 34). New TB drugs are urgently needed because of the complexity, the duration and the toxicity of the current TB drug regimens and the major problem of DR-TB.

Global TB Drug Pipeline ¹

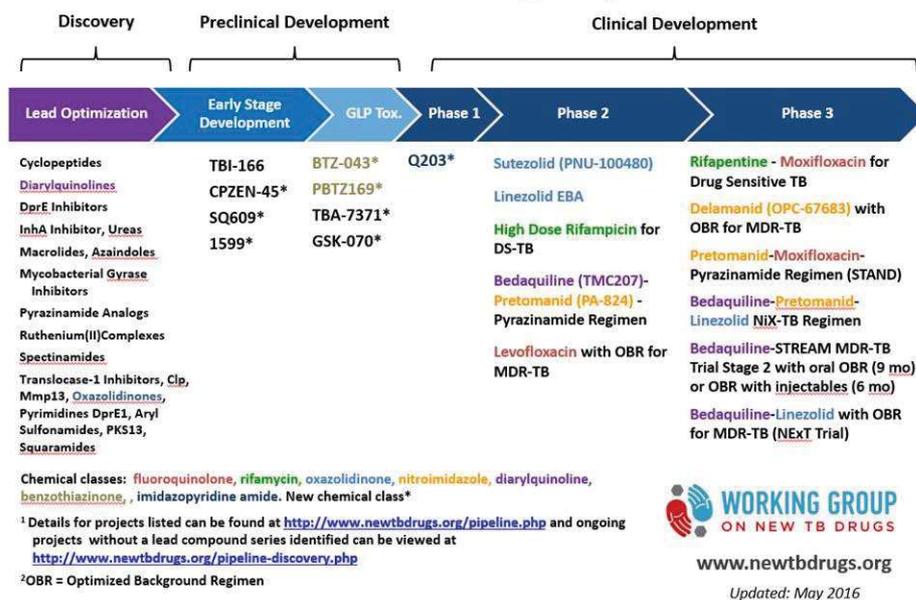


Figure 1.2. Global TB drug pipeline in 2016

(Source: <http://www.newtbdrugs.org/pipeline.php>)

1.2.4 TB Treatment

- *Standard treatment regimen for drug-sensitive and drug-resistant TB*

Treating TB takes much longer time than treating other types of bacterial infection and requires a combination of at least four distinct drugs. Indeed, TB patients must take antibiotics for at least six to nine months (35, 36). The drugs and the length of treatment depend on patient's profile including age, health condition, drug-sensitive/-resistant status and the location of infection in the body. TB patients have to strictly comply with the treatment regimen. Stopping treatment or skipping doses can allow the emergence of resistance.

To date, the current standard treatment regimen recommended by WHO consists of 2 months of INH, RIF, PZA and EMB, followed by 4 months of INH and RIF (2HRZE/4HR) for new TB patients (4). This regimen has not changed in nearly 40 years because it is efficient for the treatment of drug-sensitive TB despite the substantial drawbacks of treatment duration, complexity and toxicity (37). Nevertheless, the lack of treatment compliance is frequent because some patients abandon therapy when they start to feel better. For the retreatment of TB patients, the

eight-month regimen 2SHREZ/1HREZ/5HRE is then applied. Besides, the regimens for treatment of mono- and poly-resistant (either resistant to INH or RIF) TB are not yet standardized. They are based on the DR patterns (DST results) (38).

For MDR-TB patients, a combination of FLDs (except INH and RIF) and SLDs are generally used for 20 to 30 months (38). In all settings, WHO and the national guidelines for MDR-TB treatment suggest personalized regimens based on DST results. Globally, most of treatment regimens contain a minimum of four drugs including one FLD (PZA or EMB), one SLID (AMK/KAN or CAP), one drug belonging to FQ group (in decreasing frequency of use: OFX/LFX/MFX/GFX) and one drug from group 4 (often CS or PAS) (4, 38). The drugs belonging to group 5 should be included when four active drugs from previous groups are not available.

An inappropriate treatment for MDR-TB is one of the main factors that can lead to XDR-TB. Since XDR-TB is resistant to the most powerful FLDs and SLDs, treatment options are seriously limited. In this case, the group 5 and the new approved drugs are often key drugs in XDR treatment regimen. In general, the XDR-TB regimen should be prescribed as followed: one SLID (KAN or AMK or CAP), one drug of the last generation of FQ (LEV or MFX), at least two drugs of group 4 (ETH, PAS or CS), one drug of group 5 (BDQ or DLM or CFZ) and one drug of group 1 (PZA or EMB) (4, 38).

Recently, a standardized regimen for treating MDR-TB cases has been used in several African and Central Asian countries (WHO 2016). This regimen allowed shortening treatment course with duration of 9- 12 months. The treatment regimen consisted of 4-6 months of Kan-Mfx-Pto-Cfz-Z-H^h-E, followed by 5 months of Mfx-Cfz-Z-E and had 82% success rate. From these data, WHO updated the treatment guidelines for DR-TB and also included a recommendation to use shorter MDR-TB regimen under specific conditions. This regimen is expected to benefit MDR-TB patients worldwide. However the regimen needs to be strictly controlled, otherwise XDR could rapidly emerge.

➤ *New treatment regimens*

The new regimens are mostly or entirely composed of new drugs having potent activity against *M. tuberculosis* and no cross-resistance with the existing

agents. The goal is to shorten and simplify the TB treatment in general. Several new regimens for drug-susceptible and/or drug-resistant TB are in Phase II or Phase III trials (30, 31, 33, 39, 40). The new generation of FQs, MFX and GFX are currently being tested for replacing EMB or INH in the treatment of drug sensitive TB, to shorten the standard treatment course from six months to four months (32, 33, 41, 42).

The re-proposed and new drugs including CFZ, LZD, BDQ, DLM, PA-824 and sutezolid (also known as PNU-100480) are currently under study as add-on therapy to the current drug-resistant treatment. Recent reports showed that the combination of BDQ, sutezolid and PA-824, with or without CFZ has greater sterilizing activities than that of the first-line regimen RIF, INH and PZA (30, 31, 33, 39, 40). Other studies showed that the combination of BDQ and PZA with either PA-824 or sutezolid or CFZ had a greater bactericidal and sterilizing activities also compared to the first-line regimen, RIF, INH and PZA (39, 40). This suggests that the new regimens may offer potential shorter treatment duration (4 months) against PZA-susceptible isolates, but still constitute effective short-course regimens (6 months) against isolates resistant to PZA (39, 40). The combination of BDQ, PA-824, CFZ and PZA is currently in clinical trial (ClinicalTrials.gov, registration no. NCT01691534).

1.2.5 BCG vaccine, new vaccines and perspectives

The ‘Bacillus Calmette-Guérin’ (BCG) vaccine, an attenuated form of *Mycobacterium bovis*, is currently the only available TB vaccine and has been introduced in 1921 (43). This vaccine is one of the most widely used of all current vaccines. It can reach more than 80% of all newborn children and infants in countries where it is a part of the national childhood immunization program (BCG vaccine, the WHO, 2011, www.who.int/biologicals/areas/vaccines/). It has been reported in 2004 that about 100 million children received BCG vaccine each year ([http://www.who.int/vaccine_safety/initiative/tools/BCG Vaccine rates information sheet.pdf](http://www.who.int/vaccine_safety/initiative/tools/BCG_Vaccine_rates_information_sheet.pdf)). The BCG vaccine provides protection against TB meningitis and the disseminated form of the disease in infants and young children (43, 44). It is unclear if the BCG vaccine has an overall effect in reducing TB. Previous studies indicated that BCG vaccine efficacy against pulmonary diseases varies geographically (43, 44).

Overall, the vaccine is relatively safe, inexpensive and requires only one injection. However, vaccine-induced protection wanes with time and is likely to be lost after 10–20 years (43). Furthermore, it does not prevent primary infection and, more importantly, does not prevent reactivation of latent pulmonary infection, the principal source of bacillary spread in the community. Furthermore, a previous study showed that BCG vaccine could induce *M. bovis* BCG disease in HIV-infected children (45). The other limitation of BCG vaccine is the reactivity to the tuberculin skin test after vaccination (43, 44). This test cannot distinguish a positive response caused by *M. tuberculosis* infection from that caused by BCG vaccination or non-tuberculous bacterial infection. Currently, BCG vaccination is mainly used for infants and children who reside in settings in which the likelihood of *M. tuberculosis* transmission is high (44). Thus, development of new, safe, effective and affordable vaccines to replace the BCG vaccine is therefore essential.

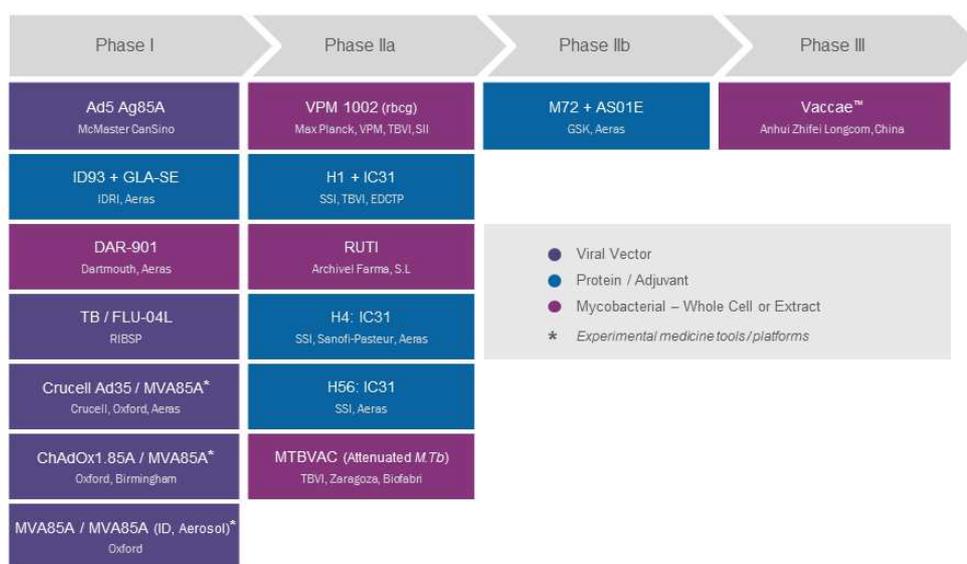


Figure 1.3. Global TB vaccine pipeline in 2014
 (Source from <http://www.aeras.org/annualreport2014>)

According to AERAS report in 2014, 15 vaccine candidates were in Phase I or Phase II clinical trials (**Figure 1.3**). The most promising approaches are the recombinant BCG vaccines, the attenuated strains of *M. tuberculosis* and DNA vaccines. Although recent progresses have been made in the development of new TB vaccines, the population structure of *M. tuberculosis* and the host-specific pathogen adaptation may need to be considered when engineering and evaluating new vaccine candidates (46).

1.2.6 General characteristics, lineage classification, evolution and distribution of *Mycobacterium tuberculosis*

A. Characteristics of *M. tuberculosis*

M. tuberculosis was first discovered in 1882 by Robert Koch (47). This pathogen belongs to the slow-growing bacterial group, characterized by a 18 to 24 hour division rate (48). Consequently, the growth on LJ medium requires at least 3-4 weeks (48). Faster results can be obtained using solid Middlebrook medium with growth supplement (OADC) or liquid medium (BACTEC) (20, 49-52). *M. tuberculosis* is non-pigmented, rough, dry colonies and forms a cord-like structure on LJ medium (**Figure 1.4, A**). Using Ziehl-Neelsen staining or acid-fast staining method, the tubercle bacteria are rod-shaped and bright red (**Figure 1.4, B**).

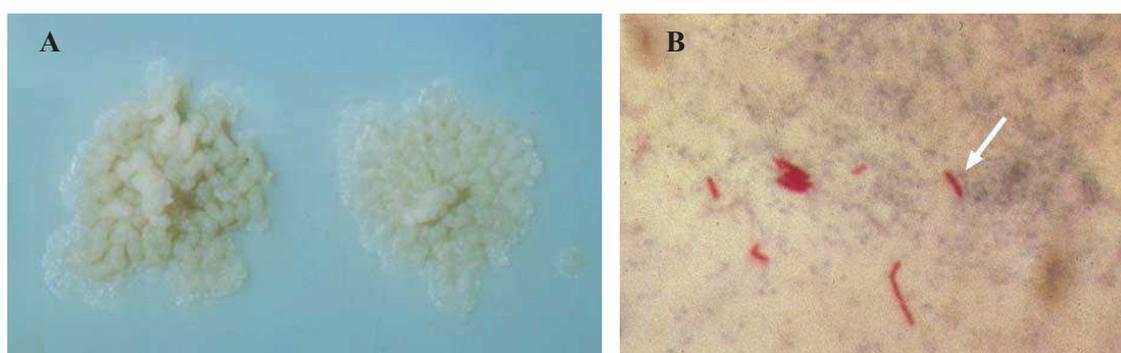


Figure 1.4. Visualization of *M. tuberculosis* colonies on Lowenstein-Jensen medium (A) and *M. tuberculosis* visualization using the Ziehl–Neelsen stain (B)

(Source from <http://textbookofbacteriology.net/tuberculosis.html>)

Under electron microscope, the bacteria are about 2 – 4 μm in length and 0.2-0.5 μm in width (**Figure 1.5**). *M. tuberculosis* is classified as acid-fast Gram-positive bacteria due to their lack of outer cell membrane. Nevertheless, the membrane characteristics do not correspond to Gram-positive ones. Indeed, the bacteria do not retain the crystal violet dye as expected for Gram-positive bacteria, sometimes resulting in “ghost” appearance after washing with alcohol or acetone. *M. tuberculosis* has a specialized cell wall complex, which consists of four major components, mycoside, mycolic acids, arabinogalactan and peptidoglycan. Mycolic acids, the major lipids of the cell wall of mycobacteria in general, are major components of the outer permeability barrier and are responsible for the "acid-fastness" of this group of microorganisms (53). Furthermore, the fatty acids are linked with carbohydrate

components that form an unique envelope which inhibits phagolysosome fusion (48). Like many other bacteria, *M. tuberculosis* does not form spores but has the capacity to become dormant – a non-replicating state characterized by low metabolic activity and prolonged persistence (48).

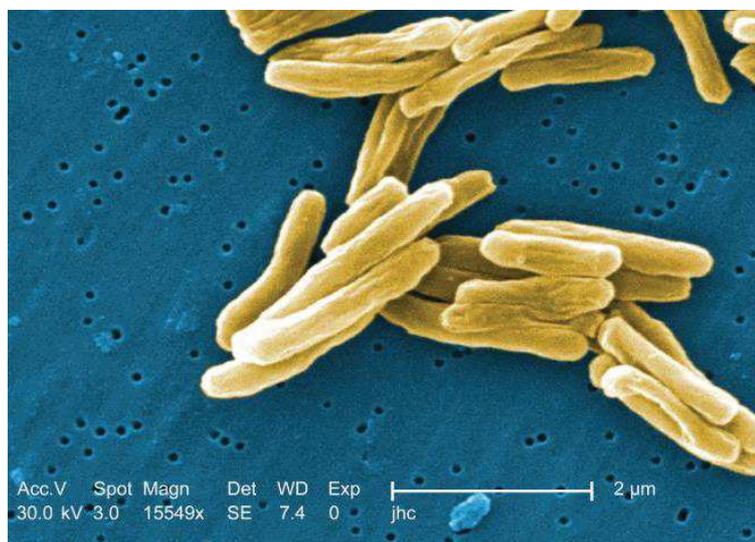


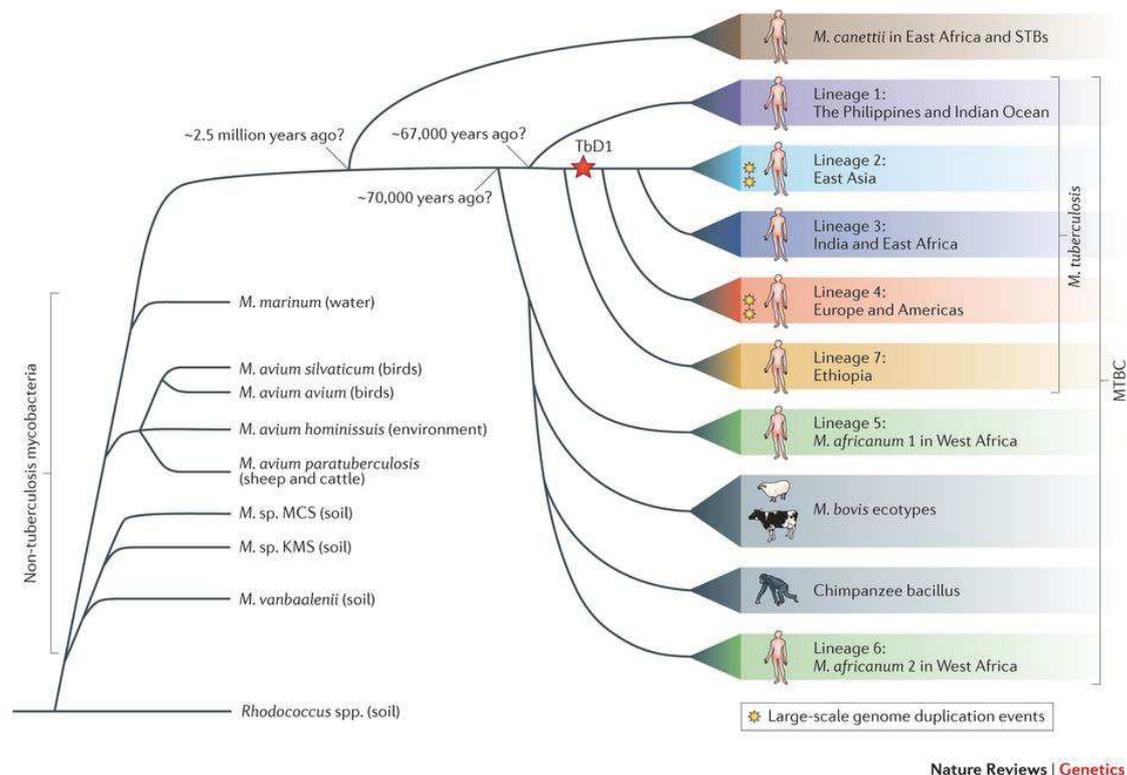
Figure 1.5. *Mycobacterium tuberculosis* scanning electron micrograph
(Source from <http://phil.cdc.gov/phil/details.asp?pid=9997>)

By 1998, the genome of *M. tuberculosis* H37Rv strain has been sequenced (54). Recently, thanks to the progresses in whole genome sequencing (WGS) technology, the sequences of many genomes have been determined covering all types of DR from pan-drug sensitive to MDR and XDR strains (55-60). The *M. tuberculosis* genome contains about 4.4 megabase pairs and around 4000 genes with a very high guanine + cytosine (GC) content (about 65%) (<http://genolist.pasteur.fr/TubercuList/>). *M. tuberculosis* is described as a clonal bacteria and the genome is highly conserved (54, 61, 62). The pathogen is characterized by a low mutation rate about 10^{-9} mutation/bacterium/cell division (63-65). The genome evolutionary rate is very low, estimated between 0.4 - 0.5 SNP/genome/year (55, 66).

B. Evolutionary history and clonal expansion of MTBC

M. tuberculosis is a member of the *M. tuberculosis* complex (MTBC), which comprises three human-adapted species (including 8 lineages), *M. tuberculosis* (5 lineages), *Mycobacterium africanum* (2 lineages) and *Mycobacterium canettii* and several animal-sourced lineages including *Mycobacterium bovis* (mainly pathogen of cattle), *Mycobacterium caprae* (pathogen of sheep and goats), *Mycobacterium*

microti (pathogen of voles) and *Mycobacterium pinnipedii* (pathogen of seals and sea lions) (11, 46, 67-71). Phylogenetic analyses indicated that the MTBC is the clonal progeny of an ancestral strain of *M. canettii*, belonging to the group of ‘smooth tubercle bacilli’ (Figure 1.6) (67, 71, 72). The genome of the MTBC members is highly conserved with 99.9% identity (62, 71-73).



Nature Reviews | Genetics

Figure 1.6. Evolutionary relationships between selected mycobacteria and members of the *Mycobacterium tuberculosis* complex.

(From Galagan (2014) (72):The animal-adapted *Mycobacterium bovis* ecotypes branch from a presumed human-adapted lineage of *Mycobacterium africanum* that is currently restricted to West Africa. Human-adapted *M. tuberculosis* strains are grouped into seven main lineages. The dates of branching events are only crude estimates. TbD1 indicates the deletion event specific for *M. tuberculosis* lineages 2, 3 and 4. Evolutionary distances are not to scale. All species shown are from the genus *Mycobacterium*.)

Among the eight human-adapted lineages, the 5 lineages belonging to *M. tuberculosis* are *M. tuberculosis* lineage 1 (The Philippines and Indian-Ocean), *M. tuberculosis* lineage 2 (East-Asian), *M. tuberculosis* lineage 3 (East-African-Indian), *M. tuberculosis* lineage 4 (Euro-American) and *M. tuberculosis* lineage 7 (Ethiopia) (46, 68, 69). From several detailed phylogenetic analyses, the lineages 1, 5 and 6 were defined as ancient lineages with *M. canettii* as the more ancestral branch; lineages 2, 3,

4 as modern lineages and lineage 7 appears to be intermediate between the ancient and modern lineages (10, 62, 71, 72).

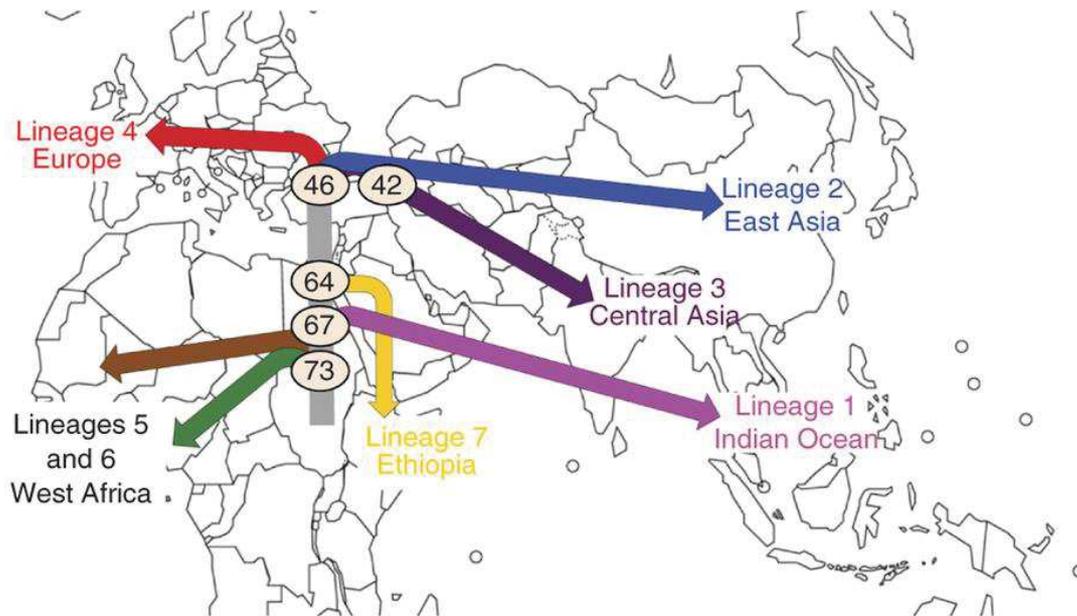


Figure 1.7. Out-of-Africa migration and Neolithic expansion of human MTBC (from Comas *et al* (10). Note: Major splits are annotated with the median value (in thousands of years) of the dating of the relevant node.)

The study of Comas *et al.* analyzed the whole genome of 259 strains representative of the global diversity of MTBC and showed that, the MTBC emerged about 70,000 years ago in Africa, accompanied migrations of modern humans out of Africa and expanded as a consequence of the human population density increase during the Neolithic period (**Figure 1.7**) (10). It is obvious now that MTBC has evolved together with humans for a long time and that they have thus influenced reciprocally their evolution (74). Among these lineages, the ancient lineage 1 emerged as early as 67,000 years ago, coinciding with the initial migration of humans out of Africa towards the Indian Ocean (10). The modern lineages 2, 3, 4 would have emerged about 46,000 years ago, coinciding with the second wave of human migration towards the Middle East, Europe and East Asia. The lineage 7 would have emerged 64,000 years ago and is still strictly associated with the Horn of Africa (10, 68). Finally, the lineages 5 and 6 seem to have emerged earlier than other lineages and as lineage 7 stayed on the African continent (10).

C. Global distribution and *M. tuberculosis* lineages

Detailed phylogenetic analyses suggested that during these migration events, the *M. tuberculosis* lineages would have adapted to different human populations (10, 62). Indeed, the lineages are strongly associated with geographical areas, their names reflecting the geographical origin of the *M. tuberculosis* population (**Figure 1.8**) (62).

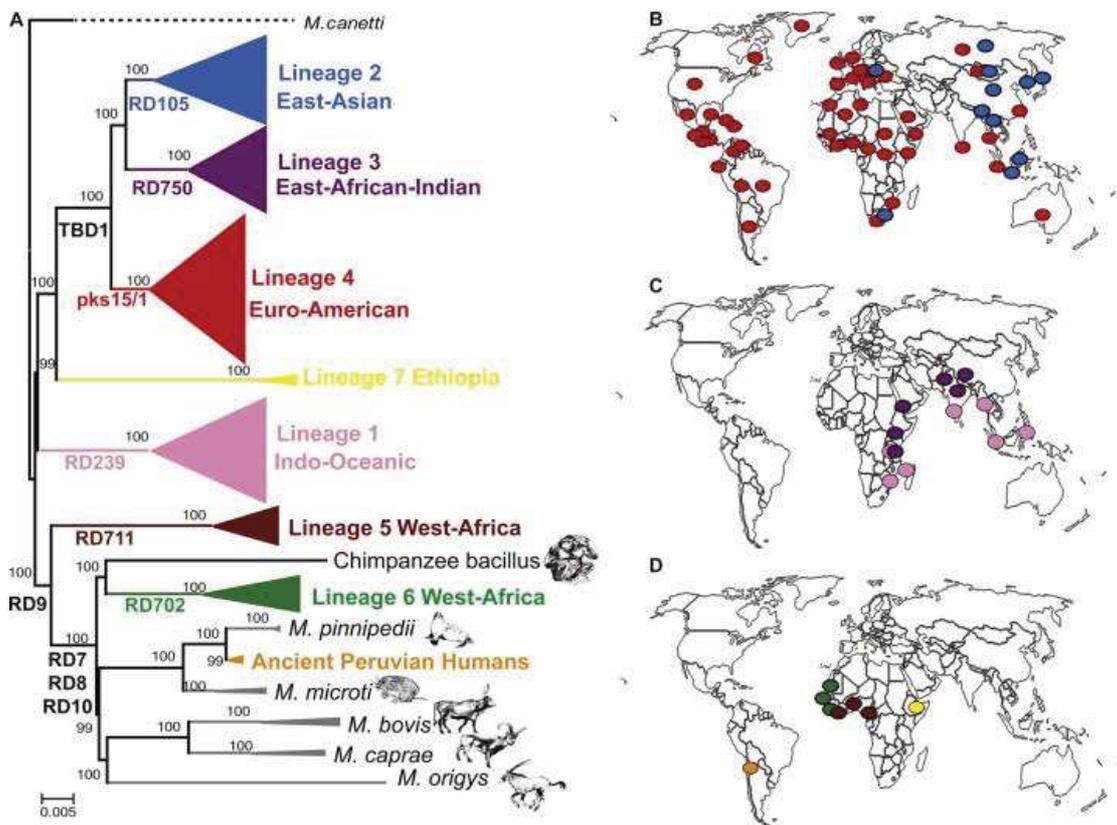


Figure 1.8. Phylogeny of the MTBC and distribution of the 7 main *M. tuberculosis* lineages

(Source from Coscolla and Gagneux (62). (A) Node support after 1000 bootstrap replications is shown on branches and the tree is rooted by the outgroup *M. canettii*. Large Sequence Polymorphisms (LSPs) are indicated along branches. Scale bar indicates the number of nucleotide substitutions per site. (B–D) Dominant MTBC lineages per country. Each dot corresponds to 1 of 80 countries represented in the 875 MTBC strains from the global strain collection analyzed by Gagneux *et al.* (10). The yellow dot represents the Lineage 7 in Ethiopia and the orange one the extinct MTBC strains from Peru, respectively. Panel (B) shows the most geographically widespread lineages, panel (C) the intermediately distributed lineages, and panel (D) the most geographically restricted lineages.)

The ancient lineage 1 (Indo-Oceanic, mainly EAI family) is reported in East Africa, but also spread all around the Indian Ocean and is frequently reported in Southeastern and Southern Asia, accounting for over 33 – 73% of total cases (75, 76).

This family is also prevalent in Northern Europe, Middle East and Central Asia, and in Oceania (22 – 25% of total cases) (75, 76).

Lineage 2 (East-Asian, mainly Beijing family) is one of the most virulent *M. tuberculosis* lineages and is spreading all over the world (75-77). More specifically, the Beijing family is predominant in East and South East Asia and in the countries of former Soviet Union, accounting for over 50 - 85% of total cases (75, 76). This family is also highly prevalent in Oceania, Africa (except the West) and in North America (more than 17% of total cases) (75, 76). However, this lineage is less detected in the other regions of the world, such as in Northern Europe, India, Central and South America, and in Middle East (less than 10% of total cases) (75, 76). The Beijing family was found at very high frequency with more than 85% in the Beijing region of China (78). This family is also found in high proportion in Mongolia, South Korea, Hong Kong, Taiwan, Vietnam, Thailand, and Malaysia (75, 76, 79). Overall, this lineage accounted for 13% of all *M. tuberculosis* lineages (76).

The lineage 3 (East-African-Indian, mainly CAS family) is essentially localized in the Southern and Western Asia (9 – 30% of total cases, mainly in the South) (75, 76). This lineage is also found in the Eastern and Northern Africa (7 – 12% of total cases, mainly in the East) (75, 76). In several other regions including Central and North America, Europe, Far-East-Asia, and Oceania, this lineage is less frequent (0.1 - 5% of total cases) (75, 76). This lineage is predominant in India, Iran, and Pakistan, accounting for over 50% of total cases (75, 76).

The lineage 4 (Euro-American) consists of 10 different families, in which 5 main families LAM, T, X, H and S that are widespread throughout the world (75, 76). Molecular epidemiology data showed that this lineage is the most frequent in Europe and Americas, but is also dominant in North Africa, Middle East and Oceania (10, 75, 76). However, the distribution of these specific families varies according to the regions. The T family was found in all continents, accounting for 20 – 35% of total cases (75, 76). The LAM family is the most represented in Americas (20 – 50% of total cases, mainly in the South), in Oceania (20% of total cases) and in all sub-regions of Africa (37% of total cases, except the West). The H family is the most represented family in Europe (24% of total cases) and in America (15 – 25% of total cases, mainly in the Caribbean region), while the X family is prevalent in Americas (8

– 21% of total cases, mainly in the North). Finally, the S family is found in Africa (5 – 8% of total cases, mainly in the North) and in Southern Europe (5.8% of total cases) (75, 76).

In summary, the molecular epidemiology and clinical studies showed that the most geographically widespread lineages, which are the lineages 2 and 4, are the more virulent ones (62, 80-82). In particular, Beijing family has been reported to be associated with young people, high virulence, drug resistance and MDR, relapses and treatment failures in many countries in the world (9, 57, 60, 62, 77, 83-85).

1.2.7 Mechanisms of drug resistance in *Mycobacterium tuberculosis*

As described above, MDR and XDR-TB cases seriously compromise global efforts to control TB. Despite the improvement of diagnostic and TB patient care, globally there is still a slight trend for MDR-TB cases to increase with the burden of MDR-TB either increasing faster or decreasing more slowly than the overall TB burden in each country (1).

Although the majority of FLDs and SLDs have been developed more than 50 years ago, they are still the bases of treatment regimens for all TB forms (3, 86). The modes of action of these drugs have been well established and the mechanisms of resistance have been largely documented (87, 88). Nevertheless, still today the way some isolates become drug resistant remains unexplained since many intrinsic and extrinsic factors can be involved in these processes. Besides, the emergence of MDR- and XDR-TB leads to the use of uncommon SLDs and new anti-TB agents even if the mode of action of these drugs and the mechanisms of resistance are still little known and they have sometimes no clear effects and many adverse sides (3, 86).

For the first time in many years, new anti-TB agents, BDQ and DLM, have been endorsed by WHO for the treatment of adults with MDR-TB in 2013 and 2014, respectively (12). These two drugs and several other new ones are in Phase II and III clinical trials and have shown potent *in vitro* and *in vivo* activity against both drug-susceptible and drug-resistant *M. tuberculosis* strains (89-91). The modes of action of the new anti-TB agents are different from FLDs and SLDs and the resistance mechanisms *in vitro* and *in vivo* need to be investigated. However, some resistances to

the new anti-TB drugs and cross-resistances with SLDs have been already reported in clinical *M. tuberculosis* isolates (92, 93). *M. tuberculosis* may resist antibiotic action by using different mechanisms that can be subdivided in two categories, innate or acquired drug resistances.

A. Innate drug resistance

The intrinsic resistance is the innate ability of bacteria to resist to antibiotics. In *M. tuberculosis*, intrinsic drug resistance has been attributed to the highly impermeable cell envelope and the active drug efflux mechanism (94-98). For instance, *M. tuberculosis* is intrinsically resistant to macrolides such as clarithromycin, while clofazimine and beta-lactam antibiotics have uncertain activity (Andini et al. 2006, Dooley et al 2013, Wivagg et al. 2014).

As described above, the cell envelope of *M. tuberculosis* is very complex and has characteristics of both Gram-positive and Gram-negative bacteria (97, 98). The mycolic acid layer and the multi-layer with various degrees of hydrophobicity lead to a highly impermeable cell envelope, which acts as an effective barrier for the penetration of anti-TB drugs (48, 98). The low permeability of cell wall allows this pathogen to tolerate diverse classes of drugs (48, 95, 96). However, this function is important but not a decisive determinant of drug resistance.

The efflux of anti-TB drugs by membrane transporters is an important mechanism in *M. tuberculosis* (94, 96, 99, 100). In general, the increased activity of efflux pumps (EPs) results in the reduction of intracellular levels of drugs, which can enable the survival of a bacterial subpopulation (96, 99, 100). This mechanism often confers low-level of drug resistance and can lead to high-level of drug resistance by selection of drug resistant mutants (94, 96, 100, 101). For instance, the prolonged exposure of *M. tuberculosis* to INH or EMB can induce activation of EPs, resulting in an increased resistance phenotype (99, 101). The EPs involved in drug resistance in *M. tuberculosis* are listed in the **Table 1.2**.

Table 1.2. The major drug efflux pumps in *M. tuberculosis* and affected drugs

Transporter family	Drug efflux pump(s)	Affected drug(s)
--------------------	---------------------	------------------

MFS	Rv1258c (Tap)	STR, INH, RIF, FQs, and tetracycline
	Rv1410c (P55)	STR, INH, RIF and CFZ
	Rv1634	FQs
	Rv2459 (JefA), Rv3728 and Rv3065	INH and EMB
	Rv2846c (EfpA)	INH and ETO
ABC	Rv2936-Rv2937-Rv2938 (DrrABC)	EMB, FQs and STR
	Rv2686c-Rv2687c-Rv2688c	FQs
	Rv0194	STR, ampicillin, novobiocin, chloramphenicol
	Rv0341-Rv0342-Rv0343 (IniBAC)	INH and EMB
	Rv1218c	novobiocins, pyrazolones, pyrroles
SMR	Rv3065 (Mmr)	INH, erythromycin and acriflavine
RND	MmpL7	INH
	MmpL5-MmpS5	BDQ and CFZ
	MmpL3	SQ109
Regulatory protein	whiB7	INH

ABC: ATP-binding cassette; MFS: major facilitator super-family; RND: resistance nodulation division, SMR: small multidrug resistance. See the reviews for details (94, 96, 102).

Although all the anti-TB drugs cannot be eliminated by the efflux systems (103), the use of EP inhibitors as an adjuvant therapy might be considered as an interesting option (104-106). For instance, Adams *et al* demonstrated that verapamil reverses tolerance to most anti-TB drugs, including the new agents such as MFX, LZD, PA-824, and BDQ, and inhibits intracellular *M. tuberculosis* growth (107). Recently, Li *et al* also showed that verapamil and chlorpromazine could significantly increase RIF susceptibility in mono-RIF resistant *M. tuberculosis* (108).

B. Acquired drug resistance

Unlike many bacteria, *M. tuberculosis* follows a pure clonal evolution model without horizontal transfers. Thus, acquired drug resistance in this pathogen is mainly caused by spontaneous mutations in drug resistance-associated genes (63). Herein, I will present in details the molecular mechanisms of resistance to the drugs widely used in clinical settings. I will also briefly present the new approved drugs as well as

the new preclinical drugs. The mode of action and the resistance mechanism for each drug as well as the mutation rate for each drug-resistance gene are described in the **Table 1.3**.

Table 1.3. Description of the modes of action of anti-TB drugs as well as the genes involved in drug resistance, the mutation rates and the mutation frequencies in clinical drug-resistant *Mycobacterium tuberculosis* isolates

Drug	Drug action	Gene involved in drug resistance	Encoding for	Mutation frequency (%)	Mutation rate [#]
Group 1					
RIF	Binding to the β -subunit of the RNA polymerase, inhibition of the elongation of messenger RNA	<i>rpoB</i>	β -subunit of RNA polymerase	90 – 100	9.8×10^{-9} to 2.3×10^{-10}
INH	- Activation by a catalase peroxidase enzyme	<i>katG</i>	Catalase peroxidase	40 – 97	3.2×10^{-7} to 2.6×10^{-8}
	- Inhibition of the synthesis of mycolic acids through binding to NADH-ACP-reductase	<i>inhA</i> and its promoter	Fatty acid enoyl acyl carrier protein reductase A (InhA)	8 – 64	
EMB	Inhibition of an arabinosyl transferase involved in cell wall synthesis	<i>embB</i>	Arabinosyl transferase	47 – 89	1.0×10^{-7}
PZA	- Activation by the pyrazinamidase - Disruption of membrane energetics that inhibits membrane transport	<i>pncA</i>	Pyrazinamidase	44 – 97	$1-1.5 \times 10^{-5}$
Group 2					
STR*	Inhibition of protein synthesis by interaction with the 16S rRNA and the S12 ribosomal protein	<i>rrs</i>	16S rRNA	12 – 26	2.95×10^{-8}
		<i>rpsL</i>	S12 ribosomal protein	40 – 68	
		<i>gidB</i>	7-Methylguanosine methyltransferase	5 – 13	
AMK, KAN & CAP	Inhibition of protein synthesis	<i>rrs</i>	16S rRNA	40 – 90	1.4×10^{-8} to 3.9×10^{-7}
KAN	Inhibition of acetyltransferase	<i>eis</i>	aminoglycoside acetyltransferase	28 – 80	3.85×10^{-7} to 1.36×10^{-8}
CAP	Inhibition of methylation of 16S rRNA & 32S rRNA	<i>tlyA</i>	2'-O-methyltransferase	4 – 13	$4.9-7.8 \times 10^{-8}$
Group 3					
OFX, LFX, MFX & GFX	Inhibition of the topoisomerase II (DNA gyrase), inhibition of DNA supercoiling	<i>gyrA</i>	DNA gyrase subunit A	70 – 90	2×10^{-8} to 2.3×10^{-9}
		<i>gyrB</i>	DNA gyrase subunit B	0 – 11	

Group 4					
ETO	- Activation by a monooxygenase (<i>ethA</i>) - Inhibition of the synthesis of mycolic acids by forming an adduct with NAD that inhibits the enoyl-ACP reductase. - EthA is regulated by the transcriptional repressor EthR	<i>inhA</i> <i>ethA</i> <i>ethR</i>	- Fatty acid enoyl acyl carrier protein reductase A (InhA) - Monooxygenase (EthA) - Transcriptional repressor EthR, NADH-ACP	33 – 62 46 – 72 0 – 4	10 ⁻⁷
CS	Blocking of the activity of D-alanine and D-cycloserine and thus inhibition of the synthesis of peptidoglycan	<i>alr</i> <i>ald</i> <i>cycA</i>	Alr protein Ald protein <i>cycA</i> encodes a D-alanine transporter	NA	NA
PAS	Inhibition of the dihydropteroate synthase and folate biosynthetic pathway	<i>thyA</i> <i>folC</i>	thymidylate synthase A (ThyA) FolC protein	37 – 50	10 ⁻⁸
Group 5					
CFZ	Production of reactive oxygen species, inhibition of energy production, membrane disruption	<i>rv0678</i> <i>pepQ</i>	-Transcriptional regulator (Rv0678) of MmpL5-MmpS5 -PepQ protein	NA	5x10 ⁻⁶
LZD	Inhibition of protein synthesis	50S ribosomal subunit	<i>rrl</i>	2 – 11	1.1x10 ⁻⁸ to 5x10 ⁻⁹
New drugs					
BDQ	Inhibition of ATP synthase	<i>atpE</i> <i>rv0678</i>	The c protein of ATP synthase	NA	4.7x10 ⁻⁸ to 8.9x10 ⁻⁹
DLM	-Inhibition of methoxy- and keto-mycolic acid -Activation by the bacteria	<i>fgd</i> , <i>ddn</i> , <i>fbiA</i> , <i>fbiB</i> , <i>fbiC</i>	F420 cofactors	NA	4.19x10 ⁻⁷ to 6.44x10 ⁻⁸
PA-824	Inhibition of the synthesis of proteins and cell wall lipids Activation by the bacteria	<i>fgd</i> , <i>ddn</i> , <i>fbiA</i> , <i>fbiB</i> , <i>fbiC</i>	F420 cofactors	NA	6.7-9.0x10 ⁻⁷
SQ-109	Inhibition of cell wall synthesis	<i>mmpL3</i>	trehalose monomycolate (MmpL3)	NA	2.55x10 ⁻¹¹
AZD-5847	Inhibition of protein synthesis	50S ribosomal subunit	<i>rrl</i>	NA	5.5x10 ⁻⁸
Sutezolid (PNU-100480)	Inhibition of protein synthesis	50S ribosomal subunit	<i>rrl</i>	NA	10 ⁻⁸

Note: Only the most commonly used FLDs and SLDs and the new developed anti-TB drugs are described in this table. NA: not available. See the following reviews for details (87, 109, 110)

C. *Molecular mechanisms of resistance to FLDs and SLDs*

❖ **Isoniazid**

INH was introduced in 1952 as an anti-TB agent and is one of the cornerstones of the standard treatment regimen for drug-sensitive TB patients (35). This drug has also been widely used for the treatment of latent TB infections (111, 112). Among anti-TB drugs, INH has the strongest early bactericidal activity (EBA) against rapid growing tubercle bacilli, but is not active against non-replicating bacilli or under anaerobic conditions (113-115). Nevertheless, resistance to INH is commonly observed, associated with various drug-resistance patterns and showed a higher resistance rate than other FLDs (except STR) (16).

Mechanism of INH resistance has been associated with multiple genes (87, 88), but the role of some genes remains unclear (116). INH is a pro-drug that requires activation of the catalase-peroxidase enzyme encoded by *katG* gene, while the primary target of INH inhibition is thought to be the InhA enzyme (enoyl-acyl carrier protein reductase), encoded by *inhA* gene (117). The main mechanism of INH resistance is due to mutations in the two main genes *katG* and *inhA*, accounted for 89-100% of INH-resistant isolates (117-119). Mutations in *katG* gene lead either to a reduction or a loss of activity of the KatG enzyme, while mutations in the promoter or coding region of *inhA* gene either leads to the overexpression of InhA enzyme or affects the active site (117). The frequency of mutations in these two genes varies according to the country (88).

The mutation frequency in the *katG* gene ranges between 40 - 95% of INH-resistant clinical isolates, among which the Ser315Thr mutation accounted for 32 - 95% of all INH-resistant isolates (116, 118-121). This mutation has been reported worldwide and has been associated with high-level of INH resistance (MIC \geq 1mg/L), MDR and low fitness cost (low impact on the organism's ability to survive and reproduce) (120, 122, 123). Many other mutations have also been detected at low frequency (116-118). Besides many other infrequent mutations associated with low- or high-level of INH resistance, deletion of a part or of the whole *katG* gene, generally linked to high-level of INH resistance, was detected in few clinical INH-resistant isolates (118, 124).

Mutations in *inhA* and its promoter often confer low level of INH resistance [56]. Approximately 0-5% of INH resistant *M. tuberculosis* isolates have mutations in the *inhA* coding region, whereas 8-64% have mutations in the promoter (116, 119). The most common mutations in the promoter region were detected at positions -8, -15, -17 and in *inhA* coding region were observed at codons 21, 94 and 95 (116, 117, 119). A study from South Africa showed very high frequency of *inhA* mutations (especially *inhA*-15 (C-T)) in XDR-TB cases (85.5%), compared to 48.4% in MDR-TB and 30.1% in INH-resistant non-MDR TB cases (119). *InhA* mutations can also confer resistance to ethionamide and prothionamide (117, 125).

Systematic review showed that detection of the two most frequent mutations, *katG*315 (Ser-Thr) and *inhA*-15 (C-T) accounted for more than 76.0% of INH-resistant isolates (119, 126, 127). Mutations in other genes such as *ahpC*, *ndh*, *kasA* and *furA* have also been reported to be associated with INH resistance, but at low frequency, often combined with *katG* mutations and also found in sensitive isolates (116, 117). This underlines that these mechanisms need to be further investigated (116, 117).

❖ Rifampicin

RIF was discovered in the 1960s and is one of the most potent anti-TB drugs that greatly shortened the duration of TB chemotherapy from 12 – 18 months to 9 months (35). RIF shows not only EBA but also sterilizing activity against slow-growing and even non-replicating bacterial populations (35, 114, 115, 128). Moreover, together with INH, these drugs kill more than 99.0% of tubercle bacilli in TB patients within the initial two-month intensive phases of the therapy. The residual dormant bacilli are normally eliminated during the continuation phase of the treatment course (35, 114). For these reasons, RIF remains a frontline anti-TB drug and is widely used in the TB control program worldwide, despite the emergence and spread of RIF-resistant *M. tuberculosis*, particularly MDR strains (12).

The mechanism of action is to inhibit the mycobacterial transcription by targeting DNA-dependent RNA polymerase encoded by the β subunit of *rpoB* (129, 130). RIF binds in a pocket of the RNA polymerase β subunit deep within the DNA/RNA channel (129, 130). Previous studies demonstrated that mutations in the

four distinct regions of *rpoB* encompassing cluster I (CI, codons 505-537), cluster II (CII, codons 562-575), cluster III (CIII, codons 684-690) and N-terminal region (codons 143-148) were associated with RIF resistance in *M. tuberculosis* (note: *E. coli* *rpoB* codon system) (131-136). 90-100% of RIF-resistant clinical isolates displayed mutations in the 81-bp hot spot region also called Rifampicin Resistance-Determining Region (RRDR) corresponding to codons 507-533 within the cluster I of *rpoB* gene (131-136). Mutations found in the other regions of *rpoB* may be responsible for approximately 5% of the RIF-resistant isolates (137, 138). Mono-resistance to RIF is quite rare and over 90% of RIF-resistant isolates were also resistant to INH (134, 139). Therefore, resistance to RIF is considered as a surrogate marker for MDR-TB (16, 131).

Mutations in *rpoB* are very diverse, single, double and multiple mutations, codon deletions and insertions were detected (131-136). In the RRDR region, the codons, 510, 511, 513, 514, 516, 526, 531 and 533 are directly involved in the interactions with RIF and mutations in these codons generally lead to RIF resistance (129, 130). Mutations at these codons accounted for over 90% of the RIF-resistant isolates (130). Particularly, mutations at the three main codons 516, 526 and 531 have been frequently observed (80%) in RIF-resistant isolates, and thus have been used as main markers for detection of RIF-resistant *M. tuberculosis* isolates (131-136, 140). Nevertheless, mutations at codons 511 and 533 often confer low level of RIF resistance and are also found in RIF-sensitive isolates (130).

Outside the RRDR, mutation at codon 572 located in the CII is associated with low-level of RIF resistance, while mutation at codon 170 within N-terminal region showed high-level of RIF resistance (137, 141-143). These mutations accounted for 1 - 4% of RIF-resistant isolates and up to 100% of RIF-resistant ones without mutations in the RRDR (134, 137, 138). However, recent study showed a high frequency (30%) of *rpoB* 572 (Ile-Phe) mutations in MDR clinical strains (28). The commercial kits including LPA and Xpert MTB/RIF assays do not detect these last mutations.

❖ Pyrazinamide

PZA, a nicotinamide analog, was discovered in 1952 and introduced into TB chemotherapy in the early 1950s (35). This drug is an important FLD used with INH and RIF for shortening TB treatment from the previous 9–12 months to 6 months (35, 144). PZA is the only FLD maintained in the new regimens in trial for treating all TB forms (3, 34). This drug is believed to exert the greatest bactericidal activity against the population of persistent bacilli contained within macrophages or in acidic pH environment in the lesions that is not killed by other drugs (35, 145). PZA has high sterilizing activity *in vivo*, but no activity against tubercle bacilli in normal culture conditions at neutral pH (145). Global prevalence of PZA resistance is little known because of no standardized DST method (22).

PZA is a pro-drug that needs to be converted to its active form, pyrazinoic acid (POA), by the enzyme *pyrazinamidase* (PZase) coded by the *pncA* gene (146). The proposed mechanism of PZA action is that POA, its active moiety, disrupts bacterial membrane energetics and thus interferes with energy production, necessary for survival of *M. tuberculosis* in acidic site of infection (146). Mutations in the *pncA* gene and its promoter are the major mechanism of PZA resistance (147, 148). The *pncA* mutations are diverse including non-synonymous mutations, nucleotide insertions or deletions and total *pncA* deletion (149, 150). These mutations are scattered in the coding and promoter region throughout *pncA* gene without any prevailing mutations (149, 150). A recent systematic review revealed more than 600 mutations affecting 171/187 codons of *pncA* gene (149). Nevertheless, three hot spot regions in *pncA*, containing catalytic and metal-binding sites of the PZase enzyme and including codons 3–17, 61–85, and 132–142 have been identified (147, 151, 152). Almost all *pncA* mutations cause reduction or loss of PZase activity leading to high-level of PZA resistance (149, 153, 154). Mutations in *pncA* promoter may disturb the translation of the *pncA* gene leading to low or high-level of PZA resistance (147, 149, 155). The frequency of *pncA* mutations varies by settings, ranging between 40 - 99% of PZA-resistant isolates (147, 148, 156). Besides, about 10% of PZA-susceptible clinical isolates also revealed *pncA* mutations (149, 150). Since phenotypic PZA-susceptibility testing was less reliable, discordance between phenotypic and genotypic data is generally obtained (23, 157). Overall, systematic reviews showed that more

than 80% of PZA-resistant isolates revealed high confident *pncA* mutations, which could be linked to PZA resistance (149, 150).

Recently, it was shown that some PZA-resistant clinical isolates without *pncA* mutations had mutations in the *rpsA* gene encoding ribosomal protein S1 (RpsA protein), which has also been proposed as a target of PZA (158). Mutations in *rpsA* gene were reported to be associated with a low-level of PZA resistance (158-160). Nevertheless, *rpsA* mutations are less frequent, generally accompanied with *pncA* mutations and also found in PZA-sensitive isolates, suggesting that the role of *rpsA* in PZA resistance needs to be further investigated (159-163). Besides, some PZA resistant isolates revealed mutations in *panD* gene, which encodes for aspartate decarboxylase enzyme (PanD) (164, 165). Nevertheless, some recent studies could not find any mutations in *panD* gene among PZA-resistant isolates without mutations in *pncA* and *rpsA* genes, suggesting no correlation between *panD* mutations and PZA resistance (160). Finally, a small number of PZA-resistant isolates did not show any mutation in *pncA*, *rpsA* and *panD*, supporting the existence of other mechanisms such as POA efflux (151).

❖ Ethambutol

EMB was first introduced for treatment of TB in combination with INH and SM in 1960s (35). Nevertheless, this drug was stopped since the two potent FLDs, RIF and PZA were adopted into the standard 6-month short-course therapy in 1970s (35, 37). Since 1995, because of the high rate of INH resistance, EMB was added in the 6-month standard regimen, which is currently recommended by WHO for treating drug-sensitive TB patients (166). EMB is a bacteriostatic drug that is active against growing bacilli and has no effect on non-replicating bacilli (35). This drug is less effective against *M. tuberculosis* than INH and RIF. Similar to RIF, mono-EMB resistance is rare in clinical samples (167). EMB is commonly used in combination with other anti-TB drugs for treating all TB forms including MDR and XDR TB (EMB-sensitive cases confirmed by DST) (3, 86). Even if the mechanism of action of this drug is not fully understood, we know that the drug inhibits arabinosyl transferase encoded by *embA*, *embB* and *embC* genes involved in mycobacterial cell wall synthesis, suggesting that mutations in these genes are associated with EMB resistance (168-174).

Mutation in *embB* gene is the main mechanism of EMB resistance accounting for 50 - 89% of EMB-resistant isolates (168, 173, 175, 176). Mutations in *embA* and *embC* were less frequent, ranging between 3 - 7% of EMB-resistant isolates, and often accompanied by *embB* mutations (171, 172, 177). Mutations in the EMB resistance-determining region (ERDR) of *embB* gene, encompassing codons 298-318, were commonly observed in EMB-resistant mutants (168, 170). Among them, *embB306* mutations were the most common, accounting for 50-90% of EMB-resistant isolates. Several studies showed an association of *embB306* mutations with MDR (167, 175, 178, 179). However, the *embB306* mutations were also found at high frequency (10 - 45%) in EMB-susceptible isolates, particularly in isolates resistant to other FLDs. This aspect questions the real role of this codon in EMB resistance (167, 175, 178, 180, 181). Mutagenesis experiments have confirmed that *embB306* mutations increase MICs (174, 179, 182). Epidemiological data and *in vitro* studies have also confirmed that mutations in two other common codons *embB3406* and *embB3497* were associated with EMB resistance (168, 169, 173-175, 177, 183). The frequency of these mutations was up to 51% in non *embB306* mutants (175). Similarly to *embB306* mutations, mutations in these two codons were also found in EMB-sensitive isolates (175, 183). In brief, targeting these 3 mutations *embB306*, *embB3406* and *embB3497* can detect 50 - 80% of EMB-resistant isolates (168, 173, 175, 176). This is all the more important that the EMB-susceptibility testing is difficult to perform and poorly reproducible (184).

Recently, mutations in *ubiA* gene (Rv3806c) have been reported leading to an increased MICs of EMB (169). These mutations were associated with high-level of EMB resistance. This study also found that, the synonymous mutations in *aftA* gene (Rv3792) could also increase the expression of *embC*, resulting in EMB resistance (169).

❖ Streptomycin

STR was discovered in 1943 and was the first antibiotic used in the treatment of TB in early 1940s (35). However it was used as mono-therapy at that time that led to rapid emergence of STR-resistant strains. STR activity against *M. tuberculosis* has shown to be approximately equivalent to EMB, and therefore it can be replaced by

EMB in the standard treatment regimen (35). STR kills actively growing tubercle bacilli, but it is inactive against non-growing or intracellular bacilli (35).

STR interacts directly with the 30S subunit of the ribosome, thereby interfering with bacterial protein biosynthesis (185-187). The main targets of STR are 16S rRNA encoded by *rrs* gene and ribosomal protein S12 encoded by *rpsL* gene (187). STR interferes with the protein synthesis by binding with the phosphate backbone of the 16S rRNA in the main domain encompassing the 530 and 915 loops of *rrs* gene (185-187). This binding results in forming both salt bridges and hydrogen bonds, preventing the contact with the S12 ribosomal protein and eventually leading to a misreading of the genetic code during translation (187). Mutations in *rpsL* and *rrs* genes are major mechanisms of STR resistance (188, 189). Mutations in *rpsL* gene are the most common (19 - 78%), and often associated with high level of STR resistance. While mutations in *rrs* gene are less frequent (10 - 28%) and often associated with low level of STR resistance (188-190). In *rpsL* gene, STR resistance-associated mutations were found mainly at two codons 43 and 88 (88). Whereas, STR resistance-associated mutations in *rrs* gene were found in several positions such as 513, 514 and 517 of the 530 loop and 905, 906, 907 and 908 of the 915 loop (88, 188, 191-193). For both genes, the frequencies of mutations significantly vary according to settings (188, 190-193). Taken together, approximately 48 - 93% of the STR-resistant isolates revealed STR-resistant mutations in *rpsL* and/or *rrs* gene (188, 190, 193).

It has been observed that mutations in *gidB*, encoding for a 7-methylguanosine methyltransferase specific for the 16S rRNA, can confer low-level of STR resistance in isolates without mutations in *rpsL* and *rrs* gene (194, 195). Mutations in *gidB* gene are diverse and distributed throughout the gene and found at high frequency (33%) in STR-resistant isolates (195). However, mutations in *gidB* are often accompanied by mutations in *rpsL* and/or *rrs* gene and were also found in STR-sensitive isolates, suggesting that the role of *gidB* mutations in STR resistance need to be further investigated (176, 190, 194).

❖ **Second-line injectable drugs**

SLIDs including the aminoglycosides (KAN and AMK) and the polypeptides (CAP and viomycin (VIO)), together with FQs are the mainstay in the treatment of

MDR and XDR-TB cases (196). These drugs show high extracellular bactericidal activity, but some of them have also intracellular bactericidal activity (35, 197). Nevertheless, KAN and AMK have poor activity against slowly multiplying bacilli, while CAP show significant activity against non-replicating *M. tuberculosis* (198). It is worth noting that there is an incomplete cross-resistance between the SLIDS (199-202). Most of the strains that acquire resistance to AMK are resistant to both KAN and CAP, while strains acquiring resistance to KAN show different levels of cross-resistance to AMK and CAP (22). Besides, mono-KAN, or CAP resistances have also been detected (203).

Although they belong to different drug classes, all of them display the same mechanism of action by inhibiting the protein synthesis (199, 200, 204). The aminoglycosides inhibit protein synthesis by modification of the ribosomal structures in the 16S rRNA, encoded by *rrs* gene (199, 205). CAP and VIO bind across the ribosomal interface involving helix 44 (H44) of 16S rRNA and helix 69 (H69) of the 23S rRNA, resulting in the inhibition of the protein synthesis (206). In addition, the *tlyA* gene encodes a 2'-O-methyltransferase and mutations at positions 1409 of H44 and 1920 of H69 inhibiting methylations resulting of resistance to CAP and VIO (206).

Mutations in the 1400 – 1500 region of *rrs* gene seem to be major mechanism of resistance to SLIDs, accounting globally for 30 - 90% of drug-resistant isolates (176, 183, 201, 202, 207-209). The mutations at positions 1401 (A-G) and 1484 (G-T) of *rrs* are linked to high-level of resistance to all SLIDS, while the mutation at position 1402 (C-T) is associated with high-level of resistance to KAN and CAP (176, 183, 201, 202, 207, 208). The mutations at position *rrs*1401 were present in the majority of drug-resistant *rrs* mutants (183, 201, 202, 207, 208). However, high frequency (40%) of CAP-susceptible isolates carrying the *rrs*1401 has been reported (210).

Overexpression of *eis* gene encoding for the aminoglycoside acetyltransferase enzyme has also been reported to be associated with a low-level of KAN resistance (211). Mutations found in the promoter of *eis* gene was observed in 29 - 80% of KAN-resistant isolates, but also found in 3% of KAN-sensitive ones (183, 211, 212).

The combination of mutations in *rrs* and *eis* could cover up to 86% of KAN-resistant isolates (183).

Mutations within *tlyA* are infrequent, scattered along the gene, accounting for only 4 - 13% of CAP-resistant clinical isolates (183, 208, 213). Nevertheless, a study from Portugal showed 57.7% of XDR isolates with mutations in this gene, particularly the insertion at position 755 (G-T) accounting for 42.3% (209). The *tlyA* mutations were also found in CAP-sensitive isolates, and some studies did not find any mutations in *tlyA* in CAP-resistant isolates (183, 208). In addition, the *tlyA* mutations were often accompanied by *rrs* mutations and not all mutations in *tlyA* lead to CAP resistance, suggesting that *tlyA* plays a minor role in CAP resistance (183, 213).

❖ Fluoroquinolones

Among the FQs, OFX is as second-generation FQ, LFX is the third-generation, MFX and GFX are the fourth-generation (3, 214). Several studies showed variable level of cross-resistances between them (86, 215, 216). However, clinical results showed that strains resistant to OFX can be still treated with the later FQ generations (215, 216). MFX and GFX have greatest bactericidal activities (only slightly lower than INH), followed by LFX and OFX (217, 218). Nevertheless, all the FQs have a greater extended EBA than INH (217). In addition, MFX has a bactericidal activity against slow growing bacilli and also a sterilizing activity against persistent bacilli that are tolerant to high RIF concentrations (218). MFX and GFX are currently in clinical trials for treatment of drug-susceptible TB and are proposed as first-line antibiotics with the goal of shortening duration of TB treatment to 4 months (31-33, 41, 42).

In *M. tuberculosis*, the principal target of the FQs is the DNA gyrase, a type II DNA topoisomerase encoded by *gyrA* and *gyrB* genes. Mutations in the highly conserved FQ resistance-determining regions (QRDRs) of these genes were associated with FQ resistance (219-224). The QRDRs were determined from codon 74 to 133 of *gyrA* and from codon 461 to 501 of *gyrB* (219, 225). High frequency of *gyrA* mutations were observed in FQ-resistant clinical isolates, accounting for 70 - 90%, while *gyrB* mutations were infrequent represented only 0 - 11% of FQ-resistant

isolates. Combined *gyrA-gyrB* mutations could detect 80 - 95% of FQ-resistant isolates (6, 226-229).

In *gyrA* gene, mutations in codons 94 and 90 were the most common (6, 226-229). Nevertheless, *gyrA94* mutations were detected at higher frequency with various changes (Asp-Gly/Ala/Tyr/Asn/His) often associated with high-level of FQ resistance and low fitness cost compared to mutations in codon 90 (Ala-Val) (228, 230-232). The mutation *gyrA91* (Ser-Pro) associated with low-level of FQ resistance was less common than the mutations at codons 90 and 94 but was found at higher frequency (42.3%) in XDR samples from Portugal (209). The infrequent mutations at codons 74, 80, 88 and 89 in *gyrA*, associated with low or high-level of FQ resistance, were also reported (227-229, 233). The combination of the two mutations *gyrA80* (Thr-Ala) and *gyrA90* (Ala-Gly) caused hyper-susceptibility to FQs (224). Various mutations of *gyrB* gene were associated with high or low-level of FQ resistance (6, 224, 226, 228, 229). The mutations occurred independently in *gyrB* and *gyrA* genes, but sometimes they were detected in combination (228, 234). Finally, double mutations found in *gyrA* gene and in both *gyrA* and *gyrB* genes revealed high level of FQ resistance (224, 228). Previous studies showed that there is no clear correlation between the type of mutations in the *gyrA* and *gyrB* genes and the MIC level of FQs (224, 227, 228). Mutations in the QRDR of *gyrA* gene were strongly associated with OFX resistance, but the level of OFX resistance may not be predicted based on the mutation patterns in this gene (224). Determining the resistance levels of individual mutations in *M. tuberculosis* is imperative to ensure that strains with a low-level of FQ resistance can be effectively treated with high-dose of FQs or the newest generation of FQs.

❖ Resistance to other SLDs

Almost all the modes of action and mechanisms of resistance to SLDs including ethionamide, 4-aminosalicylic acid, CS, CFZ and LZD are not yet fully understood. The investigated molecular mechanisms of these drugs are described in the Table 1.3. In brief, mutations in *ethA* and in *inhA* or *inhA* promoter region are the main molecular mechanisms of ETH resistance (117, 235). Nevertheless, mutations in the *ethR* (*ethA* regulator) and *mshA* (encoding for a glycosyl transferase) have also been reported to be associated with ETH resistance (235, 236). Overall, 81% of the ETH-resistant isolates had mutations in one of these genes (236).

Mutations in the thymidylate synthase A, encoded by the *thyA* gene involved in thymine biosynthesis in the folate pathway have been associated with resistance to PAS (237-239). In addition, mutations in *ribD*, *dfrA* and *folC* genes were found in both PAS-resistant laboratory strains and clinical isolates, suggesting that they may be involved in PAS resistance (239-241). However, only 61.1% of PAS-resistant isolates revealed mutations in *folC*, *thyA*, and *ribD* genes, suggesting that EPs could confer PAS resistance (238, 241). Various mutations in the promoters or coding sequences of five genes including *alr*, *ddlA*, *ald*, *cycA*, and *pykA* have been suggested to be associated with CS resistance. The clinical strains with mutations in *ald* and *alr* exhibited increased MICs of CS (242). However, clinical isolates with non-synonymous mutations in either *pykA* or *cycA* or *ddlA* have not been tested for MICs of CS. Thus, the link of these genes with CS resistance in *M. tuberculosis* needs to be further elucidated.

For CFZ resistance, Zhang *et al* found that 97% of *M. tuberculosis* H37Rv mutants resistant to CFZ had a mutation in *rv0678* (243). Moreover, mutations in this gene also confer cross-resistance to BDQ (92, 244). Recently, mutations in the *rv2535c* (*pepQ*) and *rv1979c* genes were also associated with cross-resistance to CFZ and BDQ (245). It was reported that 50% of the *in vitro* selected LZD-resistant mutants carried mutations in *rrl* gene conferring LZD resistance (246). Recent studies showed that mutations in *rplC* gene were found in LZD-resistant H37Rv strain selected *in vitro* and in LZD-resistant clinical isolates suggesting the link with LZD resistance (247).

D. Molecular mechanisms of resistance to new anti-TB drugs

As mentioned above, the new anti-TB drugs including AZD5847, BDQ, DLM, Pretomanid and SQ109 are being tested in Phase I, II or III clinical trials. The modes of action of these drugs are new targets, compared to the FLDs and SLDs. For instance, the mode of action of BDQ is to inhibit the bacterial adenosine triphosphate (ATP) synthase of *M. tuberculosis* (91). The molecular mechanism of BDQ resistance was described as mutations in the *atpE* gene coding for the c protein of ATP synthase. Nevertheless, mutations in *rv0678* gene led to cross-resistance between BDQ and CLZ (92, 244, 248). The recent study of Almeida *et al* found loss-of-function mutations in a new non-target-based *pepQ* gene. This gene encodes for a putative

Xaa-Pro aminopeptidase and mutations in this gene confer low-level resistance to BDQ and CFZ in *M. tuberculosis* (245).

Pretomanid and DLM are prodrugs that need to be activated by a deazaflavin (F420 cofactor)-dependent nitroreductase (Ddn) encoded by *ddn* (*rv3547*) gene and by the F420-dependent glucose-6-phosphate dehydrogenase (Fgd) encoded by *fgd* (*rv0407*) gene. Their active forms target to inhibit the biosynthesis of *M. tuberculosis* cell wall (90, 249-251). Besides, it has also been reported that inactivation of *fbiA*, *fbiB* or *fbiC* genes, that are involved in the F420 biosynthetic pathway, lead to the loss of F420 activity, cause of the resistance to these drugs (250, 252).

Although SQ109 is an analog of EMB, the mechanism of action is not the same (253-256). SQ109 targets MmpL3 (Rv0206c), a membrane transporter of trehalose monomycolates involved in cell wall biosynthesis of *M. tuberculosis* (255-257). Lun *et al* confirmed that *mmpL3* gene is the target of SQ109 and that mutations in this gene confer high-level of SQ109 resistance (258). Recently, Li *et al* reported that SQ109 also targets enzymes involved in menaquinone biosynthesis, electron transport, inhibiting respiration and ATP biosynthesis, suggesting that the mode of action of SQ109 is very complex (259).

In conclusion, knowledge on drug-resistance mechanisms is essential for both the development of diagnostic tools and of new and more effective anti-TB agents.

1.2.8 Evolution of drug resistance in *M. tuberculosis*

Review paper

Title: Insights into processes driving the evolution of drug resistance in *Mycobacterium tuberculosis*

Short title: Evolutionary factors of drug resistance in *M. tuberculosis*

Authors and affiliations:

Nguyen Quang Huy^{1,2,3,4#}, Bañuls Anne-Laure^{1,2,4*} and Nguyen Thi Van Anh^{2*}

¹ UMR MIVEGEC (5290 CNRS-224 IRD-Université de Montpellier), Institute of Research for Development, Montpellier, France

² Department of Bacteriology, National Institute of Hygiene and Epidemiology (NIHE), Hanoi, Vietnam

³ Department of Biotechnology-Pharmacology, University of Science and Technology of Hanoi, Hanoi, Vietnam

⁴ LMI Drug Resistance in South East Asia (DRISA), NIHE, Hanoi, Vietnam

* Both authors equally contributed to the work.

Author email addresses:

NQH: huynguyen.biotech@gmail.com, ALB: anne-laure.banuls@ird.fr and NTVA: vananhtdt@gmail.com.

† Corresponding Author: Nguyen Quang Huy, Department of Biotechnology-Pharmacology, University of Science and Technology of Hanoi (USTH), Vietnam Academy of Science and Technology (VAST), No 18. Hoang Quoc Viet road, Cau Giay district, 1000, Hanoi, Vietnam. E-mail: huynguyen.biotech@gmail.com

Abstract

We currently face successful transmission of drug resistance, including multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, *Mycobacterium tuberculosis* strains in human population threatening global TB control. Different parameters and evolutionary processes influence the drug resistance emergence and spread. Unlike many bacteria, *M. tuberculosis* drug resistance is mainly acquired through mutations in drug resistance-associated genes. The panel of mutations is highly diverse depending on the genes and *M. tuberculosis* genetic background. This indicates different evolutionary trajectories towards multiple drug resistance, even if some mutation patterns are preeminent. This review deals with the intrinsic processes that may influence the evolution of drug resistance in *M. tuberculosis* such as mutation rate, drug resistance associated mutations, fitness cost, compensatory mutations and epistasis. These knowledge help better predict the risk for *M.*

tuberculosis to become multiple-drug resistant and develop new tools and strategies to combat the emerging MDR and XDR-TB.

Introduction

Tuberculosis (TB), an infectious airborne disease mainly caused by *Mycobacterium tuberculosis*, is one of the world's deadliest infectious diseases. In 2015, TB infected approximately 10.4 million people and killed 1.8 million among them (1). The currently recommended treatment regimen for new cases of drug-susceptible TB is a six-month regimen of a combination of four first-line anti-TB drugs (FLDs) including isoniazid, rifampicin, ethambutol and pyrazinamide (4). This effective regimen developed in the early 1970s showed a high cure rate greater than 98% in clinical trials (37, 260). The regimen has not changed in nearly 50 years and currently gave a global treatment success rate of 83% for new TB cases (1). The use of this combination for treating TB in the last 5 decades had led to the global emergence of the most dangerous form of FLD-resistant TB, which is multidrug-resistant (MDR) TB (16, 261). MDR-TB is caused by *M. tuberculosis* strains resistant to the two more potent FLDs, isoniazid and rifampicin. Treating MDR-TB requires the use of second-line anti-TB drugs (SLDs) that are more expensive and toxic than FLDs and may lead to the development of extensively drug-resistant (XDR) TB. XDR-TB is caused by MDR strains that are also resistant to at least one of the three second-line injectable drugs (SLIDs: kanamycin, amikacin and capreomycin) and one of the fluoroquinolones (ofloxacin, levofloxacin, moxifloxacin or gatifloxacin). In 2015, 480 000 MDR-TB cases were estimated worldwide and approximately 10% of them developed XDR-TB (1).

The mode of action of the major first and second-line drugs and the mechanisms of drug resistance are now largely described in *M. tuberculosis* (87, 262). The lineage-specific biological characteristics, the remarkable capacity of adaptation and the variety of extrinsic and intrinsic processes contribute to the emergence and spread of MDR and XDR strains (62, 82, 232, 263-268). In this review, we focus on the intrinsic factors that may influence the evolution of drug resistance in *M. tuberculosis* such as mutation rate, drug resistance-associated mutation, fitness cost, compensatory mutation and epistasis. A better understanding of the role of these

intrinsic factors is necessary to get insights into the evolutionary trajectories of drug resistance in *M. tuberculosis*.

Evidences of drug resistance evolution in *M. tuberculosis*

Mutation rate and drug resistance acquisition

M. tuberculosis is mainly a clonal microorganism with a highly conserved genome and a long history of co-evolution with humans (10, 72, 74). This pathogen is characterized by a global low mutation rate (about 10^{-9} mutations/bacterium/cell division), ranging between 0.4 - 0.5 SNPs/genome/year and the divergence was rarely found to be higher than five SNPs per three years (55, 66). Despite this low mutation rate, drug-resistant TB cases due to acquisition of mutations, especially MDR- and XDR-TB cases are continuously globally increasing.

Besides the drug resistance attributed to the specific characteristics of the cell envelope of *M. tuberculosis* and the active drug efflux mechanism (94-96), the acquisition of drug resistance through chromosomal mutations is the major mechanisms in *M. tuberculosis* (72, 87, 95, 130, 269). For genes associated with resistance to FLDs (except pyrazinamide, no data available), second-line injectable drugs and fluoroquinolones, mutation rate ranges from 10^{-6} to 10^{-9} mutations/bacterium/cell division (63-65, 110, 270). The mutation rate seems also differ according to the concentration of drug, the drug-resistance pattern and the genetic background of the bacteria (110, 270). Regarding the association between mutation rate and genetic background, Ford *et al* demonstrated that *M. tuberculosis* lineage 2 (East Asia lineage, mainly Beijing) has a global higher mutation rate in the drug resistant associated genes than *M. tuberculosis* lineage 4 (Euro-American lineage), leading to higher rate of drug resistance acquisition (270).

In vitro, the frequency of spontaneous drug-resistant mutants was determined to be between 10^{-5} and 10^{-8} bacilli/generation (124, 153, 271). Since the genes responsible for resistance to the various anti-TB drugs are generally not linked, the risk of emergence of spontaneous double, triple and quadruple mutants is theoretically extremely low, ranging from about 10^{-14} (for both isoniazid and rifampicin) to 10^{-25} (for isoniazid, rifampicin, ethambutol and pyrazinamide). Clinical data showed that the population size in an active pulmonary disease ranged between 10^7 and 10^{10} bacilli

(16). Therefore, in theory the chance of drug resistance acquisition in a pan-susceptible bacteria is negligible when four effective FLDs are used in combination. Furthermore, it is known that resistance mutations may impose a fitness cost since they target essential biological functions in the cell (272, 273). Consequently, the spread of drug resistant strains and especially MDR and XDR strains should be limited. However, MDR-TB is still emerging and spreading suggesting the existence of favoring processes. As example, the study of Colijn *et al.* showed a probability of resistance acquisition to both isoniazid and rifampicin, even before therapy initiation, as high as 10^{-4} to 10^{-5} per bacterium population (274). In Gao *et al.*, 62/1671 (3.7%) pan-susceptible clinical isolates (susceptible to isoniazid, rifampicin, streptomycin and ethambutol) acquired different resistance patterns during the standard short course chemotherapy under DOT guidelines (Directly Observed Treatment) (275). Among the 62 acquired drug resistance cases, approximately 10% were resistant to four drugs, 22.6% were resistant to three drugs, 21% were resistant to two drugs and the remaining 46.8% were mono-drug resistant. The two last examples underlined that the theoretical rate of drug resistance acquisition does not reflect the *in vivo* reality. Bacterial populations can be located in different environments and tissues in the body or in different physiological states (48, 267). Thus even if a patient received an optimal chemotherapy, the bacteria can be in contact with suboptimal drug concentrations due to the various drug capacities of penetration according to the tissues and also to the different patient's capacities of drug absorption (pharmacokinetic variability) (48, 267). Therefore, the risk of drug resistance increases in these peculiar cases. In addition, Ford *et al* demonstrated that macaques infected with *M. tuberculosis* strains belonging to lineage 2 increased the risk (approximately 22-fold) of *de novo* MDR acquisition before treatment compared to macaques infected with the lineage 4, probably due to a higher mutation rate in lineage 2 (270).

Thus, many factors can influence the mutation rate in drug resistance-associated genes, leading to high variability of mutation rates and higher values than theoretically expected.

Intra-host heterogeneous drug-resistant population

The intra-host bacterial evolution is one of the fundamental aspects of the drug resistance emergence and spread (57, 276, 277). The existence of heterogeneous drug-resistant bacilli populations within patients is a well-known phenomenon that may impact the evolution of drug resistance (263, 265, 267, 277, 278). Meacci *et al* investigated drug resistance evolution of a *M. tuberculosis* population from a noncompliant patient during more than 12 years of active disease (276). The authors identified a MDR *M. tuberculosis* population from one single parental strain. This MDR population included discrete subpopulations of different variants of drug resistance-associated genes. These data suggest that the intra-host bacteria population evolved over time by acquiring and accumulating different resistance-associated mutations to isoniazid, rifampin and streptomycin leading to different coexisting subpopulations with different drug susceptibility profiles in one patient. In another study, Sun *et al* showed dynamic changes of drug resistance-associated mutations in *M. tuberculosis* populations during different drug-resistance acquisition stages (279). These authors found four to five transient drug resistance mutants in single sputum, but the fittest resistant mutant was ultimately fixed. Eldholm *et al*, tracking the evolution of XDR strain from a susceptible ancestor in a single patient, showed that drug resistance-associated mutations were acquired multiple times by individual clones (277). Finally, only one prevailed clone expanded and replaced the other clones. Recently, Black *et al* showed heterogeneous evolution within and between single colonies isolated from mono-rifampicin resistant clinical isolates and an evolution of isoniazid resistance in these colonies. The authors found high genetic diversity in the mono-rifampicin resistant colonies compared to parental isolates. Furthermore, among the mono-rifampicin resistant colonies acquiring isoniazid resistance, the authors observed the emergence and the disappearance of numerous variants in the genome. These observations suggest continuous genome evolution with multiple independent mutational events in the bacterial population despite the evolutionary bottleneck imposed by the isoniazid selective pressure (263). Finally, some mutations were fixed and become dominant, while others were lost during the selection pressure. All these findings suggest that the genome of *M. tuberculosis* is more dynamic than previously thought and highlight the high potential of drug resistance acquisition and of adaptation of these bacteria under drug selective pressure.

Characteristics and diversity of drug resistance-associated mutations

The frequency and type of mutations vary according to various parameters such as the geographic region, the drug-resistance pattern and the genetic background of the bacteria (88, 191, 280-284). Despite the large diversity of mutation patterns, particular mutations are predominant (Table 1) (87, 88). For instance, hundred *rpoB* mutations have been reported but more than 80% of rifampicin-resistant isolates displayed mutations in three main codons (*rpoB*531, 526 and 516) (130). Among more than 300 mutations found in *katG* gene, the *katG*315 (Ser-Thr) mutation is observed in up to 95% of INH-resistant clinical isolates (117). However, the prevalence of *katG*315 mutation ranges between 32 – 95% of isoniazid-resistant isolates according to different geographic regions (116, 121, 191).

Table 1. The most frequent drug resistance-associated mutations found in clinical drug-resistant *Mycobacterium tuberculosis* isolates

Drug(s)	Gene associated with drug resistance	Frequent mutation (amino acid/nucleotide change)	Mutation frequency in clinical drug-resistant isolates (%)	Reference
isoniazid	<i>katG</i>	315 (Ser-Thr)	32 – 95	Hazbon et al. 2006, Soolingen et al. 2000, Mokrousov et al. 2002, Lipin et al 2007
	<i>inhA</i>	-15 (C-T)	8 – 71	
rifampicin	<i>rpoB</i>	531 (Ser-Leu)	41 – 74	Telenti et al. 1993, Pozzi et al. 1999, Hillemann et al. 2005, Qian et al. 2002, Campbell et al. 2011
		526 (His-Tyr)	6 – 24	
streptomycin	<i>rpsL</i>	526 (His-Asp)	2 – 30	Nhu et al, 2012, Sreevatsan et al. 1996, Lipin et al. 2007
		516 (Asp-Val)	5 - 18	
		43 (Lys-Arg)	35 – 62	
		88 (Lys-Arg)	13 – 28	
ethambutol	<i>rrs</i>	514 (A-C)	3 – 12	Sreevatsan et al. 1997, Shi et al. 2011, Campbell et al. 2011
	<i>embB</i>	306 (Met-Val)	40 – 60	
fluoroquinolones	<i>gyrA</i>	94 (Asp-Gly)	25 – 60	Duong et al. 2010,

				Campbell et al. 2011 Von Groll et al. 2009
kanamycin, amikacin & capreomycin	<i>rrs</i>	90 (Ala-Val) 1401 (A-G)	12 – 30 30 – 90	Li et al. 2014 Campbell et al. 2011, Du et al. 2013, Perdigão et al. 2009

In addition, different mutations in a single gene or in different genes can produce similar drug-resistance phenotypes (87, 88) and can be associated with different levels of drug resistance (122, 231, 285, 286). For instance, resistance to rifampicin is associated with many different mutations only in *rpoB* gene, while resistance to isoniazid can be due to mutations in several genes such as *katG*, *inhA* and *inhA* promoter, *ndh* and *kasA* (87, 88, 117). The *rpoB* mutations such as *rpoB531* (Ser-Leu) and *rpoB526* (His-Tyr/Arg/Asp) are associated with high level of rifampicin resistance, while mutations *rpoB511* (Leu-Pro), *rpoB533* (Leu-Pro) and *rpoB572* (Ile-Phe) are generally associated with low level of rifampicin resistance (130). In the case of isoniazid resistance, *katG* mutations are often associated with high level of isoniazid resistance, while *inhA* mutations are associated with low level of isoniazid resistance (117, 120, 123). Even different amino acid changes occurred at the same position in drug resistance-associated gene, they can lead to different level of drug resistance. For instance, the *rpoB526* His-Leu/Asn changes are linked to a lower level of rifampicin resistance compared to the *rpoB526* His-Tyr/Arg changes. Besides, phenomena of cross-resistance were also clearly demonstrated in *M. tuberculosis* consisting in one mutation leading to the resistance to several drugs. As examples, the *rrs1401* (A-G) substitution can be associated with resistance to kanamycin, amikacin, and capreomycin (200, 204, 287) and *inhA-15* (C-T) mutation to isoniazid and ethionamide (117). Recently, some studies reported that mutations in *rv0678* gene encoding for efflux pump MmpL5 are associated with clofazimine and bedaquiline cross-resistance in both, laboratory and clinical *M. tuberculosis* isolates (92, 244, 248). It is worth noting that clofazimine is a second-line drug currently re-proposed and widely used for MDR treatment and bedaquiline is a new drug currently in phase III clinical assay also used for the treatment of MDR-TB patients. Finally, mutations in different regions of the same gene can be associated with different drug resistances. This is the case of *rrs* gene, since mutations in the 530 loop and 915 region are associated with streptomycin resistance (188, 190), while mutations in the

1400 – 1500 region are linked to resistance to kanamycin, amikacin and capreomycin (200, 201, 204).

In addition, drug resistance-associated mutations may provide advantage that can lead to the acquisition of other drug resistances (179, 232, 268). For instance, Safi *et al* showed that *embB306* (ATG-CTG) *M. tuberculosis* mutants had growth advantages compared to parental sensitive strain at sub-MICs of isoniazid or rifampicin in mono-cultures and at sub-MICs of isoniazid in competition assays (179). The *embB306* (ATG-CTG) *M. tuberculosis* mutants were also more resistant to the additive or synergistic activities of isoniazid, rifampicin or ethambutol used in different combinations (179). Furthermore, multiple-drug resistant isolates acquire drug resistance-associated mutations more easily than mono-drug resistant isolates or susceptible isolates, suggesting a cumulative effect of the mutations specifically associated to drug resistance (116, 277, 288-290). For instance, *katG315* (Ser-Thr) mutation was more frequently observed in MDR isolates than in isoniazid-mono resistant isolates (116, 290). Hazbon *et al* concluded that *embB306* mutations may favor the emergence of resistance to other antibiotics and increase the ability of drug-resistant isolates to be transmitted between subjects (178). A later study of Shen *et al* showed that MDR isolates had higher proportion of *embB306* mutations than in non-MDR isolates (167). Furthermore, in a recent study, Nguyen *et al* suggest a cumulative effect of drug resistance-associated mutations since the quadruple drug resistant strains showed a significant higher potential to be pre-XDR or XDR than MDR isolates or triple drug resistant isolates in agreement with previous studies (268, 288).

Besides, correlation between drug resistance-associated mutations and genetic background of *M. tuberculosis* lineages has been largely reported, as well as variations in frequencies according to the geographical regions (84, 132, 281-284, 291). As example, among isoniazid resistance-associated mutations, *katG315* (Ser-Thr) is more prevalent in lineage 2 than in other lineages (83, 121, 280, 292). Concerning lineage 1, isoniazid drug resistance is more associated with *inhA-15* C-T mutation (118, 280, 292). Similarly, the *rpoB531* (Ser-Leu) mutation is more associated with lineage 2 than with other lineages, while the *rpoB516* (Asp-Val) is associated with lineage 4 (LAM family) (83, 191, 281, 282, 293). Furthermore, for

each *M. tuberculosis* lineage, the prevalence of drug resistance-associated mutations varies according to the regions. As example, the frequency of *rpoB*531 (Ser-Leu) mutation ranged between 71% to 84% in the Beijing population from East Europe and Russia, whereas it ranged between 41% and 45% in the Beijing population in Korea and Japan (132, 281-284). Similarly, frequency of *katG*315 (Ser-Thr) was found up to 95% in the Beijing population from East Europe and Russia, whereas it ranged between 51% and 65% in the Beijing population from Korea and Japan. These differences might reflect the adaptation of *M. tuberculosis* sub-lineages to the different human populations and treatment strategies applied in the country (10, 72, 74).

Overall, the panel of mutations can be highly diverse according to the genes and the genetic background of the *M. tuberculosis*, highlighting that the evolution of drug resistance is multifactorial in the bacteria. This indicates different evolutionary trajectories towards multiple drug resistance, even if some mutation patterns are preeminent. Nevertheless, mutations are expected to produce a fitness cost because they target important biological functions.

Fitness cost of drug resistance-associated mutations

Since the drug targets are generally involved into important biological functions, mutations in these genes are expected to impart a biological cost that results in a reduced fitness of resistant strains in comparison with sensitive strains in absence of antibiotics (272, 273). Accordingly, several studies described drug-resistant *M. tuberculosis* mutants characterized by a fitness decrease (122, 130, 294, 295). However, the magnitude of the fitness cost depends on the mutations and the genetic background of the strains (122, 123, 230, 295, 296). Theoretically, mutations arise spontaneously at a constant rate but only beneficial mutations would be positively maintained in population and transmitted in community (297, 298).

Clinical epidemiological and *in vitro* data showed that, the *in vitro* selected drug-resistant mutants did not always reflect the *in vivo* drug resistances. Furthermore, the predominant mutations in clinical isolates are frequently not

associated with a substantial reduction in growth rate and virulence (124, 294, 299, 300). Previous studies demonstrated that mutations such as *katG*315 (Ser-Thr), *rpoB*531 (Ser-Leu), *rpsL*43 (Lys-Arg) and *gyrA*94 (Asp-Gly) conferring resistance to isoniazid, rifampicin, streptomycin and fluoroquinolones, respectively, are not only associated with high level of drug resistance but also with a low fitness cost in both clinical and laboratory-derived mutants (123, 230, 294-296). Some clinical isolates carrying these mutations showed even no fitness cost (122, 294, 296). As expected, these mutations are most commonly found in clinical drug-resistant isolates because they confer a fitness advantage to the bacteria compared to other mutations (83, 130, 288, 298).

In contrast, mutations associated with high fitness cost detected in *in vitro* resistant mutants were rarely found in clinical drug-resistant isolates (122, 124, 231, 299). For example, the mutation *rpoB*529 (Arg-Gln) leading to the highest fitness cost among rifampicin resistance-associated mutations has never been found in clinical rifampicin-resistant isolates (122, 130). Many other nucleotide deletions within the rifampicin resistance-determining region of *rpoB*, frequently detected in *in vitro*-selected rifampicin-resistant mutants were also rarely found in clinical isolates (130, 299). Another example, mutations such as frame-shift nucleotide deletions or insertions in *katG* gene that lead to complete loss of catalase–peroxidase enzyme function were found much less in clinical mutants than in *in vitro* ones (124). All these observations suggest that the prevalent mutations in clinical isolates are positively selected because of their high level of drug resistance and low fitness cost (297, 298).

In the case of mutations in *pncA* gene, the high diversity of mutations detected in clinical isolates (over six hundred different mutations) can be explained by two different hypotheses. First, mutations associated with PZA resistance in *pncA* gene may not cause any fitness deficit since *pncA* is not an essential gene (*M. tuberculosis* can survive without this gene by using another metabolic pathway (149, 153)). In this case, each mutation would have the same probability to be selected and transmitted. Second, all the mutations would cause high fitness cost that impaired the *M. tuberculosis* transmission of these mutants strains (301). This situation can explain the lack of *pncA* mutant clusters that reflect a low potential of transmission. Nevertheless,

clusters of *pncA* mutants were found in some specific outbreaks of MDR and XDR clones in South Africa (288, 302). All these data, high diversity of mutations and existence of clusters even if it is rare, suggest that all the *pncA* mutations should have the same fitness cost and thus the same probability to be transmitted (149). Nevertheless, it is necessary to validate this assumption by further experimental evolution studies.

Since the fitness cost imparted by drug resistance acquisition also varies according to the lineages, several studies investigated the influence of genetic background on the cost of drug resistance mutations in *M. tuberculosis* (118, 122, 280). In the study by Gagneux *et al*, the authors found differences in fitness cost for rifampicin resistance-associated mutation *rpoB526* (His-Asp) between lineage 2 and lineage 4. Conversely, the *rpoB531* (Ser-Leu) mutants of both lineages revealed similar costs (122). In another study, Gagneux *et al* reported that the *inhA-15* (C-T) and the *katG315* (Ser-Thr) mutations are known to be associated with low/no cost linked to lineage 1 and lineage 4, respectively. On the contrary, the isoniazid-resistant non *katG315* (Ser-Thr) mutations appear to be linked to lineage 2 and to be likely to abrogate *katG* activity resulting in high biological cost (118). From Gagneux *et al*, the lineage 2 might be better adapted to compensate the loss or reduced activity of KatG enzyme in the context of isoniazid resistance (122, 230). This hypothesis can also explain that Beijing lineage is generally strongly associated with resistance to isoniazid regardless the isoniazid resistant-associated mutations in *katG* and the country (84, 118, 120, 121, 280, 281).

Compensatory mutations

The compensatory mutations have the ability to alleviate a loss of fitness produced by drug resistance-associated mutations (296, 303, 304). The role of compensatory mutations has been demonstrated in many other bacteria (304, 305). In *M. tuberculosis*, knowledge on compensatory evolution is still limited and mainly focused on isoniazid or rifampicin resistance (296, 306-309). Nevertheless, mechanisms of compensatory evolution listed in the Table 2 were proposed for some other drug resistances.

Table 2. Mechanisms of drug resistance, fitness costs and compensatory processes in*Mycobacterium tuberculosis*

Gene (drug resistance)	Mechanism of resistance caused by gene mutation	Fitness cost	Compensatory mechanism	Reference
<i>katG</i> (INH)	Reduction of prodrug activation	Reduction of protection from oxidative damage	Overexpression of <i>ahpC</i> by mutations in its promoter	Sherman et al 1996, Gagneux et al 2006
<i>inhA</i> promoter (INH)	Overexpression of NADH-ACP-reductase	No fitness cost	No	This study
<i>inhA</i> (INH)	Reduction of its affinity for drug	Reduction of fatty acid biosynthesis	Secondary mutation in promoter (suggested)	This study
<i>rpoB</i> (RIF)	Decrease of DNA polymerase affinity for drug	Reduction of DNA transcription efficiency	Secondary mutation in <i>rpoA</i> or <i>rpoC</i> or <i>rpoB</i>	Comas et al. 2012
<i>rpsL</i> (STR)	Reduction of ribosomal target affinity for drug	Impairment of ribosome performance	Unknown	Bottger et al 1998
<i>embB</i> (EMB)	Decrease of arabinosyl transferase affinity for drug	Reduction of cell wall biosynthesis efficiency	Secondary mutation in <i>embA</i> or <i>embC</i> (suggested)	This study
<i>pncA</i> (PZA)	Reduction or loss of PZA prodrug activation	Unknown	Unknown	
<i>gyrA</i> & <i>gyrB</i> (FQs)	Reduction of DNA gyrase affinity for drug	Reduction of supercoiling of DNA, DNA replication and transcription efficiency	Secondary mutation in <i>gyrA</i> or <i>gyrB</i> (Suggested)	This study
<i>rrs</i> (STR, KAN, AMK, CAP)	Reduction of ribosomal target affinity for drug	No fitness cost	No	Nessa et al 2011
<i>eis</i> (KAN)	Overexpression of aminoglycoside acetyltransferase	No	No	This study

In 1996, it has been shown that mutations in *ahpC* promoter leading to overexpression of *ahpC* gene (encoding an alkyl hydroperoxide reductase) can partially compensate the loss of KatG enzyme activity (309). However, *ahpC* mutations are rare and not genetically clustered with *katG* mutations, indicating that acquiring *ahpC* promoter mutations is not essential for the transmission of isoniazid-resistant strains (118). Later, since almost all the laboratory-generated mutants harboring a rifampicin resistance-associated mutation in RRDR showed a significant fitness deficit compared to their drug-susceptible ancestors when grown in the absence of rifampicin, it was believed that fitness cost linked to rifampicin resistance

can be reduced by compensatory mutations in clinical isolates (122, 130, 294, 295, 306, 307). Non-synonymous mutations in the *rpoA* and *rpoC* encoding the α and β ' subunits of RNA polymerase, respectively, were suspected to play the role of fitness-compensatory mutations in rifampicin-resistant mutants (306, 307). Comas *et al* found that 27.1% (89/239) of MDR clinical strains having a mutation in *rpoB* also carried a non-synonymous mutation in *rpoA* or *rpoC* (94). Furthermore, the use of *Salmonella* as model organism and genetic reconstructions demonstrated that mutations not only in *rpoA* and *rpoC*, but also in *rpoB* genes were associated with an increased growth rate (303, 310). Furthermore, many previous studies showed that rifampicin-resistant *M. tuberculosis* clinical isolates carried multiple (double, triple and quadruple) mutations in *rpoB* gene, suggesting a compensatory evolutionary mechanism to improve the fitness cost of the original resistance mutation (305). It is worth noting that, the compensatory mutations in *rpoA* and *rpoC* genes were more commonly identified in the dominant MDR and XDR clones in high MDR-TB burden countries suggesting that the highly drug-resistant mutants carrying these mutations were successfully transmitted in the population (83, 85, 288, 306, 307, 311). Moreover, these studies showed that the *rpoC* mutations were significantly associated with *rpoB*531 Ser-Leu mutation, suggesting cooperation between compensatory mutations and drug resistance-associated mutation (83, 307, 311). This may explain that the *rpoB*531 Ser-Leu is the most common mutation observed in rifampicin-resistant *M. tuberculosis* clinical isolates and impart a low fitness cost. Furthermore, the compensatory mutations seem to be associated with Beijing strains and especially with the *rpoB*531 Ser-Leu mutants (83, 311). Nevertheless, the frequency of compensatory mutations differs according to the Beijing genotype clades, suggesting strong link between drug resistance mutations, compensatory mutations and genetic background (83).

Although the SLD resistance is more and more a problem of public health with the emergence of pre-XDR and XDR-TB cases, the fitness cost and the mechanisms of compensatory mutations in SLD resistance still need to be investigated. Thus, further studies need to be addressed in order to better evaluate the risk of spread of evolutionary successful SLD resistant isolates.

Role for epistasis in drug resistance evolution in *M. tuberculosis*

Epistasis is known to play an important role in the evolution of organisms in general and of drug resistance in particular (312, 313). Epistasis generally defines the interactions between two genes or several genes. Depending on the effects of the combined mutations (deleterious or beneficial), epistasis can be negative or positive (312, 313). In the case of negative epistasis, the total cost of multiple-drug resistance is higher than the cost of single-drug resistance and thus the multiple-drug resistance mutants would be less fit than their single-drug resistant counterparts and therefore will disappear in the absence of drug pressure (313, 314). On the contrary, when positive epistasis occurs, the total cost of multiple-drug resistance is lower than the cost of the single resistance even in the absence of drug pressure. The multiple-drug resistant mutants, in this case, will become dominant in the population (312-314).

Although still little is known about epistasis in *M. tuberculosis*, there are evidences suggesting that this process could play an important role in the emergence and evolution of MDR and XDR *M. tuberculosis* strains (315). The interaction between drug resistance mutations may restore or even increase fitness of drug-resistant mutants compared to drug-susceptible strains. As example, Spies *et al* demonstrated that, the double mutants *rpsL43* (Lys-Arg)/*katG315* (Ser-Thr), *rpsL43* (Lys-Arg)/*rpoB531* (Ser-Leu), and *rpoB531* (Ser-Leu)/*katG315* (Ser-Thr), more frequently detected in clinical isolates, grow faster than drug-susceptible strains (264). This suggests that the interaction between these mutations may provide fitness advantage for the double mutants. Indeed, these double mutations have been proven to increase the fitness of drug-resistant *E. coli* and to drive evolution of multiple drug resistance acquisition (314). Nevertheless, recent study of Salvatore *et al* found that the MDR strains carrying the double mutations *katG315* (Ser-Thr) and *rpsL43* (Lys-Arg) were less detected in multiple-case households, compared to single-case household MDR strains suggesting a negative epistasis (290). Since the *katG315* mutation unchanged the virulence and transmission of isoniazid-resistant strains, the authors suggested that the *rpsL43* mutation might impart a cost on the transmissibility of drug-resistant bacteria. Nevertheless, since the combination of *katG315* and *rpsL43* mutations was commonly found in drug-resistant clinical isolates, particularly in Beijing strains, it is thus possible that the epistasis effect is also regulated by the genetic background of the strains.

In another study, Borrell *et al* described epistatic interaction between mutations associated with resistance to ofloxacin and rifampicin using *M. smegmatis* as model organism (232). They showed that 35% (6/17) of the double drug-resistant mutants carrying mutations associated with rifampicin and fluoroquinolone resistances, *rpoB526* (His-Pro/Arg/Tyr) and *gyrA94* (Asp-Asn), *rpoB526* (His-Pro/Arg) and *gyrA94* (Asp-Gly), and *rpoB526* (His-Pro) and *gyrA94* (Asp-Tyr) had a significantly higher fitness than the corresponding single drug-resistant mutants (232). This study also showed that the specific *gyrA94* (Asp-Gly) mutation was associated with improved fitness in all of the double mutants, irrespective of the *rpoB* mutation. It is worth noting that these peculiar combinations of mutations were the most common among MDR and XDR clinical isolates in South Africa, which is one of the highest MDR-TB burden country in the world. Nevertheless, these authors also found some double mutants with fitness defect, which is the sign of negative epistasis. In TB treatment, rifampicin, classified as a FLD is always used before fluoroquinolones that are classified in SLD. Consequently, *rpoB* mutations are generally acquired before *gyrA* mutations. Following this route, we may predict the mutation paths leading to *rpoB-gyrA* double mutants resulting from epistatic interaction (268). However, fluoroquinolones have been widely used for treating other infectious diseases increasing the burden of selective pressure and compromising their efficacy in the TB treatment. Consequently, *M. tuberculosis* strains could be exposed to fluoroquinolones before rifampicin. It would be interesting to explore the epistasis effect in this drug pressure configuration and the *gyrA-rpoB* mutation combinations acquired by the bacteria.

It is worth noting that, in some cases without drug pressure, drug-resistant mutants may reveal higher relative fitness than the drug-sensitive strains owing to the epistatic interaction between a drug-resistant mutation and a compensatory mutation such as *rpoB531* and *rpoC* mutations (312). By using *M. smegmatis* model, Song *et al* demonstrated that the recombinants strains carrying both *rpoB531* (Ser-Thr) and *rpoC* mutations had higher growth rate and showed also a higher relative fitness than their counterparts bearing only *rpoB531* (Ser-Thr) mutation (308). However, these interactions have not been yet explored in *M. tuberculosis*. Nevertheless, a proportion of clinical drug-resistant mutants carrying compensatory mutations had low or no

fitness cost, suggesting that the interaction between these two types of mutations could also exist in this pathogen.

Concluding remarks

While *M. tuberculosis* can be intrinsically resistant to drugs thanks to the drug extrusion capacity by activation of drug efflux systems, the main mechanism of resistance is the acquisition of chromosomal drug-resistance-associated mutations. This mechanism is essential for the bacteria because it can maintain the resistances in the bacterial populations. However, the evolution of drug resistance in *M. tuberculosis* is driven by various factors impacting this mechanism. Mutation frequencies and the mutation type can be affected by the drug resistant-patterns and the genotypes. Different mutations can cause different levels of drug resistance and biological fitness cost even if they are located in the same genes. The most frequent drug resistance mutations found in clinical isolates worldwide are often associated with high level of drug resistance and/or low/no fitness costs, or with other mutations (drug resistance-associated mutations or compensatory mutations). These points strongly suggest that epistatic interactions drive the evolution of drug resistance in *M. tuberculosis*. Furthermore, all these evolutionary processes not only maintain drug-resistant strains in the population but also favor their transmission in host population. Thus, MDR and XDR strains are emerging worldwide probably due to compensatory mechanisms, reduced fitness cost and epistasis.

Nevertheless, still today some drug resistance mechanisms remain unclear and many mechanisms of fitness-compensatory evolution and epistasis have not been yet investigated in this pathogen. Therefore, further works are needed to better know all the forces that drive the evolution of drug resistance in *M. tuberculosis* in order to better control the emergence and rapid spread of highly drug-resistant strains.

Disclosure: The authors declare no conflict of interest.

1.2.9 The tuberculosis situation in Vietnam

A. TB burden, drug-resistant TB and risk factors

Till now, Vietnam is one of the countries with the highest TB and MDR-TB burden (12). The incidence, prevalence and mortality rates were estimated at 130, 180 and 17 per 100 000 population, respectively (12). Nevertheless, Vietnam was one of the 9 high TB burden countries, which met all the three WHO's 2015 targets: 50% reduction in incidence, prevalence and mortality rates compared with that in 1990 (12). The overall treatment success rate for all TB cases, including new and retreated cases, was 89% in 2013, slightly higher than the global rate (83%) (12). TB has been associated with male (76%) and with working age (60%), indicating that TB is also a social and economic burden in this low-income country (316).

The most recent National anti-TB drug resistance survey in 2011 showed that, the proportion of TB cases with resistance to any FLD drug was as high as 32.7% and 54.2% among new cases and previously treated cases, respectively (316). Among resistances to FLDs, INH and STR resistances were the most commonly detected. Resistance to STR was lower among new cases than among previously treated cases (27.4% vs. 42.2%), as was INH resistance (18.9% vs 44.7%). Resistances to RIF and EMB were low among new cases (4.1% and 3.4%, respectively), but were high among previously treated cases (23.1% and 11.9%, respectively). The proportion of cases with resistance to at least two drugs was 10% and 14%, respectively. Compared to the previous National survey in 2005, the proportion of MDR-TB cases increased from 2.7% to 4% among new cases and from 19% to 23% among previously treated cases between 2005-2011 (316). Based on the results of the last survey in 2011, the annual estimated number of MDR-TB cases is about 5100.

In 2014, the Vietnam National TB Program (NTP) had started to replace the 8-month regimen (2HRZS/6HE), which had been used since 1990 for new TB cases, by a 6-month regimen with the inclusion of EMB in the continuation phase (2HRZE/4HRE). Similarly, the 8-month regimen 2SHRZE/1HRZE/5HRE had been introduced for previously treated TB cases (316).

In late 2009, the Vietnam NTP initiated the Programmatic Management of Drug-resistant TB (PMDT) to provide SLD treatment to MDR-TB patients (317). Before the launching of PMDT, the SLDs have been used without official guidelines (318) and sources of supply and quality of drugs have not been controlled. Thus suboptimal treatments were suspected since pre-XDR and XDR-TB were detected (data presented in this thesis). In 2011, the prevalence of resistance to any SLDs was 18% among MDR-TB cases, the proportion of pre-XDR and XDR-TB were 18% and 6%, respectively (319).

Although the number of MDR-TB cases detected and enrolled in PMDT increased between 2013-2015 from 18.5% to 42%, an estimated 58% of MDR-TB cases have not been diagnosed (319, 320), implying an inadequate-controlled transmission of MDR-TB in the population. The PMDT guidelines for MDR-TB treatment suggest that treatment regimen should consist of a 6-month intensive phase, with the use of a combination of at least 6 drugs, chosen among the drug PZA, EMB, KAN/CAP, LFX, PTO and CS/PAS, followed by a 12-months continuation phase, with the use of a combination of at least 5 drugs, chosen among the drug PZA, EMB, LFX, PTO and CS/PAS (317). A retrospective cohort study on treatment outcomes of MDR-TB patients during 2010-2012 showed that, favorable outcomes were attained in 73% of patients, that was higher than the global average rate (50%) (321). In fact, Vietnam is one of the five countries with high MDR-TB burden in the world that achieved a MDR-TB treatment success rate of more than 70% (12).

Noticeably, the new anti-TB drug BDQ is available in Vietnam since late 2015. The drug safety and efficacy have been evaluated through a cohort study, in which a donation program with the delivery of medicines to the studied patients (who have pre-XDR- and XDR-TB) has also been initiated. This event also kicked off the implementation of a regimen that was shorten from 18-24 months to 9 month, and thus cost-effective for MDR-TB treatment.

Regarding the diagnosis of drug resistant TB, solid culture is a standard method for DST. Besides, liquid culture using BACTEC-MGIT system, LPAs and Xpert®MTB/RIF (Xpert) assay which are endorsed by WHO were also implemented by NTP (321). The proportions of MDR-TB patients diagnosed with culture-based DST, LPAs and Xpert were respectively 33%, 55% and 12% (321). Only 5.5% new

TB cases and 96% previously treated cases were subjected to DST for RIF only or both RIF and INH. Among the confirmed MDR-TB cases, 78% were tested for FQ and SLID resistance (12). These figures underline a large proportion of undetected drug resistant TB cases in Vietnam.

B. Molecular characteristics of drug resistance in clinical M. tuberculosis isolates in Vietnam

Gene sequencing have been done to explore molecular characteristics of drug resistance mainly to INH and RIF and in clinical *M. tuberculosis* isolates mainly collected from the South of Vietnam (6, 190, 322). For instance, an earlier study revealed that 89% of INH-resistant isolates harbored either *katG315* or *inhA-15* mutations and that 95.2% of RIF resistant isolates carried mutations in the RRDR of *rpoB* gene (322). A recent study also showed a high prevalence (85.4%) of *katG315* mutation (Ser-Thr) in INH-resistant isolates from the North (323). Mutations associated with STR resistance were also investigated in a collection of clinical isolates from the South by sequencing *rpsL*, *rrs* and *gidB* genes (190). The study found 93.1% of SM-resistant isolates carrying mutations in *rpsL* and *rrs* gene. The authors could not identify links between *gidB* gene mutations and SM resistance, because *gidB* mutations were found in both resistant and susceptible isolates. Another study determined 82.6% of FQ-resistant isolates having FQ resistance-associated mutations in *gyrA* and *gyrB* genes (6).

Mutations associated with drug-resistance in *M. tuberculosis* were also detected by commercial kits. For example, a study showed that Genotype MTBDR*plus*, which is used for detecting MDR-TB, identified 75.3% INH-resistant isolates carrying a mutation at *katG315* position and 28.2% carrying a mutation at *inhA-15* position (324).

So far, gene mutations responsible for resistance to PZA, EMB, new FQ generations and SLIDs have not been reported in Vietnam. Associations between phenotypic drug-resistant patterns and drug resistance-associated mutations have never been explored to investigate the interactions among genetic elements in the evolution of drug resistance. Other potential driving factors of the drug resistance

evolution such as genetic background of the bacteria and compensatory mutations also have little or not been studied.

C. Associations between *M. tuberculosis* lineages and drug resistance

Studies of molecular epidemiology of TB showed that Beijing and EAI families are predominant in Vietnam, together responsible for about 80% of the TB cases (5, 325-327). Their distribution varies according to geographic areas. For Beijing family, the prevalence was 39% - 54% in the South, 30% - 38% in the North and 28% in the Center region (5, 326-330). This family was more predominant in urban (53% - 82%) than in rural areas (35% - 40%) (5, 6, 190, 326-329). On the contrary, EAI family was more predominant in rural areas (50%) compared to urban areas (21%) (8, 330). Their prevalence in the North was 20% - 38.5%, in the South was 36% - 50% and in the Centre was 59% (5, 8, 330, 331). Other families including H, LAM, T, U, MANU, Zero and X were also detected, but with low prevalence (8, 326, 329).

Beijing family was reported to be more associated with drug resistance, high level of drug resistance, younger ages, treatment failure, relapses and TB transmission (5-7, 325-327). Whereas, the EAI family strains were more associated with older ages, drug sensitiveness and low level of drug resistance (5, 326, 327, 330).

Up to now, little is known about the association between genetic determinants of drug resistance and *M. tuberculosis* families in Vietnam except two studies reporting the association of Beijing strains with FQs and SM resistance based on genetic mutation analyses (6, 190).

Chapter 2. MATERIALS AND METHODS

2.1 Study population

From clinical *M. tuberculosis* cultures stored in National Institute of Hygiene and Epidemiology (NIHE) in Hanoi, Vietnam, 260 clinical isolates were included in this study. These samples were collected from three regional TB reference hospitals of Vietnam (National Lung Hospital in the North [NLH], Hue general Hospital in the Centre [HGH] and Pham Ngoc Thach Hospital in the South [PNT]) by Vietnam NTP in between 2005 – 2009, before the PMDT launched in Vietnam (in the late 2009). The samples were chosen according to their FLD-susceptibility patterns (INH, RIF, STR and EMB resistance) and families. For each FLD-resistance pattern, we selected isolates according to the *M. tuberculosis* family determined by spoligotyping. For each family, when the number of isolates was less than 10 per FLD-resistance pattern, all the isolates were exhaustively taken, when the number was more than 10, around 10 isolates were randomly selected. The details on the samples are listed in the Table A2 in appendix.

2.2 Ethics approval of research

This study has been performed in accordance with the Declaration of Helsinki. Since the study used only isolates that were routinely collected from patients, informed consent to participate was not required. The Ethical Review Committee at the National Institute of Hygiene and Epidemiology (NIHE) approved the study procedures.

2.3 Bacterial culture

From the original stocks stored at -80°C, all the samples were sub-cultured on LJ medium. After 2 - 3 weeks of growth, the cultures were harvested and used for all the experimentations described below. All the bacterial cultures were performed in the Biosafety Laboratory level 3 of NIHE.

2.4 Drug susceptibility testing (DST)

Drug susceptibility testing (DST) is the determination of susceptibility to drugs used in treatment: a negative result means that the isolates are sensitive to the tested drugs and the patient with that isolates has a high probability of treatment success, while a positive result means that the isolates are resistant to the drug under study and thus the patient with that isolates have high possibility of treatment failure.

The objectives here were: to determine the drug susceptibility to FLDs and SLDs by using the Canetti-Grosset technique; to develop a new technique based on proportional agar microplate assay to reduce the turn-around time (see paper 2 below at the end of this chapter) (332, 333). The Canetti-Grosset technique is the gold standard for DST of *M. tuberculosis* and the technique was previously described in the WHO 2003 report (333). This test is based on the determination of the "growth proportion" of *M. tuberculosis* colonies in drug-containing medium relative to that in drug-free medium, using the same inoculum. An isolate was considered as drug-sensitive when no growth or a growth proportion lower than 1% was observed and drug-resistant when the growth proportion was higher than or equal to 1%. However, this technique is time consuming (4 – 8 weeks for getting final DST result), costly and difficult to implement.

In this context, we developed a proportional microplate DST method for a faster detection of drug resistance in *M. tuberculosis* based on M7H11 medium for FLDs and on M7H10 for SLDs. The details of this technique and the study results are described in the published paper below at the end of this chapter: "Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay" (332).

2.4.1 FLD-susceptibility testing

All the 260 *M. tuberculosis* isolates were tested for susceptibility to the 4 first line anti-TB drugs INH, RIF, EMB and STR by both the proportional Canetti-Grosset technique (at NLH, PNT and NIHE) and the proportional microplate assay (at NIHE). The critical concentrations were applied for the two techniques on LJ medium for Canetti-Grosset technique and on 7H11 medium for the proportional microplate

assay, as recommended by WHO 2012 (see Table A1 in appendix) (332, 333). The H37Rv laboratory strain was included as control.

2.4.2 SLD-susceptibility testing

SLD-susceptibility testing was also adapted on proportional microplate assay by using M7H10 supplemented with OADC (the DST are not standardized for SLDs on M7H11 medium) (WHO 2012, see Table A1 in appendix for details). The critical concentrations were used for the tested drugs as follows: KAN (5 mg/L), AMK (4 mg/L), CAP (4 mg/L), OFX (2 mg/L), LVX (1 mg/L), MXF (0.5 and 2 mg/L), and GAT (1 mg/L) (WHO 2012, see Table A1 in appendix). For MXF susceptibility, growth at concentrations ≥ 0.5 mg/L and < 2 mg/L indicated low level of resistance and growth at concentrations ≥ 2 mg/L indicated high level of resistance. SLD-susceptibility testing was carried out on a subsample of 105 FLD-resistant (mainly MDR) and 20 FLD-sensitive isolates. The H37Rv laboratory strain was included as control.

2.5 DNA preparation

A full loop of *M. tuberculosis* colonies grown on Löwenstein-Jensen medium was harvested and suspended in 1mL TE buffer (10mM Tris-HCl, 1mM EDTA). After killing of the bacteria at 95°C for 45 min (repeated twice), the suspension was centrifuged and the DNA-containing supernatant was transferred in a new tube and stored at -20°C until use.

2.6 Molecular Genotyping

The two genotyping methods for *M. tuberculosis*, Spoligotyping and MIRU-VNTR were performed on the whole sample.

2.6.1 Spoligotyping typing

Spoligotyping method is based on the polymorphism in the direct repeat (DR) locus (member of the CRISPR region [clustered regularly interspaced short palindromic repeats]) (334). This region consists of 36 base pair DR copies

interspersed by non-repetitive 35–41 base pair sequences called spacers (**Figure 2.1**) (335). 94 spacer sequences were described, but 43 spacers are commonly used for the genotyping (334). The polymorphism is based on the presence or absence of these spacers. Spoligotyping is a PCR-based method that amplifies the spacers. The method that we used is based on reverse line blot hybridization technique. The PCR products are hybridized to a membrane with 43 covalently bound synthetic oligonucleotides representing the different spacers selected from *M. tuberculosis* H37Rv (spacers 1–19, 22–32, and 37–43) and *M. bovis* BCG (spacers 20-21 and 33–36) (**Figure 2.1**) (335). The details of this technique are described in (3). The patterns (presence and absence of spacers) were coded as a binary number. The data were then compared with the international database on SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE)(4). This technique allowed determining the *M. tuberculosis* families present in our sample.

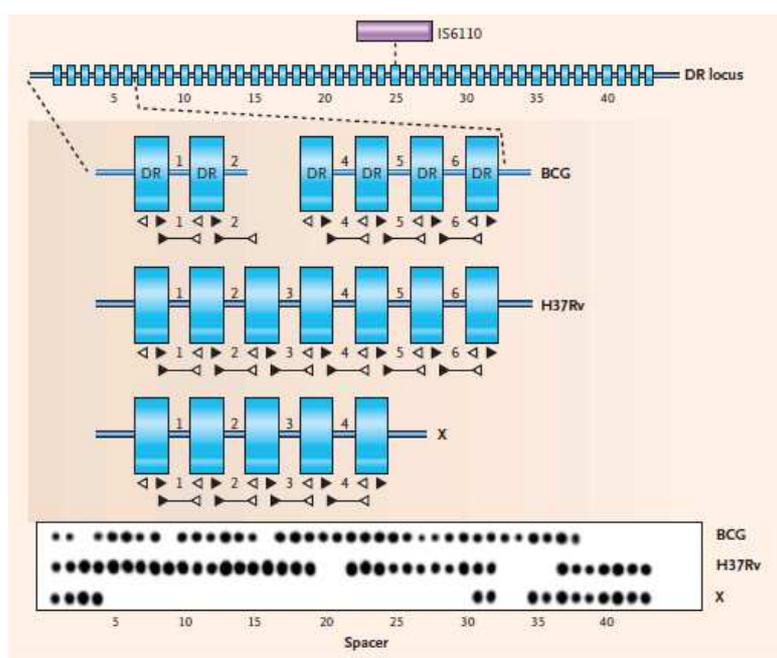


Figure 2.1. Schema of spoligotyping technique
(Source from Barnes and Cave 2003 (327))

2.6.2 24-locus MIRU-VNTR typing

Mycobacterial Interspersed Repetitive Units of Variable Number of Tandem Repeats (MIRU-VNTR) has become a major method for the epidemiological tracking of *M. tuberculosis* strains, as it is highly discriminatory and reproducible (335-337).

These loci correspond to “macrosatellite” elements. The repetitive units are 40–100 base pairs in length and are located in 41 loci scattered throughout the genome of *M. tuberculosis* (335, 338). The polymorphism is based on the variability in the number of copies of the repeat unit. Nowadays, the current recommendations are to use 24 loci to have a global high discrimination power (337, 339, 340). MIRU-VNTR is PCR-based method using primers specific for the flanking regions of the different loci (Figure 2.2) (335).

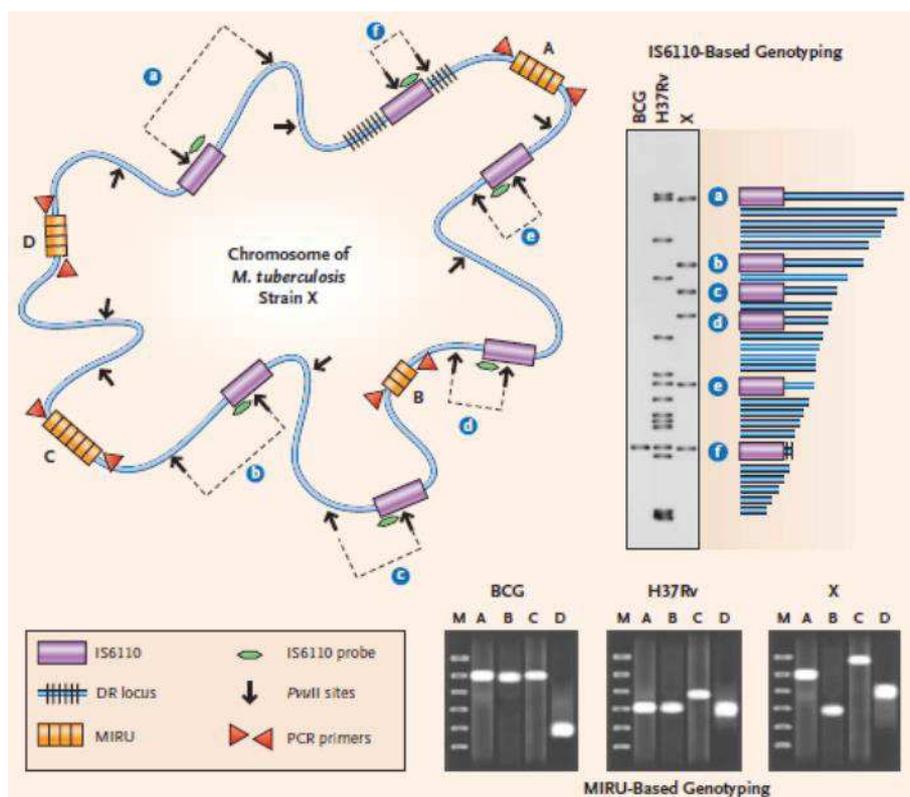


Figure 2.2. Schema of the different molecular markers used for *M. tuberculosis* genotyping of which MIRU-VNTR typing
(Source from Barnes and Cave 2003 (327))

After the amplification of each locus, the products were visualized by agarose gel electrophoresis. The methodology was performed according to the procedure detailed in (337). Since the length of the repeat unit is known, the size of the PCR products gave the number of copies of the repeat unit. The data were coded as numerical value reflecting the number of repeats in tandem at each locus. The results can be analyzed using the specialized bioinformatics web tool *MIRU-VNTRplus* (<http://www.miru-vntrplus.org/>) containing a collection of strains representing the major *M. tuberculosis* lineages (336, 341).

2.7 Detection of drug resistance-associated mutations by DNA sequencing

The main genes associated with resistance to FLDs and SLDs were PCR-amplified and sequenced. For the FLD resistance, the following genes and gene fragments were studied: *katG* gene, *inhA* gene and the *inhA* promoter (INH resistance); *rpoB* gene (RIF resistance); *rpsL* gene and *rrs*-F2 fragment (STR resistance); *embB* gene (EMB resistance); *pncA* gene and its promoter (PZA resistance). For the SLD resistance, the following genes and gene fragments were analyzed: *gyrA* and *gyrB* genes (FQ resistance), *rrs*-F2 fragment (SLID resistance). The list of primers, PCR conditions and DNA sequencing are described in the **Table 2.1** (see papers 3 and 4 in Chapter 3 for details).

Each sequence was treated independently using the Bioedit software (version 7.1.10). The consensus sequence was generated. Multi-sequence alignment was then performed. Point mutations were identified by comparison with the sequence of the *M. tuberculosis* H37Rv reference strain available in GenBank (NC.000962.3). Sensitivity and specificity values were determined by comparison of phenotypic and genotypic data for each drug (except for PZA). The two tailed-Fisher's exact test was used to compare the mutation frequencies between drug-resistant patterns and between *M. tuberculosis* families. *P*-values <0.05 were considered statistically significant. The odds ratio and 95% confidence interval (95% CI) were calculated to quantify the association of FLD resistance patterns with mutation frequency.

Table 2.1. Primers used for DNA amplification and sequencing of genes involved in anti-TB drug resistance and compensatory mutation in *M. tuberculosis*

Drug(s)	Gene/gene promoter	Primer sequence	Annealing T°C	Length (bp)	Target region
RIF	<i>rpoB</i> -F1	F- <i>rpoB</i> 1: 5'-GTCGACGCTGACCGAAGAAG-3'	62°C	1148	Clusters I (including RRDR), II, III
		R- <i>rpoB</i> 1: 5'-TCTCGCCGTCGTCAGTACAG-3'			
	<i>rpoB</i> -F2	F- <i>rpoB</i> 2: 5'-TAGTTGCGTGCCTGAGATCC-3'	60°C	1174	Promoter and N-terminal region
		R- <i>rpoB</i> 2: 5'-TGGTCTGACCCTCGTGCAAG-3'			
INH	<i>katG</i>	F- <i>katG</i> 1: 5'-CCAACCTCCTGGAAGGAATGC-3'	58°C	1169	Full length gene
		R- <i>katG</i> 1: 5'-AGAGGTCAGTGCCAGCAT-3'			
	F- <i>katG</i> 2: 5'-ACGAGTGGGAGCTGACGAA-3'	60°C	1229		
	R- <i>katG</i> 2: 5'-AACCCGAATCAGCGCACGT-3'				
<i>inhA</i>	F- <i>inhA</i> 1: 5'-GCGACATACCTGCTGCGCAA-3'	60°C	300	Promoter region	
	R- <i>inhA</i> 2: 5'-ATCCCCCGTTTCTCCGGT-3'				
	F- <i>inhA</i> 3: 5'-GACACAACACAAGGACGCA-3'	60°C	1008	Full length gene	
	R- <i>inhA</i> 4: 5'-TGCCATTGATCGGTGATACC-3'				
STR	<i>rpsL</i>	F- <i>rpsL</i> : 5'-GCGCCCAAGATAGAAAAG-3'	58°C	451	Full length gene
		R- <i>rpsL</i> : 5'-CAACTGCGATCCGTAGA-3'			
	<i>rrs</i> -F1	F-S1: 5'-GAGAGTTTGATCCTGGCTCAG-3'	58°C	973	Loops 530 & 915
		R-S1: 5'-CCAGGTAAGGTTCTTCGCGTTG-3'			
EMB	<i>embB</i>	F- <i>embB</i> : 5'-TGACCGACGCCGTGGTGATA-3	62°C	1312	ERDR & flanking sequences
		R- <i>embB</i> : 5'-GCCATGAAACCGCCACGAT-3			
PZA	<i>pncA</i>	F- <i>pncA</i> : 5'-CTTGCGGCGAGCGCTCCA-3'	63°C	709	<i>pncA</i> and its promoter
		R- <i>pncA</i> : 5'-TCGCGATCGTCGCGCGTC-3'			
KAN, AMK & CAP	<i>rrs</i> -F2	F-S2: 5'-GCGCAGATATCAGGAGG-3'	58°C	918	1400-1500 region
		R-S2: 5'-CGCCACTACAGACAAG-3'			
FQs	<i>gyrA</i> & <i>gyrB</i>	F- <i>gyrAB</i> : 5'-GCAACACCGAGGTCAAATCG-3'	62°C	1296	QRDRs of <i>gyrA</i> & <i>gyrB</i>
		R- <i>gyrAB</i> : 5'-CTCAGCATCTCCATCGCCAA-3'			
Compensatory mutation	<i>rpoA</i> (RIF)	F- <i>rpoA</i> : 5'-AACCGATCCCAGTTCGTGAT-3'	60°C	856	<i>rpoA</i> gene
		R- <i>rpoA</i> : 5'-GCAGCTTGATCTTCACCTCG-3'			
	<i>rpoC</i> (RIF)	F- <i>rpoC</i> : 5'-AGTCGCTTCCGATCTGCTC-3'	60°C	952	RpoA-RpoC interaction region
	R- <i>rpoC</i> : 5'-TTGAGCTTGTCGACGGTCTG-3'				
	<i>ahpC</i> and its promoter (INH)	F- <i>ahpC</i> : 5'-CCGCAACGTCGACTGGCTCATA-3'	62°C	835	<i>ahpC</i> promoter and AhpC

2.8 Detection of compensatory mutations in RIF-resistant and INH-resistant isolates by DNA sequencing

The *ahpC* gene and its promoter was amplified and sequenced for all INH-resistant isolates. The *rpoA* and *rpoC* genes were amplified and sequenced in all RIF-resistant isolates. The list of primers, PCR conditions and DNA sequencing were described in the Table 2.1. The analysis of sequences and mutations were performed according to the procedure described above using Bioedit software and sequence comparison with the *M. tuberculosis* H37Rv reference strain.

2.9 Phylogenic analysis

A Neighbor Joining based phylogenetic tree was built from DSW distances (based on the 24-locus MIRU-VNTR set) (342) with *M. canettii* as root using MIRU-VNTR*plus* (<http://www.miru-vntrplus.org/>) (341). The tree was edited by the Treedyn software (<http://www.treedyn.org/>), including mapping mutations, drug resistance patterns, families and genotype clusters (343).

2.10 PhyloType analysis

This analysis had for objectives to better understand the evolutionary pathways that follow the isolates to become MDR and XDR. We used the PhyloType method (<http://phylotype.org/>) that helps to quickly inspect phylogenies and combine them with extrinsic traits (e.g. geographic location, risk group, presence of a given resistance mutation) (344). This method allows determining groups that share specific traits, called phylotypes, from a phylogeny having taxa (strains) annotated with extrinsic traits. Phylotypes are subsets of taxa with close phylogenetic relationships and common trait values. We thus performed a combined analysis of the phylogenetic tree build from MIRU-VNTR data with all the available data (phenotypes, genetic mutations, families, genotypes, drug resistance mutations) in order to identify how the isolates evolved from sensitive to XDR form. P -value ≤ 0.05 was statistically significant (344).

Chapter 3. RESULTS AND DISCUSSIONS

In order to reach the objectives of this PhD thesis, which aims to acquire knowledge on drug resistance in clinical *M. tuberculosis* isolates in Vietnam and to understand how this bacteria evolved from sensitive to highly drug-resistant form by molecular analysis, this chapter has been subdivided in four parts, each addressing specific questions about drug resistance.

The first part is aimed to develop a DST procedure with a reduced turn-around, a cheaper cost and a specificity and sensitivity comparable to DST on LJ solid medium. Indeed, rapid and accurate detection of drug-resistant *M. tuberculosis* is one of the priorities to combat the emergence of multiple drug-resistant TB including MDR and XDR TB worldwide. The traditional proportional method for DST based on LJ medium cultures takes 6 – 8 weeks for getting final result. Liquid culture-based DST method is efficient and fast, but expensive and does not allow detecting the contaminations. The results of this study are presented in the paper 2 entitled “Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay” which is published on Clinical Microbiology and Infection (see the attached paper 2).

The second part is focused on the study of highly FLD resistant clinical isolates by phenotypic and genetic approach. The questions to be solved were: What is the risk for quadruple-FLD resistant isolates to be pre-XDR and XDR and what are the genetic processes leading to these high level of drug resistance? This study is detailed in paper 3 entitled “Quadruple-First line Drug Resistance in *Mycobacterium tuberculosis* in Vietnam: What can we learn from genes?” published on Infection, Genetics and Evolution (see the attached paper 3).

The third part deals with the molecular study of PZA (key drug in current TB and MDR-TB treatment) resistance, which is unknown in Vietnam. The questions in this part were: What is the proportion of clinical *M. tuberculosis* isolates carrying *pncA* mutations associated with PZA resistance in Vietnam; Are there specific correlations between PZA resistance, FLD resistant patterns and *M. tuberculosis* families? The data are detailed in paper 4 entitled “Molecular analysis of pyrazinamide resistance in *Mycobacterium tuberculosis* in Vietnam highlights the

high rate of pyrazinamide resistance-associated mutations in clinical isolates” accepted for publication on Emerging Microbes and Infections (see the attached manuscript).

Finally, the last part is focused on the evolution of drug resistance in *M. tuberculosis* in Vietnam. The questions addressed here were: How *M. tuberculosis* evolved from sensitive to highly drug resistant status including mono-resistance, MDR, pre-XDR and XDR; Do the clinical isolates display specific genetic characteristics (such as low fitness cost mutations or fitness-compensatory mutations) explaining the successful spread of highly drug resistant strains in Vietnam? From the collected data can we predict the evolution of drug resistance? This four part is entitled: “Evolution of drug resistance in *Mycobacterium tuberculosis* in Vietnam”.

Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay

V. A. T. Nguyen¹, H. Q. Nguyen^{1,2,3}, T. T. Vu¹, N. A. T. Nguyen¹, C. M. Duong¹, T. H. T. Tran¹, H. V. Nguyen⁴, D. A. Dang¹ and A.-L. Bañuls^{1,2}

1) Department of Bacteriology, National Institute of Hygiene Epidemiology, Hanoi, Vietnam, 2) MIVEGEC (IRD 224-CNRS 5290-Université de Montpellier), Centre IRD, Montpellier, France, 3) Department of Biotechnology Pharmacology, University of Science and Technology of Hanoi and 4) Department of Microbiology, National Lung Hospital, Hanoi, Vietnam

Abstract

Multidrug-resistant tuberculosis is a major issue worldwide; however, accessibility to drug susceptibility testing (DST) is still limited in developing countries, owing to high costs and complexity. We developed a proportion method on 12-well microplates for DST. The assay reduced the time to results to <12 days and <10 days when bacterial growth was checked with the naked eye or a microscope, respectively. Comparison with the Canetti–Grosset method showed that the results of the two assays almost overlapped (kappa index 0.98 (95% CI 0.91–1.00) for isoniazid, rifampicin, streptomycin; and kappa index 0.92 (95% CI 0.85–0.99) for ethambutol). The sequencing of genes involved in drug resistance showed similar level of phenotype–genotype agreement between techniques. Finally, measurement of the MICs of rifampicin and ethambutol suggests that the currently used critical ethambutol concentration should be revised, and that the current molecular drug susceptibility tests for rifampicin need to be re-evaluated, as *in vitro* rifampicin-sensitive isolates could harbour drug resistance-associated mutation(s).

Clinical Microbiology and Infection © 2015 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Keywords: Drug resistance, drug susceptibility testing, *M. tuberculosis*, MIC, proportional microplate assay, sequencing

Original Submission: 6 March 2015; **Revised Submission:** 4 August 2015; **Accepted:** 27 August 2015

Editor: M. Drancourt

Article published online: 5 September 2015

Corresponding author: V. A. T. Nguyen, Laboratory of Tuberculosis, Department of Bacteriology, National Institute of Hygiene and Epidemiology, Hanoi 10000, Vietnam
E-mails: vananhtdt@gmail.com, vananh@nihe.org.vn

Introduction

Multidrug-resistant tuberculosis (MDR-TB), i.e. tuberculosis (TB) resistant to at least isoniazid and rifampicin, the two essential first-line anti-TB drugs [1], is a major challenge. However, in the countries with the highest MDR-TB burden, less than one-fifth of patients with MDR-TB are detected, owing to the lack of diagnostic capacity [2], leading to treatments without prior drug susceptibility testing (DST) [3–5]. Sputum

conversion is achieved in only 33–38% of retreatment cases in such settings [5,6], which is a much lower rate than that achieved with DST-based treatments (38% vs. 79%) [5].

Liquid medium-based DST with automated culture systems such as BACTEC 460 TB and BACTEC MGIT 960 [7] is efficient and fast, but expensive. Other disadvantages are invisible contaminations, overgrowth of atypical mycobacteria, inability to check the colony morphology and to determine the MIC, and the need for multiple tubes or bottles [8]. GeneXpert, a fast molecular-based test, was endorsed by the WHO for the identification of suspected MDR-TB cases [9]. However, the high cost hampers its routine use in developing countries [9]. Moreover, the suspected MDR-TB cases detected by GeneXpert still need to be confirmed with a reference standard technique [10], such as proportional DST on Löwenstein–Jensen medium [11–13], which takes 6–8 weeks [14].

The proportional method on M7H10 agar developed by the National Committee for Clinical Laboratory Standards is the reference standard drug susceptibility test for mycobacteria in the USA [15,16]. It is also used in The Netherlands and elsewhere, but on microplates instead of in culture tubes or bottles, providing a low-cost, high-throughput, fast and easy-to-read test with high sensitivity, specificity, and reproducibility [8,17]. Recent studies have shown that some drug-resistant *Mycobacterium tuberculosis* isolates do not grow or grow very slowly on M7H10 medium, leading to false drug-sensitive results. Therefore, it was recommended to replace M7H10 with M7H11 agar, which is supplemented with a pancreatic digest of casein, to facilitate the growth of fastidious *M. tuberculosis* cultures [18,19].

Here, we describe a proportional method for DST in 12-well microplates with M7H11 medium supplemented with OADC and low-magnification microscopic monitoring of bacterial growth to reduce turn-around time. We compared the results with those obtained with the conventional Canetti–Grosset proportional method. We also sequenced the genes involved in drug resistance to validate the DST profiles, and measured MICs in *M. tuberculosis* isolates with conflicting DST and sequencing results.

Materials and methods

Ethics statement

The National Institute of Hygiene and Epidemiology (NIHE) ethics review committee, Vietnam, approved the study protocol.

M. tuberculosis isolates

Two hundred and twelve *M. tuberculosis* isolates were randomly selected at the National Lung Hospital (NLH) in Hanoi, which has the highest rates of MDR-TB.

DST

The sensitivities of all *M. tuberculosis* isolates to the four drugs used as first-line TB treatment in Vietnam during the study period (isoniazid, rifampicin, streptomycin, and ethambutol) were tested with the Canetti–Grosset proportional technique, as previously described [14], at the NHL, and also with the new proportional microplate technique (described below) at the TB Laboratory, NIHE, Vietnam. All experiments were performed in biosafety level 3 laboratories. The critical inhibitory drug concentrations (CIDs) recommended by the WHO were used (Table S1). Both methods determine the ‘growth proportion’ of *M. tuberculosis* colonies in drug-containing medium relative to that in drug-free medium, with the same inoculum. An isolate was considered to be drug sensitive when no growth

or a growth proportion of <1% was observed, and drug resistant when the growth proportion was $\geq 1\%$.

Preparation of DST microplates

The M7H11 agar (Difco, Maryland, USA) was prepared according to the manufacturer’s instructions, and enriched with 10% OADC (Difco). Freshly prepared anti-TB drugs (Sigma; INH: India, RMP, STM, EMB: China) at 100 × CIDC were added (final concentration of 1 × CIDC), or not added, to the agar, which was then distributed at 3 mL/well in 12-well microplates (Thermo Scientific Nunc), as described in Fig. S1. Plates were labelled, kept at 4°C, and used within 1 month.

M. tuberculosis inoculation in DST microplates

Bacterial colonies from fresh cultures (4 weeks) were homogenized by vortexing in 15-mL Falcon tubes containing water and glass beads. Larger particles were allowed to settle. The supernatants were then transferred to new glass tubes, and bacterial stock solutions (10^8 CFU/mL) were prepared with McFarland standard No. 2 for adjustment. Bacterial solutions of 10^5 CFU/mL and 10^3 CFU/mL were prepared by making serial ten-fold dilutions from the stock solutions in water. Fifty microlitres of 10^5 CFU/mL bacterial solution was added to each well of the DST microplate, except for control 2, where 50 μ L of 10^3 CFU/mL bacterial solution was inoculated. Three sides of the microplate were sealed with tape, and plates were incubated at 37°C in an incubator. Culture contamination and *M. tuberculosis* growth were monitored by eye and under a microscope at low resolution (10 \times) from day 1 to day 14, and recorded on a follow-up form.

DST microplate reading

Results were read by naked eye or microscopy. The minimum number of colonies in control 2 had to be at least ten for interpretation of the assay. Isolates were considered to be sensitive when, in drug-containing wells, no colonies or fewer colonies than in control 2 were observed. Isolates were considered to be resistant if the number of colonies in drug-containing wells was higher than or equal to the number of colonies in control 2 (Fig. 1).

Sequencing of drug resistance genes

PCR amplification and DNA sequencing. The genetic elements of resistance to rifampicin (a 1148-bp fragment in *rpoB* containing the hotspot region), isoniazid (the entire sequences of *katG* and *inhA*, and the *inhA* promoter), streptomycin (the entire sequences of *rpsL* and *rrs*) and ethambutol (a 1312-bp fragment in *embB* containing the hotspot region) were PCR amplified and sequenced. The primers and amplified regions are shown in Table S2 [20–25]. The PCR conditions (with HotStarTag) were

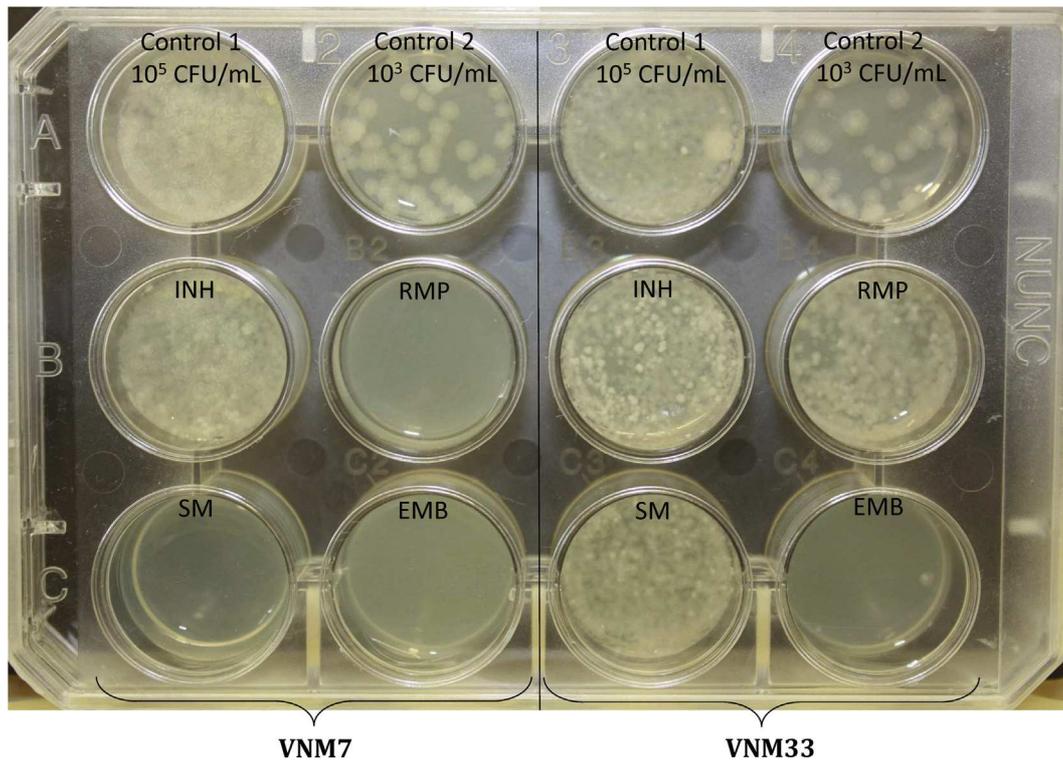


FIG. 1. Reading of results in a 12-well microplate drug susceptibility testing assay. VNM7: *Mycobacterium tuberculosis* isolate resistant to isoniazid (INH) (left half of the 12-well plate); VNM33: *M. tuberculosis* isolate resistant to INH, rifampicin (RMP), and streptomycin (SM) (right half of the 12-well plate). Photograph taken at day 11 of culture.

as follows: 15 min of Taq activation at 95°C, 35 cycles of 95°C for 1 min, 58–62°C (Table S2) for 1.5 min, and 72°C for 2 min, and 72°C for 5 min. PCR products were sequenced by Eurofins MWG Operon or 1st BASE.

Sequence analysis

Each sequence was analysed with Bioedit and ClustalW softwares. Point mutations were identified by comparison with the sequence of the *M. tuberculosis* H37Rv reference strains in GenBank (NC 000962).

Determination of rifampicin and ethambutol MICs

MICs were determined by use of the M7H11 microplate assay with 0.031, 0.062, 0.124, 0.248, 0.496, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/L rifampicin, and 2, 3, 4, 5, 6, 7 and 7.5 mg/L ethambutol. The highest concentrations used for MIC testing were the CIDCs recommended by the WHO.

Data analysis

Data were analysed with STATA 12 (Statacorp LP, TX, USA). Sensitivity, specificity and Cohen's kappa coefficient with two-sided 95% CIs were calculated for the proportional microplate DST assay relative to the Canetti–Grosset method. A

kappa coefficient of 0.81–0.99 was considered to indicate almost perfect between-test agreement, and a kappa value of 1.0 was considered to indicate perfect agreement [26].

Results

As four *M. tuberculosis* isolates were contaminated by fungi during subculture and DST, only 208 isolates were analysed.

DST

DST results obtained with the Canetti–Grosset and the 12-well microplate methods showed between-test discrepancies in only eight *M. tuberculosis* isolates, with only one drug susceptibility difference in most of the cases (Table S3 and 2 × 2 table in Table S4). The sensitivity, specificity and kappa coefficients for detection of multidrug resistance and resistance to the four drugs showed almost perfect agreement between methods (Table 1).

Time to results with the proportional microplate DST assay

Visual growth monitoring of the first 140 *M. tuberculosis* isolates from day 1 to day 14 indicated that DST results could be read at

TABLE 1. Sensitivity, specificity and kappa coefficients for the comparison of the proportional 12-well microplate and Canetti–Grosset drug susceptibility testing assays

Drug resistance	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Kappa coefficient (\pm SE)
RMP	100 (94.5–100)	98.6 (95.0–99.8)	0.98 (0.91–1.00)
INH	99.0 (94.7–100)	99.0 (94.8–100)	0.98 (0.91–1.00)
SM	100 (96.8–100)	98.9 (94.2–100)	0.98 (0.91–1.00)
EMB	96.4 (81.7–99.9)	98.3 (95.2–99.7)	0.92 (0.85–0.99)
MDR	100 (93.8–100)	98.7 (95.3–99.8)	0.98 (0.91–1.00)

EMB, ethambutol; INH, isoniazid; MDR, multidrug resistant; RMP, rifampicin; SE, standard error.

days 5–6 for 108 isolates (77.1%) and at days 7–11 for the remaining 32 isolates (22.9%). The mean time to results was 6.3 days. By microscopic observation (10 \times), DST results could be read at days 4–5 for 115 isolates (82.1%) and at days 6–9 for 25 isolates (17.9%). The mean time to results was 4.7 days (Table 2). Fig. 2 shows the microscopic follow-up of *M. tuberculosis* growth in a control well from day 1 to day 12.

Sequencing of drug resistance genes

To validate the DST profiles, the main genes associated with resistance to the four tested anti-TB drugs were sequenced in the eight isolates with discordant DST results between techniques and in 115 randomly selected isolates.

Overall, previously described drug resistance-associated mutations were found at: codons 511, 516, 518, 522, 526, 531, 533 and 572 of *rpoB* (rifampicin resistance); at codon 315 of *katG*, at codon 21 of *inhA*, and at position –15 (C-T) of the *inhA* promoter (isoniazid resistance); at codons 43 and 88 of *rpsL*, and at positions 514, 517, 878, 1001 and 1401 of *rrs* (streptomycin resistance); and at codons 306, 330, 354, 406 and 497 of *embB* (ethambutol resistance). Synonymous mutations and mutations previously reported as not being

TABLE 2. Time to results when bacterial growth is monitored in the proportional 12-well microplate drug susceptibility testing assay by eye and under a low-magnification microscope (140 *Mycobacterium tuberculosis* isolates)

Time (days)	No. of isolates (%)	
	By eye	By microscopy
4	—	93 (66.4)
5	49 (35.0)	22 (15.7)
6	59 (42.1)	9 (6.4)
7	7 (5.0)	6 (4.3)
8	5 (3.6)	6 (4.3)
9	8 (5.7)	4 (2.9)
10	8 (5.7)	—
11	4 (2.9)	—

associated with drug resistance were also identified (Table S5) [24,25,27,28].

The frequencies of drug resistance-associated mutations among the resistant and sensitive isolates identified with the two DST assays are shown in Table 3. The level of agreement between phenotypic susceptibility and genotypic determinants was similar for the two techniques.

Gene sequencing of the eight isolates with discordant DST results showed that, among these, both methods correctly detected three of the six resistant isolates with mutations (Table 4). For the ones without mutations, we did not consider which assay results were more precise, because the known molecular determinants targeted by sequencing cannot detect 100% of the drug resistant isolates.

Determination of the MICs for rifampicin and ethambutol in isolates with phenotype–genotype discrepancies

Because of the significant number of isolates with phenotype–genotype discrepancies concerning susceptibility to rifampicin (seven of 74 isolates) and ethambutol (13 of 98 isolates), their MICs for rifampicin and ethambutol were determined. In three of the seven rifampicin-sensitive isolates with a rifampicin resistance-associated mutation, and in all three control rifampicin-sensitive isolates without detectable mutations, the MIC for rifampicin was 0.248 mg/L. The other four rifampicin-sensitive isolates with mutations had an MIC for rifampicin between 0.6 mg/L and 0.9 mg/L (Table 5).

The MICs for ethambutol in 12 randomly selected ethambutol-sensitive isolates without *embB* mutations (controls) were \leq 2 mg/L. Conversely, among the 13 ethambutol-sensitive isolates with an ethambutol resistance-associated mutation, 11 had an MIC for ethambutol of 6 mg/L, and two had an MIC for ethambutol of 5 mg/L (Table 6).

Discussion

Simplification and time reduction for *M. tuberculosis* DST with the proportional microplate assay

This proportional microplate assay is faster and simpler than the currently used Canetti–Grosset method. Indeed, the M7H11 medium promotes *M. tuberculosis* growth. Moreover, microplates allow the microscopic detection of bacterial colonies before they can be seen with the naked eye, further decreasing the time to results. Previous studies have shown that *M. tuberculosis* can be detected on M7H11 from sputum samples as early as 7 days [29–31]. A meta-analysis of the results obtained with thin-layer agar and microscopic-observation drug susceptibility assays, in which patients'

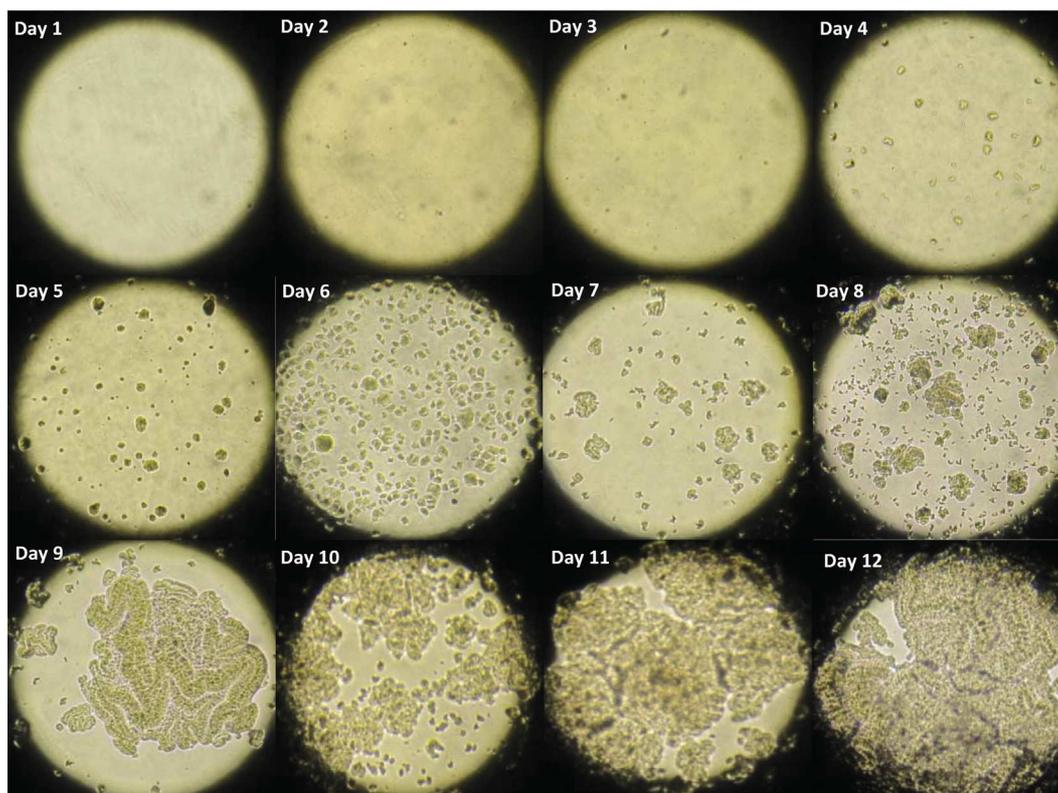


FIG. 2. *Mycobacterium tuberculosis* growth on a control well of the 12-well microplate from day 1 to day 12 (control well: 10^5 CFU/mL bacterial solution and no drug), monitored with a low-magnification ($10\times$) microscope.

specimens are inoculated directly on drug-free and drug-containing medium for DST, showed that the mean turn-around times for the two assays were 11.1 days (95% CI 10.1–12.0) and 9.9 days (95% CI 4.1–15.8), respectively [32]. With our indirect proportional microplate assay and a low-magnification microscope, primary results could be read at day 4 (or day 6 with the naked eye) and the complete results at day 9 (or day 11–12 by eye).

A 12-well plate can be used to test four anti-TB drugs in two *M. tuberculosis* isolates, whereas 12 culture tubes are needed for testing the same number of drugs and isolates with the Canetti–Grosset technique. Therefore, inoculation, growth monitoring and result reading are much easier and less labour-intensive and time-consuming. Moreover, the M7H11 medium without fresh eggs is much simpler to prepare than Löwenstein–Jensen medium. On the basis of our experience, with

TABLE 3. Frequency of mutations associated with resistance to antituberculosis drugs in the drug-resistant and drug-sensitive *Mycobacterium tuberculosis* isolates characterized with each drug susceptibility testing (DST) technique and with both techniques

<i>M. tuberculosis</i>	Mutation frequency, no. (%)			
	Drug resistance (1)	Canetti–Grosset (2)	Microplate DST (3)	Both techniques (4)
Drug-resistant isolates	RMP	44/46 (95.6)	47/49 (96)	44/46 (95.6)
	INH	73/77 (94.8)	73/77 (94.8)	73/76 (96)
	SM	62/70 (88.6)	62/71 (87.3)	62/70 (88.6)
	EMB	22/24 (91.7)	20/22 (91)	19/21 (90.5)
Drug-sensitive isolates	RMP	10/77 (13)	7/74 (9.5)	7/74 (9.5)
	INH	2/46 (4.4)	2/46 (4.4)	2/45 (4.4)
	SM	0/53 (0)	0/52 (0)	0/52 (0)
	EMB	11/99 (11.1)	13/101 (12.9)	14/10/98 (10.2)

Columns (2), (3) and (4) shows the number of isolates with mutations that confer resistance to the drug indicated in column (1) relative to the total number of isolates that are resistant/sensitive to that drug (determined with each DST techniques and with both techniques); the corresponding mutation frequency is in parentheses. EMB, ethambutol; INH, isoniazid; RMP, rifampicin; SM, streptomycin.

TABLE 4. Mutations in genes involved in drug resistance found in the *Mycobacterium tuberculosis* isolates with discrepant drug susceptibility testing results by the proportional Canetti–Grosset and 12-well microplate techniques

Isolates	Drug resistance profile		Drug resistance-associated mutations in the studied genes					
	Canetti–Grosset	Microplate DST	<i>rpoB</i>	<i>inhA/its promoter</i>	<i>katG</i>	<i>rpsL</i>	<i>rrs</i>	<i>embB</i>
VNM48	RHSE	RHS	Asp526Val	ND	Ser315Thr	Lys88Arg	ND	Met306Val
VNM53	Sensitive	H	ND	ND	ND	ND	ND	ND
VNM56	HS	RHS	Leu533Pro	ND	Ser315Thr	Lys88Arg	ND	ND
VNM151	H	RH	Leu533Pro	ND	Ser315Thr	ND	ND	Gln497Arg
VNM152	H	Sensitive	ND	ND	ND	ND	ND	ND
VNM165	RH	RHSE	Ser531Leu	ND	Ser315Thr	ND	ND	Asp354Ala
VNM180	HE	H	Leu533Pro	ND	Ser315Thr	ND	ND	Met306Val
VNM182	HE	H	ND	ND	Ser315Thr	ND	ND	Met306Val

E, ethambutol; H, isoniazid; R, rifampicin; S, streptomycin; ND, not detected.

the same amount of time and labour, the number of tests performed can be increased by at least four-fold.

Validation of proportional microplate DST for *M. tuberculosis*

For detection of multidrug resistance and resistance to isoniazid, rifampicin, and streptomycin, the sensitivity of proportional microplate DST ranged between 99.0% (95% CI 94.7–100%) and 100.0% (95% CI 96.8–100%) and the specificity ranged between 98.6% (95% CI 95.0–99.8%) and 99.0% (95% CI 94.8–100%), as compared with the proportional Canetti–Grosset technique. Similarly, the kappa coefficients between tests was 0.98 (95% CI 0.91–1.00), indicating almost perfect agreement between the techniques. Previous studies also showed that all assessments of rifampicin and isoniazid resistance with the thin-layer agar assays yielded 100% accuracy, showing a promising diagnostic technique. However, the WHO expert group agreed that more evidence is needed before the use of these assays can be recommended [32].

For ethambutol, the specificity was as high as for the other drugs (98.3%; 95% CI 95.2–99.7%), whereas the sensitivity was

lower (96.4%; 95% CI 81.7–99.9%), and consequently also the kappa index was lower (0.92; 95% CI 0.83–0.97). DST results for ethambutol are frequently less reproducible [33]. Thus, a lower kappa index does not mean that the microplate assay is less precise than the Canetti–Grosset technique. Ängeby et al. showed that the low reproducibility for ethambutol is due to the use of non-optimal critical drug concentrations that cannot discriminate between wild-type and drug-resistant isolates [33].

Sequencing showed similar phenotype–genotype agreement for the two techniques. This suggests that the precision of the proportional microplate assay is comparable to that of the proportional Canetti–Grosset technique.

The need for adjustment of the critical concentration for ethambutol susceptibility testing

On the basis of the WHO recommended critical concentrations, we missed 11 (33%) and 13 (39%) ethambutol-resistant mutants with the Canetti–Grosset technique and the microplate assay, respectively. All of the missed isolates had significantly higher MICs than those of the non-mutants (5–6 mg/L vs. ≤ 2 mg/L). These data strongly suggest that the critical

TABLE 5. Measurement by microplate assay of the MIC for rifampicin (RMP) in rifampicin-sensitive isolates that harbour or do not harbour rifampicin resistance-associated mutation(s) in *rpoB*

No.	Isolate	RMP susceptibility		RMP resistance-associated mutation(s) in <i>rpoB</i>	MIC (mg/L)
		Canetti–Grosset	Microplate assay		
1	VNM148	S	S	Leu511Pro	0.248
2	VNM162	S	S	Leu533Pro	0.248
3	VNM168	S	S	Leu533Pro	0.6
4	VNM192	S	S	Asp516Tyr	0.248
5	VNM193	S	S	Leu511Pro	0.7
6	VNM194	S	S	Leu533Pro	0.7
7	VNM208	S	S	Leu533Pro	0.9
8	VNM137	S	S	ND	0.248
9	VNM166	S	S	ND	0.248
10	VNM182	S	S	ND	0.248

ND, not detected; S, sensitive.

TABLE 6. Measurement by microplate assay of the MIC for ethambutol (EMB, China) in ethambutol-sensitive isolates harbouring or not harbouring ethambutol resistance-associated mutation(s) in *embB*

No.	Isolate	EMB susceptibility		EMB resistance-associated mutation(s) in <i>embB</i>	MIC (mg/L)
		Canetti–Grosset	Microplate assay		
1	VNM32	S	S	Gln497Arg	6.0
2	VNM69	S	S	Gln497Arg	6.0
3	VNM57	S	S	Met306Ile	6.0
4	VNM72	S	S	Met306Ile	6.0
5	VNM76	S	S	Met306Ile	6.0
6	VNM163	S	S	Gln497Arg	5.0
7	VNM164	S	S	Phe330Leu	6.0
8	VNM179	S	S	Gly406Asp	5.0
9	VNM188	S	S	Gly406Asp	6.0
10	VNM151	S	S	Gln497Arg	6.0
11	VNM48	R	S	Met306Val	6.0
12	VNM180	R	S	Met306Val	6.0
13	VNM182	R	S	Met306Val	6.0
14	VNM137	S	S	ND	<2.0
15	VNM138	S	S	ND	<2.0
16	VNM152	S	S	ND	<2.0
17	VNM153	S	S	ND	<2.0
18	VNM51	S	S	ND	<2.0
19	VNM36	S	S	ND	<2.0
20	VNM107	S	S	ND	<2.0
21	VNM178	S	S	ND	<2.0
22	VNM56	S	S	ND	<2.0
23	VNM85	S	S	ND	<2.0
24	VNM185	S	S	ND	<2.0
25	VNM96	S	S	ND	<2.0

ND, not detected; R, resistant; S, sensitive.

ethambutol concentration should be revised, especially because the currently used cut-offs are not based on clinical evidence [33].

Rifampicin-sensitive isolates carrying rifampicin resistance-associated mutations

All of the isolates that carried rifampicin resistance-associated mutations but were phenotypically rifampicin-sensitive had MICs lower than the critical concentration recommended by the WHO. Three of them had MICs as low as those of the non-mutants (0.248 mg/L). This suggests that, for these isolates, molecular-based DST could lead to false-positive results. A recent study also indicated that not only the presence but also the nature of *rpoB* mutations must be assessed for accurate diagnosis of rifampicin resistance in *M. tuberculosis* [34]. Moreover, all of the mutations found in susceptible isolates in our study have been previously reported to be associated with low rifampicin resistance [27,35–39]. This observation supports the need to re-evaluate the current molecular-based DST assays.

Conclusions

Our proportional microplate assay for *M. tuberculosis* DST is as accurate as the proportional Canetti–Grosset technique, but easier to perform. It can provide DST results as rapidly as automated liquid culture systems [7], but is much more affordable. It also allows calculation of the growth proportion

and observation of the colony morphology, thus reducing the contamination problems of liquid cultures. Therefore, it could facilitate the diagnosis of drug-resistant TB in developing countries for better control of drug-resistant TB. However, the critical ethambutol concentration may need to be adjusted to improve the precision of ethambutol susceptibility testing.

Transparency declaration

The authors declare no conflicts of interest.

Acknowledgements

We would like to thank the NLH of Vietnam for *M. tuberculosis* sampling. We are grateful to our institutions NIHE, IRD, and CNRS. We thank E. Andermarcher for work on the English. This work was supported by the research grant ‘Apply microplate assay for DST of *M. tuberculosis*’ provided by the NIHE, and by grants from the JEAI MySA and the GDRI ID-BIO.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cmi.2015.08.024>.

References

- [1] World Health Organization. The W.H.O./IUATLD global project on anti-tuberculosis drug resistance surveillance. Anti-tuberculosis drug resistance in the world. Report no. 2: prevalence and trends. Geneva: WHO; 2000.
- [2] World Health Organization. Global tuberculosis report 2013., WHO, Geneva
- [3] Quy HT, Lan NT, Borgdorff MW, Grosset J, Linh PD, Tung LB, et al. Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate? *Int J Tuberc Lung Dis* 2003;7:631–6.
- [4] Mehra RK, Dhingra VK, Nish A, Vashist RP. Study of relapse and failure cases of CAT I retreated with CAT II under RNTCP—an eleven year follow up. *Indian J Tuberc* 2008;55:188–91.
- [5] Saravia JC, Appleton SC, Rich ML, Sarria TM, Bayona J, Becerra MC. Retreatment management strategies when first-line tuberculosis therapy fails. *Int J Tuberc Lung Dis* 2005;9:421–9.
- [6] Lan NTN, Lademarco MF, Binkin NJ, Tung LB, Quy HT, Cj NV. A case series: initial outcome of persons with multidrug-resistant tuberculosis after treatment with the WHO standard retreatment regimen in Ho Chi Minh City, Vietnam. *Int J Tuberc Lung Dis* 2001;5:575–8.
- [7] Ardito F, Posteraro B, Sanguinetti M, Zanetti S, Fadda G. Evaluation of BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) automated system for drug susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2001;39:4440–4.
- [8] van Klingeren B, Dessens-Kroon M, van der Laan T, Kremer K, van Soolingen D. Drug susceptibility testing of *Mycobacterium tuberculosis* complex by use of a high-throughput, reproducible, absolute concentration method. *J Clin Microbiol* 2007;45:2662–8.
- [9] Piateka AS, Cleeffb MV, Alexanderc H, Coggind WL, Rehrb M, Kampenb SV, et al. GeneXpert for TB diagnosis: planned and purposeful implementation. *Glob Health Sci Pract* 2013;1:18–23.
- [10] Falzon D, Jaramillo E, Schunemann HJ, Arentz M, Bauer M, Bayona J, et al. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. *Eur Respir J* 2011;38:516–28.
- [11] Ani AE, Daylop YB, Agbaji O, Idoko J. Drug susceptibility test of *Mycobacterium tuberculosis* by nitrate reductase assay. *J Infect Dev Ctries* 2009;3:16–9.
- [12] Dang TM, Nguyen TN, Wolbers M, Vo SK, Hoang TT, Nguyen HD, et al. Evaluation of microscopic observation drug susceptibility assay for diagnosis of multidrug-resistant tuberculosis in Viet Nam. *BMC Infect Dis* 2012;12:49.
- [13] World Health Organization. Guidelines for surveillance of drug resistance in tuberculosis. 4th ed. Geneva: WHO; 2009.
- [14] Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler HT, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull World Health Organ* 1963;29:565–78.
- [15] Piersimoni C, Olivieri A, Benacchio L, Scarparo C. Current perspectives on drug susceptibility testing of *Mycobacterium tuberculosis* complex: the automated nonradiometric systems. *J Clin Microbiol* 2006;44:20–8.
- [16] National Committee for Clinical Laboratory Standards. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes. Approved standard M24-A. Wayne, PA: NCCLS; 2003.
- [17] Wedajo W, Schon T, Bedru A, Kiros T, Hailu E, Mebrahtu T, et al. A 24-well plate assay for simultaneous testing of first and second line drugs against *Mycobacterium tuberculosis* in a high endemic setting. *BMC Res Notes* 2014;7:512.
- [18] Versalovic J. Manual of Clinical Microbiology. 10th ed. Washington, DC: ASM Press; 2011.
- [19] Cohn ML, Waggoner RF, McClatchy JK. The 7H11 medium for the cultivation of mycobacteria. *Am Rev Respir Dis* 1968;98:295–6.
- [20] Pretorius GS, van Helden PD, Sirgel F, Eisenach KD, Victor TC. Mutations in katG gene sequences in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* are rare. *Antimicrob Agents Chemother* 1995;39:2276–81.
- [21] Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopec E, Zwolska Z, Kirikae F, Toyota E, et al. Detection of multidrug resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 2007;45:179–92.
- [22] Rigouts L, Nolasco O, de Rijk P, Nduwamahoro E, Van Deun A, Ramsay A, et al. Newly developed primers for comprehensive amplification of the rpoB gene and detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 2007;45:252–4.
- [23] Lee AS, Ong DC, Wong JC, Siu GK, Yam WC. High-resolution melting analysis for the rapid detection of fluoroquinolone and streptomycin resistance in *Mycobacterium tuberculosis*. *PLoS One* 2012;7:e31934.
- [24] Lipin MY, Stepanshina VN, Shemyakin IG, Shinnick TM. Association of specific mutations in katG, rpoB, rpsL and rrs genes with spoligotypes of multidrug-resistant *Mycobacterium tuberculosis* isolates in Russia. *Clin Microbiol Infect* 2007;13:620–6.
- [25] Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011;55:2032–41.
- [26] Viera AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. *Fam Med* 2005;37:360–3.
- [27] Andres S, Hillemann D, Rusch-Gerdes S, Richter E. Occurrence of rpoB mutations in isoniazid-resistant but rifampin-susceptible *Mycobacterium tuberculosis* isolates from Germany. *Antimicrob Agents Chemother* 2014;58:590–2.
- [28] Hazbon MH, Bobadilla del Valle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, et al. Role of embB codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. *Antimicrob Agents Chemother* 2005;49:3794–802.
- [29] Battaglioli T, Rintiswati N, Martin A, Palupi KR, Bernaerts G, Dwiwardiani B, et al. Comparative performance of thin layer agar and Lowenstein–Jensen culture for diagnosis of tuberculosis. *Clin Microbiol Infect* 2013;19:E502–8.
- [30] Irfan S, Hasan R, Kanji A, Hassan Q, Azam I. Rapid detection of *Mycobacterium tuberculosis* in sputum samples by microscopic observation methods. *Infect Dis J Pakistan* 2008;17:10–3.
- [31] Welch DF, Guruswamy AP, Sides SJ, Shaw CH, Gilchrist MJ. Timely culture for mycobacteria which utilizes a microcolony method. *J Clin Microbiol* 1993;31:2178–84.
- [32] Minion J, Leung E, Menzies D, Pai M. Microscopic-observation drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis* 2010;10:688–98.
- [33] Angeby K, Jureen P, Kahlmeter G, Hoffner SE, Schon T. Challenging a dogma: Antimicrobial susceptibility testing breakpoints for *Mycobacterium tuberculosis*. *Bull World Health Organ* 2012;90:693–8.
- [34] Ocheretina O, Escuyer VE, Mabou MM, Royal-Mardi G, Collins S, Vilbrun SC, et al. Correlation between genotypic and phenotypic testing for resistance to rifampin in *Mycobacterium tuberculosis* clinical isolates in Haiti: investigation of cases with discrepant susceptibility results. *PLoS One* 2014;9:e90569.
- [35] Zaczek A, Brzostek A, Augustynowicz-Kopec E, Zwolska Z, Dziadek J. Genetic evaluation of relationship between mutations in rpoB and resistance of *Mycobacterium tuberculosis* to rifampin. *BMC Microbiol* 2009;9:10.

- [36] Ohno H, Koga H, Kohno S, Tashiro T, Hara K. Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. *Antimicrob Agents Chemother* 1996;40: 1053–6.
- [37] Taniguchi H, Aramaki H, Nikaido Y, Mizuguchi Y, Nakamura M, Koga T, et al. Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* 1996;144:103–8.
- [38] Van Deun A, Barrera L, Bastian I, Fattorini L, Hoffmann H, Kam KM, et al. *Mycobacterium tuberculosis* strains with highly discordant rifampin susceptibility test results. *J Clin Microbiol* 2009;47:3501–6.
- [39] Huitric E, Werngren J, Jureen P, Hoffner S. Resistance levels and *rpoB* gene mutations among in vitro-selected rifampin-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob Agents Chemother* 2006;50: 2860–2.

Supplementary Tables:

Supplementary Table S1. Minimum inhibitory concentrations (MIC) for drug susceptibility testing in *M. tuberculosis* isolates

Drug	Solvent	MIC ($\mu\text{g}/\text{mL}$)	
		L-J*	M7H11**
Isoniazid	H ₂ O	0.2	0.2
Streptomycin	H ₂ O	4	2
Ethambutol	H ₂ O	2	7.5
Rifampicin	DMSO	40	1

Note: * L-J: Löwenstein-Jensen medium; ** M7H11: Middlebrook 7H11 agar

Supplementary Table S2. Primers used for PCR amplification and sequencing of genes involved in anti-TB drug resistance in *M. tuberculosis*

Drug	Sequenced gene	Primer sequence	Annealing T°C	Amplicon position (Length)	Target region
RMP	<i>rpoB</i>	F-B2: 5'-GTCGACGCTGACCGAAGAAG-3'	62°C	1053 – 2200 (1148 bp)	N-terminal II, RRDR* and Cluster II, III
		R-B2: 5'-TCTCGCCGTCGTCAGTACAG-3'			
INH	<i>katG</i>	Fragment 1 F-G1: 5'-CCAACTCCTGGAAGGAATGC-3'	58°C	(-21) – 1147 (1169 bp)	Full length gene
		R-G1: 5'-AGAGGTCAGTGGCCAGCAT-3'			
	Fragment 2	F-G2: 5'-ACGAGTGGGAGCTGACGAA-3'	60°C	1016 – 2244 (1229 bp)	
		R-G2: 5'-AACCCGAATCAGCGCACGT-3'			
<i>inhA</i>	Fragment 1	F-PiA: 5'-GCGACATACCTGCTGCGCAA-3'	60°C	(-220) – 80 (300 bp)	Promoter
		R-PiA: 5'-ATCCCCCGGTTTCTCCGGT-3'			
	Fragment 2	F-CRA: 5'-GACACAACACAAGGACGCA-3'	60°C	(-20) – 987 (1008 bp)	Full length gene
		R-CRA: 5'-TGCCATTGATCGGTGATACC-3'			
SM	<i>rpsL</i>	F-L: 5'-GCGCCCAAGATAGAAAAG-3'	58°C	(-27) – 424 (451)	Full length gene

		R-L: 5'-CAACTGCGATCCGTAGA-3'		bp)	
<i>rrs</i>	Fragment 1	F-S1: 5'-GAGAGTTTGATCCTGGCTCAG-3'	58°C	9 – 981 (973 bp)	Loop 530 and loop 915
		R-S1: 5'-CCAGGTAAGGTTCTTCGCGTTG-3'			
	Fragment 2	F-S2: 5'- GCGCAGATATCAGGAGG-3'	58°C	689 – 1606 (918 bp)	1400 region
		R-S2: 5'- CGCCCACTACAGACAAG-3'			
EMB	<i>embB</i>	F-EMB: 5'-TGACCGACGCCGTGGTGATA-3	62°C	833 – 2144 (1312 bp)	ERDR** and flanking sequences
		R-EMB: 5'-GCCATGAAACCGCCACGAT-3			

Note: RRDR*: rifampicin resistance determining region; ERDR**: ethambutol resistance determining region

Supplementary Table S3. DST results using the proportional Canetti-Grosset technique and the proportional 12-well microplate assay (n= 208 *M. tuberculosis* isolates)

<i>M. tuberculosis</i> isolate	Drug sensitivity							
	Canetti-Grosset				Microplate DST			
	RMP	INH	SM	EMB	RMP	INH	SM	EMB
VNM1	S	S	R	S	S	S	R	S
VNM2	S	S	R	S	S	S	R	S
VNM3	S	R	S	S	S	R	S	S
VNM4	S	R	S	S	S	R	S	S
VNM5	S	S	R	S	S	S	R	S
VNM6	S	S	R	S	S	S	R	S
VNM7	S	R	S	S	S	R	S	S
VNM8	S	S	S	S	S	S	S	S
VNM9	S	S	S	S	S	S	S	S
VNM10	S	S	R	S	S	S	R	S
VNM11	S	S	R	S	S	S	R	S
VNM12	S	S	R	S	S	S	R	S
VNM13	S	S	S	S	S	S	S	S
VNM14	S	S	R	S	S	S	R	S

VNM15	S	S	R	S	S	S	R	S
VNM16	S	S	R	S	S	S	R	S
VNM17	R	R	R	S	R	R	R	S
VNM18	S	S	S	S	S	S	S	S
VNM19	S	S	R	S	S	S	R	S
VNM20	S	S	R	S	S	S	R	S
VNM21	S	S	R	S	S	S	R	S
VNM22	S	S	R	S	S	S	R	S
VNM23	S	S	R	S	S	S	R	S
VNM24	S	S	R	S	S	S	R	S
VNM25	S	S	R	S	S	S	R	S
VNM26	S	R	S	S	S	R	S	S
VNM27	R	R	R	S	R	R	R	S
VNM28	S	S	S	S	S	S	S	S
VNM29	S	R	R	S	S	R	R	S
VNM30	R	R	R	S	R	R	R	S
VNM31	S	S	R	S	S	S	R	S
VNM32	R	R	R	S	R	R	R	S
VNM33	R	R	R	S	R	R	R	S
VNM34	S	S	R	S	S	S	R	S
VNM35	S	R	S	S	S	R	S	S
VNM36	S	S	R	S	S	S	R	S
VNM37	S	S	R	S	S	S	R	S
VNM38	S	S	S	S	S	S	S	S
VNM39	S	S	S	S	S	S	S	S
VNM40	R	R	R	S	R	R	R	S
VNM41	S	S	S	S	S	S	S	S
VNM42	S	S	R	S	S	S	R	S
VNM43	S	S	R	S	S	S	R	S

VNM44	S	R	S	S	S	R	S	S
VNM45	S	S	S	S	S	S	S	S
VNM46	S	S	S	S	S	S	S	S
VNM47	R	R	R	R	R	R	R	R
VNM48	R	R	R	R	R	R	R	S
VNM49	S	S	S	S	S	S	S	S
VNM50	S	S	S	S	S	S	S	S
VNM51	S	R	S	S	S	R	S	S
VNM52	R	R	R	R	R	R	R	R
VNM53	S	S	S	S	S	R	S	S
VNM54	S	S	S	S	S	S	S	S
VNM55	S	S	S	S	S	S	S	S
VNM56	S	R	R	S	R	R	R	S
VNM57	R	R	R	S	R	R	R	S
VNM58	S	S	S	S	S	S	S	S
VNM59	S	S	R	S	S	S	R	S
VNM60	R	R	R	S	R	R	R	S
VNM61	R	R	R	S	R	R	R	S
VNM62	R	R	R	S	R	R	R	S
VNM63	R	R	R	S	R	R	R	S
VNM64	S	S	S	S	S	S	S	S
VNM65	S	S	S	S	S	S	S	S
VNM66	R	R	R	R	R	R	R	R
VNM67	S	R	R	S	S	R	R	S
VNM68	S	S	S	S	S	S	S	S
VNM69	R	R	R	S	R	R	R	S
VNM70	R	R	R	S	R	R	R	S
VNM71	R	R	R	S	R	R	R	S
VNM72	R	R	R	S	R	R	R	S

VNM73	R	R	R	S	R	R	R	S
VNM75	R	R	R	S	R	R	R	S
VNM76	R	R	R	S	R	R	R	S
VNM74	R	R	R	R	R	R	R	R
VNM77	R	R	R	S	R	R	R	S
VNM78	S	S	S	S	S	S	S	S
VNM79	S	S	S	S	S	S	S	S
VNM80	S	S	S	S	S	S	S	S
VNM81	S	S	S	S	S	S	S	S
VNM82	S	R	R	R	S	R	R	R
VNM83	S	R	R	S	S	R	R	S
VNM84	R	R	R	S	R	R	R	S
VNM85	R	R	R	S	R	R	R	S
VNM86	S	S	R	S	S	S	R	S
VNM87	S	R	R	S	S	R	R	S
VNM88	R	R	R	R	R	R	R	R
VNM89	S	S	S	S	S	S	S	S
VNM90	S	S	S	S	S	S	S	S
VNM91	R	R	R	R	R	R	R	R
VNM92	S	S	S	S	S	S	S	S
VNM93	S	S	S	S	S	S	S	S
VNM94	S	S	S	S	S	S	S	S
VNM95	S	S	S	S	S	S	S	S
VNM96	R	R	R	S	R	R	R	S
VNM97	S	R	S	S	S	R	S	S
VNM98	S	S	S	S	S	S	S	S
VNM99	S	S	S	S	S	S	S	S
VNM100	R	R	R	R	R	R	R	R
VNM101	S	S	R	S	S	S	R	S

VNM131	R	R	R	R	R	R	R	R	R
VNM132	S	S	S	S	S	S	S	S	S
VNM133	S	R	R	R	S	R	R	R	R
VNM134	S	R	R	S	S	R	R	R	S
VNM135	S	S	S	S	S	S	S	S	S
VNM136	S	S	S	S	S	S	S	S	S
VNM137	S	S	S	S	S	S	S	S	S
VNM137	S	S	S	S	S	S	S	S	S
VNM138	S	S	S	S	S	S	S	S	S
VNM139	S	S	S	S	S	S	S	S	S
VNM140	S	S	S	S	S	S	S	S	S
VNM141	S	S	S	S	S	S	S	S	S
VNM142	S	S	S	S	S	S	S	S	S
VNM143	S	S	S	S	S	S	S	S	S
VNM144	S	S	S	S	S	S	S	S	S
VNM145	S	S	S	S	S	S	S	S	S
VNM146	S	S	S	S	S	S	S	S	S
VNM147	S	R	S	S	S	R	S	S	S
VNM148	S	R	S	S	S	R	S	S	S
VNM149	S	R	S	S	S	R	S	S	S
VNM150	S	R	S	S	S	R	S	S	S
VNM151	S	R	S	S	R	R	S	S	S
VNM152	S	R	S	S	S	S	S	S	S
VNM153	S	R	S	S	S	R	S	S	S
VNM154	S	R	S	S	S	R	S	S	S
VNM155	S	R	S	S	S	R	S	S	S
VNM156	S	S	R	S	S	S	R	S	S
VNM157	S	S	R	S	S	S	R	S	S
VNM158	S	S	R	S	S	S	R	S	S

VNM159	S	S	R	S	S	S	R	S
VNM160	S	S	R	S	S	S	R	S
VNM161	S	S	R	S	S	S	R	S
VNM162	S	S	R	S	S	S	R	S
VNM163	R	R	S	S	R	R	S	S
VNM164	R	R	S	S	R	R	S	S
VNM165	R	R	S	S	R	R	R	R
VNM166	S	R	R	S	S	R	R	S
VNM167	S	R	R	S	S	R	R	S
VNM168	S	R	R	S	S	R	R	S
VNM169	S	R	R	S	S	R	R	S
VNM170	S	R	R	S	S	R	R	S
VNM171	S	R	R	S	S	R	R	S
VNM172	S	R	R	S	S	R	R	S
VNM173	R	S	S	S	R	S	S	S
VNM174	R	S	S	S	R	S	S	S
VNM175	S	S	R	S	S	S	R	S
VNM176	S	S	R	S	S	S	R	S
VNM177	S	S	R	S	S	S	R	S
VNM178	R	R	S	S	R	R	S	S
VNM179	R	R	S	S	R	R	S	S
VNM180	S	R	S	R	S	R	S	S
VNM181	S	R	S	R	S	R	S	R
VNM182	S	R	S	R	S	R	S	S
VNM183	R	R	R	S	R	R	R	S
VNM184	R	R	R	S	R	R	R	S
VNM185	R	R	R	S	R	R	R	S
VNM186	R	R	R	S	R	R	R	S
VNM187	R	R	R	S	R	R	R	S

VNM188	R	R	R	S	R	R	R	S
VNM189	R	R	R	S	R	R	R	S
VNM190	R	R	R	S	R	R	R	S
VNM191	R	R	R	S	R	R	R	S
VNM192	S	R	R	R	S	R	R	R
VNM193	S	R	R	R	S	R	R	R
VNM194	S	R	R	R	S	R	R	R
VNM195	R	R	R	R	R	R	R	R
VNM196	R	R	R	R	R	R	R	R
VNM197	R	R	R	R	R	R	R	R
VNM198	R	R	R	R	R	R	R	R
VNM199	R	R	R	R	R	R	R	R
VNM200	R	R	R	R	R	R	R	R
VNM201	R	R	R	R	R	R	R	R
VNM202	R	R	R	R	R	R	R	R
VNM203	R	S	R	S	R	S	R	S
VNM204	R	S	R	S	R	S	R	S
VNM205	R	S	S	S	R	S	S	S
VNM206	R	S	S	S	R	S	S	S
VNM207	R	S	S	S	R	S	S	S
VNM208	S	R	R	R	S	R	R	R

Note: Isolates with discordant between-test DST results are highlighted.

Supplementary Table S4. Comparison of the DST results for the studied *M. tuberculosis* isolates obtained with the proportional Canetti-Grosset and 12-well microplate assays.

	Drug resistance	Proportional Canetti-Grosset DST			
		R	S	Total	
Proportional	RMP	R	65	2	67

Microplate	S	0	141	141
	DST	Total	65	143
INH	R	102	1	103
	S	1	104	105
	Total	103	105	208
SM	R	114	1	114
	S	0	93	94
	Total	114	94	208
EMB	R	27	1	28
	S	3	177	180
	Total	30	178	208
MDR	R	58	0	58
	S	2	148	150
	Total	60	148	208

Supplementary Table S5. Drug resistance- associated mutations in the studied genes of the studied *M. tuberculosis* isolates

A. In *M. tuberculosis* isolates with concordant DST profiles by the proportional Canetti-Grosset and 12-well microplate techniques (n=115)

No	Isolate	DST results		Mutations were found in the studied genes						
		Canetti-Grosset	Microplate DST	Gene associated with RMP resistance	Genes associated with INH resistance			Genes associated with SM resistance		Gene associated with EMB resistance
				<i>rpoB</i>	<i>katG</i>	<i>inhA</i> and its promoter	<i>rpsL</i>	<i>rrs</i>	<i>embB</i>	
1	VNM9	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT	
2	VNM28	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT	
3	VNM46	Sensitive	Sensitive	WT	WT	WT	WT	WT	Glu378Ala	
4	VNM89	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT	
5	VNM92	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT	

6	VNM108	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT
7	VNM110	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT
8	VNM118	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT
9	VNM120	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT
10	VNM123	Sensitive	Sensitive	Gln517Gln	Arg463Leu	WT	WT	WT	Glu378Ala
11	VNM125	Sensitive	Sensitive	WT	WT	WT	WT	WT	Glu378Ala
12	VNM135	Sensitive	Sensitive	WT	WT	WT	WT	WT	Glu378Ala
13	VNM137	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	WT
14	VNM138	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	WT
15	VNM139	Sensitive	Sensitive	WT	Ala61Ala & Arg463Leu	WT	WT	WT	WT
16	VNM140	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	WT
17	VNM141	Sensitive	Sensitive	WT	Asn35Asn & Gly96Gly	WT	WT	WT	WT
18	VNM142	Sensitive	Sensitive	WT	WT	WT	WT	WT	WT
19	VNM143	Sensitive	Sensitive	WT	Ser446Asn & Arg463Leu	WT	WT	WT	WT
20	VNM144	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	Glu378Ala
21	VNM145	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	WT
22	VNM146	Sensitive	Sensitive	WT	Arg463Leu	WT	WT	WT	WT
23	VNM173	R	R	His526Tyr	Arg463Leu	WT	WT	WT	WT
24	VNM174	R	R	Ser531Leu	Arg463Leu	WT	WT	WT	WT
25	VNM205	R	R	Ser531Leu & Val577Met	WT	WT	WT	WT	WT
26	VNM206	R	R	His526Tyr	Arg463Leu	WT	WT	WT	WT
27	VNM3	H	H	WT	Ser315Thr	WT	WT	WT	WT
28	VNM4	H	H	WT	Ser315Thr	WT	WT	WT	WT
29	VNM7	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Glu378Ala
30	VNM26	H	H	WT	Arg463Leu	C-15T	WT	WT	WT
31	VNM35	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	WT
32	VNM51	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Glu378Ala
33	VNM97	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Glu378Ala
34	VNM104	H	H	WT	Ser315Thr	WT	WT	WT	WT
35	VNM128	H	H	WT	Ser315Thr	WT	WT	WT	WT
36	VNM147	H	H	WT	WT	WT	WT	WT	WT
37	VNM148	H	H	Leu511Pro	Arg463Leu	C-15T	WT	C875T	Glu378Ala
38	VNM149	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	WT
39	VNM150	H	H	WT	Ser315Thr	WT	WT	WT	WT
40	VNM153	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	WT
41	VNM154	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Glu378Ala
42	VNM155	H	H	WT	Ser315Thr & Arg463Leu	WT	WT	WT	WT
43	VNM1	S	S	WT	WT	WT	Lys43Arg	WT	WT
44	VNM2	S	S	WT	WT	WT	WT	WT	WT
45	VNM10	S	S	WT	WT	WT	WT	WT	WT
46	VNM19	S	S	WT	Arg463Leu	WT	Lys88Arg	WT	WT

47	VNM22	S	S	WT	Arg463Leu	WT	WT	C517T	Glu378Ala
48	VNM36	S	S	WT	Arg463Leu	Ile21Val	Lys43Arg	C517T	WT
49	VNM86	S	S	WT	WT	WT	Lys43Arg	WT	WT
50	VNM156	S	S	WT	Ser315Thr & Arg463Leu	WT	Lys88Arg	WT	WT
51	VNM157	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	WT
52	VNM158	S	S	WT	Ser446Asn	WT	Lys43Arg	WT	WT
53	VNM159	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	WT
54	VNM160	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	Glu378Ala
55	VNM161	S	S	WT	WT	WT	WT	WT	WT
56	VNM162	S	S	Leu533Pro	Arg463Leu	WT	WT	C517T	Leu370Arg
57	VNM175	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	WT
58	VNM176	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	WT
59	VNM177	S	S	WT	Arg463Leu	WT	Lys43Arg	WT	WT
60	VNM163	HR	HR	Ser531Leu	Arg463Leu	C-15T	WT	WT	Gln497Arg
61	VNM164	HR	HR	Ser531Leu	Pro241Pro & Ser315Thr	WT	WT	WT	Phe330Leu
62	VNM178	HR	HR	Ser531Leu	WT	C-15T	WT	WT	WT
63	VNM179	HR	HR	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Gly406Asp
64	VNM87	HS	HS	WT	WT	C-15T	WT	WT	WT
65	VNM107	HS	HS	WT	Ser315Asn	WT	Lys43Arg	WT	Met557Ile
66	VNM126	HS	HS	WT	Ser315Asn	WT	Lys43Arg	C1001T	WT
67	VNM134	HS	HS	WT	Ser315Asn	WT	Lys43Arg	WT	WT
68	VNM166	HS	HS	WT	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	WT
69	VNM167	HS	HS	WT	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	WT
70	VNM168	HS	HS	Leu533Pro	Ser315Thr & Arg463Leu	C-15T	Lys43Arg	WT	WT
71	VNM169	HS	HS	WT	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	WT
72	VNM170	HS	HS	WT	Ser315Thr & Arg463Leu	WT	WT	A514C	WT
73	VNM171	HS	HS	WT	Arg463Leu	WT	Lys43Arg	WT	Met557Ile
74	VNM172	HS	HS	WT	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met557Ile
75	VNM181	HE	HE	WT	Ser315Thr & Arg463Leu	WT	WT	WT	Met306Val
76	VNM203	RS	RS	Ser531Leu	Arg463Leu	WT	WT	C517T	WT
77	VNM204	RS	RS	His526Tyr	Arg463Leu	WT	Lys43Arg	WT	Asp534Asp
78	VNM32	HRS	HRS	Ile572Phe	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Gln497Arg & Leu558Leu
79	VNM57	HRS	HRS	His526Tyr	Ser315Asn	WT	Lys43Arg	WT	Met306Ile
80	VNM60	HRS	HRS	Ser531Leu	Ser315Thr	WT	Lys88Arg	WT	WT
81	VNM62	HRS	HRS	Ser531Leu	Ser315Thr	WT	Lys43Arg	WT	WT
82	VNM69	HRS	HRS	Ser531Leu	Ser315Thr	WT	Lys43Arg	WT	Gln497Arg & Met557Ile
83	VNM70	HRS	HRS	Asp516Val	Ser315Asn	WT	WT	WT	WT
84	VNM72	HRS	HRS	His526Arg	Arg463Leu	C-15T	Lys88Arg	WT	Met306Ile & Glu378Ala
85	VNM76	HRS	HRS	His526Arg	Arg463Leu	C-15T	WT	WT	Met306Ile &

									Glu378Ala
86	VNM85	HRS	HRS	Ser522Leu & Ser531Leu	Ser315Asn	WT	Lys43Arg	WT	WT
87	VNM96	HRS	HRS	Ser522Leu & Ser531Leu	Ser315Asn	WT	Lys43Arg	WT	WT
88	VNM183	HRS	HRS	Leu511Pro	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	WT
89	VNM184	HRS	HRS	His526Tyr	Ser315Thr	WT	Lys43Arg	WT	WT
90	VNM185	HRS	HRS	Leu533Pro	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	WT
91	VNM186	HRS	HRS	His526Tyr	Arg463Leu	WT	Lys88Arg	WT	WT
92	VNM187	HRS	HRS	Leu511Pro	Ser315Thr	WT	Lys43Arg	WT	WT
93	VNM188	HRS	HRS	Leu530Met & Ser531Phe	Ser315Thr & Arg463Leu	WT	WT	A514C	Gly406Asp & Asp534Asp
94	VNM189	HRS	HRS	WT	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met557Ile
95	VNM190	HRS	HRS	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys88Thr	WT	WT
96	VNM192	HSE	HSE	Asp516Tyr	Ser315Thr & Arg463Leu	C-15T	WT	A1401G	Gly406Ala
97	VNM193	HSE	HSE	Leu511Pro & Asn518Ser	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met306Ile & Met557Ile
98	VNM194	HSE	HSE	Leu533Pro	Ser315Thr & Arg463Leu	WT	WT	WT	Met306Val
99	VNM208	HSE	HSE	Leu533Pro	Ser315Thr & Arg463Leu	WT	Lys88Arg	WT	Met306Val
100	VNM47	HRSE	HRSE	His526Asn & Leu533Ser	Ser315Asn	WT	Lys43Arg	WT	Gly406Ala
101	VNM52	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met306Val
102	VNM88	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	C-15T	WT	A1401G	Val360Met
103	VNM91	HRSE	HRSE	Asp516Val	Ser315Asn&Arg463Leu	WT	WT	G878A	Met306Val
104	VNM100	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	Leu60Leu	Lys43Arg	WT	Gln497Arg
105	VNM102	HRSE	HRSE	His526Arg	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met306Ile & Met557Ile
106	VNM103	HRSE	HRSE	His526Arg	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met306Val
107	VNM106	HRSE	HRSE	His526Asn & Leu533Ser	Ser315Asn	WT	Lys43Arg	WT	Gly406Ala
108	VNM114	HRSE	HRSE	Ser531Leu	Ser315Thr	WT	Lys43Arg	WT	Asp354Ala & Gln497Lys
109	VNM130	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys43Arg	WT	Met306Val
110	VNM195	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	WT	A514C	Met306Val
111	VNM196	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys43Arg	A1401G	WT
112	VNM198	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys88Thr	WT	WT
113	VNM200	HRSE	HRSE	His526Arg	Ser315Thr & Arg463Leu	WT	Lys43Arg	A1401G	Met306Val
114	VNM201	HRSE	HRSE	Ser531Leu	Ser315Thr	WT	WT	WT	Asp354Ala
115	VNM202	HRSE	HRSE	Ser531Leu	Ser315Thr & Arg463Leu	WT	Lys43Arg	A1401G	Met306Val



Research paper

Quadruple-first line drug resistance in *Mycobacterium tuberculosis* in Vietnam: What can we learn from genes?



Huy Quang Nguyen^{a,b,c,d,*}, Nhung Viet Nguyen^{e,f}, Lucie Contamin^{a,b,d}, Thanh Hoa Thi Tran^b, Thuong Thi Vu^b, Hung Van Nguyen^g, Ngoc Lan Thi Nguyen^h, Son Thai Nguyenⁱ, Anh Duc Dang^b, Anne-Laure Bañuls^{a,b,d,1}, Van Anh Thi Nguyen^{b,1}

^a UMR MIVEGEC (224 IRD-5290 CNRS-Université de Montpellier), Institute of Research for Development, Montpellier, France

^b Department of Bacteriology, National Institute of Hygiene and Epidemiology, Hanoi, Viet Nam

^c Department of Biotechnology-Pharmacology, University of Science and Technology of Hanoi, Hanoi, Viet Nam

^d LMI Drug Resistance in South East Asia (DRISA), NIHE, Hanoi, Viet Nam

^e Viet Nam National Tuberculosis Programme, Hanoi, Viet Nam

^f Viet Nam Association for Tuberculosis and Lung Disease, Hanoi, Viet Nam

^g Department of Microbiology, National Lung Hospital, Hanoi, Viet Nam

^h Department of Microbiology, Pham Ngoc Thach Hospital, Ho Chi Minh, Viet Nam

ⁱ Department of Microbiology, Military Medical University, Hanoi, Viet Nam

ARTICLE INFO

Article history:

Received 6 December 2016

Received in revised form 13 February 2017

Accepted 13 February 2017

Available online xxxx

Keywords:

Drug resistance-associated mutations

Drug susceptibility testing

First line drugs

Second line drugs

Mycobacterium tuberculosis

Spoligotyping

MIRU-VNTR

Extensively drug resistance

ABSTRACT

In Vietnam, a country with high tuberculosis (137/100.000 population) and multidrug-resistant (MDR)-TB burdens (7.8/100.000 population), little is known about the molecular signatures of drug resistance in general and more particularly of second line drug (SLD) resistance. This study is specifically focused on *Mycobacterium tuberculosis* isolates resistant to four first-line drugs (FLDs) that make TB much more difficult to treat. The aim is to determine the proportion of SLD resistance in these quadruple drug resistant isolates and the genetic determinants linked to drug resistance to better understand the genetic processes leading to quadruple and extremely drug resistance (XDR). 91 quadruple (rifampicin, isoniazid, ethambutol and streptomycin) FLD resistant and 55 susceptible isolates were included. Spoligotyping and 24-locus MIRU-VNTR techniques were performed and 9 genes and promoters linked to FLD and SLD resistance were sequenced. SLD susceptibility testing was carried out on a subsample of isolates. High proportion of quadruple-FLD resistant isolates was resistant to fluoroquinolones (27%) and second-line injectable drugs (30.2%) by drug susceptibility testing. The sequencing revealed high mutation diversity with prevailing mutations at positions *katG*315, *inhA*-15, *rpoB*531, *embB*306, *rrs*1401, *rpsL*43 and *gyrA*94. The sensitivity and specificity were high for most drug resistances (>86%), but the sensitivity was lower for injectable drug resistances (<69%). The mutation patterns revealed 23.1% of pre-XDR and 7.7% of XDR isolates, mostly belonging to Beijing family. The genotypic diversity and the variety of mutations reflect the existence of various evolutionary paths leading to FLD and SLD resistance. Nevertheless, particular mutation patterns linked to high-level resistance and low fitness costs seem to be favored.

© 2017 Published by Elsevier B.V.

1. Introduction

10.4 million people were estimated to be infected with tuberculosis (TB) in 2015, among which 1.8 million died (WHO, 2016). The emergence of drug resistance is currently one of the challenges in TB control. Multidrug resistant TB (MDR-TB), caused by *Mycobacterium tuberculosis* resistant at least to rifampicin (RIF) and isoniazid (INH), the two most

potent first-line anti-TB drugs (FLDs), has been found in all regions of the world (WHO, 2016). MDR-TB treatment with second-line drugs (SLDs) is complex, lengthy and costly and can lead to the emergence of extensively drug-resistant TB (XDR-TB), caused by MDR bacteria that are resistant to at least one of the fluoroquinolones (FQs) and one of the three second-line injectable drugs (SLIDs) (WHO, 2016). Among the estimated 9.5% of patients with MDR-TB who developed XDR-TB, only 28% have been treated successfully (WHO, 2016). In 2015, 117 countries reported XDR-TB cases (WHO, 2016).

In *M. tuberculosis*, the molecular bases of FLD and SLD resistance are largely documented (Sandgren et al., 2009; Zhang and Yew, 2015). The type and frequency of drug resistance-associated mutations vary by

* Corresponding author at: Department of Biotechnology-Pharmacology, University of Science and Technology of Hanoi, 18 Hoang Quoc Viet road, 1000 Hanoi, Viet Nam.

E-mail address: huynguyen.biotech@gmail.com (H.Q. Nguyen).

¹ Anne-Laure Bañuls and Van Anh Thi Nguyen contributed equally to this work.

region and according to *M. tuberculosis* genetic background (Fenner et al., 2012; Ford et al., 2013; Gagneux et al., 2006a; Sandgren et al., 2009).

In Vietnam, MDR-TB increased from 2.7% to 4.0% among new TB cases, and from 19.0% to 23.0% among retreatment TB cases between 2005 and 2011 (Nhung et al., 2015). The FLDs used for treatment of non-MDR TB include INH, RIF, streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) (Nhung et al., 2015). In the late 2009, SLDs were officially used for MDR-TB cases in the framework of the Programmatic Management of Drug-resistant TB (PMDT) (Vietnam National Tuberculosis Control Programme, 2009). Nevertheless, SLDs were used before the PMDT launch without any official guidelines (Hoa et al., 2015). The few data published on SLD resistance in Vietnam showed 17.9% of ofloxacin (OFX) resistance and 6.0% of kanamycin (KAN) and capreomycin (CAP) resistance among 84 tested MDR-TB cases leading to 5 XDR-TB cases (Nguyen et al., 2016). The genetic background of these drug resistant isolates is unknown. Like in many high TB burden countries, information on the genetic determinants of FLD and SLD resistance and the link with the genetic background of the bacteria are still lacking.

In this context, we aimed to genetically and phenotypically characterize quadruple-FLD resistant *M. tuberculosis* isolates (resistant to RIF, INH, EMB and STR) collected in Vietnam and to identify the genetic determinants of FLD and SLD resistance in these samples. This study will help define the proportion of these quadruple-FLD resistant isolates also SLD resistant (pre-XDR or XDR), understand how these isolates became resistant on a genetic point of view and investigate the mutation patterns according to *M. tuberculosis* families and MIRU-VNTR genotypes. The final goal is to better understand the genetic processes leading to quadruple drug resistance and XDR.

2. Materials and methods

2.1. *M. tuberculosis* isolates

A total of 91 quadruple-FLD (RIF, INH, EMB, STR) resistant *M. tuberculosis* isolates were selected from the collection of the Laboratory of Tuberculosis, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. These samples were collected between 2005 and 2009 (before the PMDT launch) from three regional TB reference hospitals of Vietnam: the National Lung Hospital (North), Pham Ngoc Thach Hospital (South), and Hue Central Hospital (Centre) (see Table S1.1). Fifty-five FLD-sensitive isolates from the same hospitals and period were added for comparison. Considering the MDR-TB rate of 2.7% among new cases and 19% among previously treated cases determined in Vietnam in 2005 and the 36% estimated percentage of quadruple resistant isolates among MDR-TB cases (Caws et al., 2006), for a collection of 1500 *M. tuberculosis* isolates per year (Nhung et al., 2015), we thus expect a maximum of 137 quadruple drug resistant isolates for 5 years. Consequently, the 91 isolates originated from different regions in Vietnam and different years can be considered as a meaningful sampling to study the genetic diversity in the quadruple-FLD resistant isolates.

2.2. Drug susceptibility testing

The FLD-susceptibility testing was determined by using the proportion method as recommended by WHO in two of the three TB reference laboratories (the National Lung Hospital and Pham Ngoc Thach Hospital) based on the following drug concentrations on Löwenstein-Jensen medium: INH (0.2 mg/L), RIF (40 mg/L), STR (4 mg/L) and EMB (2 mg/L) (WHO, 2003). In parallel, the proportional agar microplate assay on Middlebrook 7H11 medium with drug concentrations: INH (0.2 mg/L), RIF (1 mg/L), STR (2 mg/L) and EMB (5 and 7.5 mg/L), as described in Nguyen et al. (2015), was applied on all samples before genotyping in the National Institute of Hygiene and Epidemiology (NIHE), Hanoi. PZA susceptibility testing was not performed for the

whole sample because the technique is difficult to implement and is not routinely performed in Vietnam.

To test also the sensitivity and specificity of mutations for SLD resistance detection, SLD-susceptibility testing was carried out on a subsample of 63 FLD-resistant and 20 sensitive isolates by the proportional agar microplate assay on Middlebrook 7H10 medium using drug concentrations in NIHE: KAN (5 mg/L), amikacin (AMK, 4 mg/L), CAP (4 mg/L), OFX (2 mg/L), levofloxacin (LVX, 1 mg/L), moxifloxacin (MXF, 0.5 and 2 mg/L), and gatifloxacin (GAT, 1 mg/L) (WHO, 2012, http://www.stoptb.org/wg/gli/assets/documents/Updated%20critical%20concentration%20table_1st%20and%202nd%20line%20drugs.pdf). For MXF susceptibility, growth at concentrations ≥ 0.5 mg/L and < 2 mg/L indicated low level of resistance and growth at concentrations ≥ 2 mg/L indicated high level of resistance. The H37Rv laboratory strain was included as control for all FLD and SLD-susceptibility testing.

2.3. Genotyping methods

These samples were typed by spoligotyping to identify their families, as previously described (Kamerbeek et al., 1997), and data were compared on SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE) (Demay et al., 2012). The isolates were also characterized using the 24-locus MIRU-VNTR set to explore the genetic diversity (Supply et al., 2006). The unknown spoligotypes in SITVIT WEB were further classified thanks to the MIRU-VNTR analysis when it was possible.

2.4. DNA amplification and sequencing

The following drug resistance genes or gene promoters were PCR-amplified and sequenced (see Table S2): i) RIF resistance: *rpoB* (codons 432–814, called here *rpoB*-F1) containing RIF resistance-determining region (RRDR, codons 507–533); ii) INH resistance: full-length *katG* and *inhA* genes and *inhA* promoter; iii) STR resistance: full-length *rpsL* gene and *rrs* (973 bp containing the 530 loop and the 915 region, called here *rrs*-F1); iv) EMB resistance: *embB* fragment (codons 277–714); v) FQ resistance: fragment containing the quinolone resistance-determining regions (QRDRs) of *gyrA* (codons 74–133) and *gyrB* (codons 461–502) genes; and vi) KAN, AMK and CAP resistance: *rrs* (containing the 1400–1500 region, called here *rrs*-F2). In quadruple-resistant isolates without mutations in *rpoB*-F1, a second fragment *rpoB* (codons 1–428, called here *rpoB*-F2) was sequenced. The primers used in this study were previously described or modified using the Primer-BLAST tool (see Supplementary material, Table S2) (Campbell et al., 2011; Lee et al., 2012; Lipin et al., 2007; Nguyen et al., 2015; Rigouts et al., 2007; Shi et al., 2011). The *M. tuberculosis* codon numbering system was used for all the genes, except for the *rpoB* gene for which the *Escherichia coli* codon numbering system was applied.

The DNA and PCR mixtures were prepared as previously described (Nguyen et al., 2015). The PCR conditions were: 15 min of Taq activation at 95 °C, then 35 cycles (95 °C for 1 min, 58–63 °C (Table S2) for 1.5 min and 72 °C for 2 min), and 72 °C for 5 min. PCR products were sequenced in the forward and reverse sense by Eurofins MWG Operon (Ebersberg, Germany). Each sequence was analyzed using the Bioedit software, version 7.1.10. The sequence of the *M. tuberculosis* H37Rv reference strain (GenBank n° NC.000962.3) was used for mutation identification.

2.5. Data analysis

Sensitivity and specificity values were determined by comparison of phenotypic and genotypic data. The known mutations associated with drug resistance, the non-synonymous mutations and new mutations that were found only in drug-resistant isolates were considered for analyses. The synonymous mutations, the non-synonymous mutations previously reported not to be linked to drug resistance or found only

in sensitive isolates were not considered (see details in Tables S1.2, S1.3, S1.4, S1.5 and S1.6 in Supplementary material). The two tailed-Fisher's exact test was used for comparing the proportion of mutations between *M. tuberculosis* families; *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Spoligotyping and 24-locus MIRU-VNTR patterns

In susceptible isolates, 41 spoligotypes and 49 MIRU-VNTR patterns (46 unique and 3 clusters) were detected. In quadruple-FLD resistant isolates, 27 spoligotypes and 73 MIRU-VNTR patterns (63 unique and 10 clusters) were identified. Each cluster contained 2–8 isolates (average 3.15) (Table S3). It is worth noting that in clusters, the gene sequence analysis revealed only two Beijing isolates with the same pattern of drug resistance-associated gene mutations (see below). These data showed that even if the 24-locus MIRU-VNTR data cannot fully detect the genetic diversity within Beijing family, only two quadruple drug resistant isolates were totally identical (genotypes and phenotypes, see Table S3). To compare the genetic data between the families, we used four classes, "Beijing", "EAI" (included the EAI-like defined by MIRU-VNTR), "T" and "Others" (included the unknown and LAM isolates).

3.2. Drug susceptibility testing of SLDs

All the 20 tested FLD-sensitive isolates were also susceptible to SLDs (Table 1). Among the 63 out of 91 quadruple-resistant isolates tested, 39.7% (25 isolates) were resistant to at least one SLD. Specifically, 30.2% (19 isolates) were resistant to at least one SLID and 27% (17 isolates) were resistant to at least one FQs (1 OFX-resistant isolate and 16 OFX-MFX resistant isolates, none with high level resistance to MFX). No isolate was resistant to LFX and GFX (Table 1). Among the 25 isolates resistant to at least one SLD, 11 isolates (17.5%) were XDR (concomitantly resistant to SLIDs and FQs) and 14 isolates (22.2%) were pre-XDR (Table 1). Within the pre-XDR and XDR samples, the Beijing family was the most represented (Table 1). Nevertheless, the proportion of T family in pre-XDR and XDR was much higher than for the other families. These results should be confirmed on a larger sample of T and EAI families.

3.3. INH resistance and mutations in *katG*, *inhA* and *inhA* promoter

Among the 91 quadruple-FLD resistant isolates, 89 (97.8%) carried INH resistance-associated mutations in *katG* and/or *inhA* (Tables 2 and S1.2). The most frequent *katG* mutation was found at *katG*315 (Ser-Thr) (Table 3). Nine other *katG* mutations were also detected at low frequency (Table S1.2). The deletion at *katG*480 was never reported (Table S1.2). The mutation *katG*463 (Arg-Leu), previously confirmed not to be associated with INH resistance, was found in both drug-

resistant and sensitive isolates. In *inhA*, the most frequent mutations were at position *inhA*-15 (14.3%, *n* = 13) and at position *inhA*94 (5.5%, *n* = 5) (Table 3). The *katG* and *inhA* mutations that were never associated with INH resistance were found in susceptible isolates (Table S1.2).

Analysis of mutations in *katG*, *inhA* and *inhA* promoter revealed a sensitivity of 97.8% and a specificity of 100% for detecting INH-resistant isolates (Table 2).

3.4. RIF resistance and *rpoB* mutations

Among the 91 quadruple FLD-resistant isolates, 90 (98.9%) carried at least one RIF resistance-associated mutation in *rpoB*-F1, of which 94.5% (86 isolates) were in the RRDR (Table 2). Various types of mutations were detected such as single mutations, insertion, deletion and double mutations (Table S1.3). The most frequent *rpoB* mutations were at *rpoB*531, *rpoB*526 and *rpoB*516 positions, accounting for 86.8% (79 isolates) of the quadruple-FLD resistant isolates (Tables 3 and S1.3). The Ser531Leu (TCG-CTG) substitution was never described (Table S1.3). The only quadruple-FLD resistant isolate without any mutation in *rpoB*-F1 had Val146Phe mutation in *rpoB*-F2 (Table S1.3). Among the susceptible isolates, only one had a synonymous mutation at *rpoB*517 within the RRDR (Table S1.3).

Analysis of mutations in the *rpoB*-F1 and *rpoB*-F2 showed 100% of sensitivity and specificity for detecting RIF-resistant isolates (Table 2).

3.5. STR resistance and *rpsL* and *rrs* mutations

STR resistance-associated mutations in *rpsL* and/or *rrs*-F1 were detected in 90.1% (*n* = 82) of quadruple FLD-resistant isolates (Table 2). All the *rpsL* mutations were found at *rpsL*43 and *rpsL*88 positions, accounting for 70.3% (*n* = 64) of quadruple FLD-resistant isolates (Table S1.4). Mutations in *rrs*-F1 were detected at *rrs*514, *rrs*517, *rrs*878 and *rrs*908, accounting for 20.9% (*n* = 19) of quadruple-FLD resistant isolates (Table S1.4).

No susceptible isolate had any mutation in the *rpsL* gene, while one isolate carried a substitution at nucleotide C295T of *rrs*-F1 that was never associated with STR resistance (Table S1.4). Sensitivity and specificity for detecting phenotypic-STR resistance based on mutations in *rpsL* and *rrs*-F1 were 90.1% and 100%, respectively (Table 2).

3.6. EMB resistance and *embB* mutations

EMB resistance-associated mutations in the *embB* gene were found in 79 (86.8%) quadruple-FLD resistant isolates particularly at positions *embB*306, *embB*497, and *embB*406, accounting for 79.1% (*n* = 72) (Tables 2, 3 and S1.5). Six other EMB resistance-associated mutations were also detected. Non-synonymous mutations at positions *embB*370, *embB*378, *embB*387 and *embB*438, not associated with EMB resistance, were found in EMB resistant and sensitive isolates (Table S1.5).

Analysis of *embB* mutations showed a sensitivity of 86.8% and specificity of 100%, for detecting EMB resistant isolates (Table 2).

3.7. Mutations in multiple loci and resistance patterns of quadruple-FLD resistant isolates

Considering the seven studied genes and promoter (*rpoB*, *katG*, *inhA* and its promoter, *rpsL*, *rrs*-F1, and *embB*), a total of 76 distinct mutation patterns were identified in the 91 quadruple-FLD resistant isolates. Unique mutation patterns were found in 67 isolates, whereas 9 different mutation patterns characterized the other 24 isolates. Seventy-two isolates carried mutations associated with resistance to RIF, INH, STR and EMB, in agreement with their quadruple-FLD resistant phenotype (sensitivity = 79.1% and specificity = 100%). The most common combination included at least mutations at positions *rpoB*531 (Ser-Leu), *katG*315 (Ser-Thr), *rpsL*43 (Lys-Arg) and *embB*306 (Met-Val) (*n* = 7,

Table 1

SLD-susceptibility patterns of 63 quadruple-FLD resistant isolates according to the *M. tuberculosis* families.

Type	SLD resistance	Beijing	EAI	T	Others	Total
Pre-XDR	Sensitive to SLDs	28	7	2	1	38
	KAN	1	0	0	0	1
	CAP	1	0	0	0	1
	KAN + AMK	0	0	0	1	1
	KAN + AMK + CAP	5	0	0	0	5
XDR	OFX + MXF	4	0	2	0	6
	KAN + OFX + MXF	0	0	1	0	1
	KAN + AMK + CAP + OFX	0	1	0	0	1
	KAN + AMK + CAP + OFX + MXF	6	2	1	0	9
	Total	45	10	6	2	63

KAN: kanamycin, AMK: amikacin, CAP: capreomycin, OFX: ofloxacin; MXF: moxifloxacin.

Table 2
Sensitivity and specificity estimation for determination of SLD and FLD resistance by mutation detection in the 91 quadruple-resistant isolates.

Drug	Locus/gene	FLD-resistant isolates		FLD-susceptible isolates		Sensitivity/specificity ^a	
		With mutation	Without mutation	With mutation	Without mutation	Sensitivity (%)	Specificity (%)
RIF	<i>rpoB</i>	91 ^b	0	0	55	100	100
	RRDR of <i>rpoB</i>	86	5	0	55	94.5	100
INH	<i>katG</i>	82 ^c	9	0	55	90.1	100
	<i>inhA</i> and its promoter	14	77	0	55	15.4	100
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	89 ^c	2	0	55	97.8	100
STR	<i>rpsL</i>	64	27	0	55	70.3	100
	<i>rrs-F1</i>	19	72	0	55	20.9	100
	<i>rpsL</i> + <i>rrs-F1</i>	82	9	0	55	90.1	100
EMB	<i>embB</i>	79 ^d	12	0	55	86.8	100
OFX	<i>gyrA</i>	20 ^e	71	0	55	76.5 ^f	100
	<i>gyrB</i>	2	89	0	55	11.8 ^f	100
	<i>gyrA</i> + <i>gyrB</i>	22 ^e	69	0	55	88.2 ^f	100
KAN	<i>rrs-F2</i>	13 ^g	78	0	55	61.1 ^f	100
AMK	<i>rrs-F2</i>	13 ^g	78	0	55	68.8 ^f	100
CAP	<i>rrs-F2</i>	13 ^g	78	0	55	68.8 ^f	100
QDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA</i> and <i>rpsL</i> and/or <i>rrs-F1</i> and <i>embB</i>	72	19	0	55	79.1	100

^a Mutations not associated with drug resistance were excluded (see Table S1.1–S1.6 in the Supplemental material for details).

^b The synonymous *rpoB*517 mutation was not considered.

^c The *katG*463 mutation was not considered.

^d The *embB*370, *embB*378, *embB*387 and *embB*438 mutations were not considered.

^e The *gyrA*95 mutation was not considered.

^f SLD-susceptibility testing was performed on 63 QDR and 20 susceptible isolates (KAN resistant isolates, n = 18; AMK resistant isolates, n = 16; CAP resistant isolates, n = 16; OFX resistant isolates, n = 17; Table 1).

^g Only 11 isolates had SLD-susceptibility profile.

7.7%) (Table S1.1). The sequencing results did not allow identifying 19 isolates (20.9%) as quadruple-FLD resistant, mainly due to the non-detection of mutations associated with STR and EMB resistance.

3.8. Mutations conferring resistance to SLDs in *rrs-F2* (1400–1500 region) and *gyrA* and *gyrB* genes

Mutations in *rrs-F2* and in *gyrA* and *gyrB* genes were detected in 14.3% (n = 13) and 24.2% (n = 22) of the 91 quadruple-FLD resistant isolates, respectively (Tables 2 and S1.6). In *rrs-F2*, the most frequent mutation was at *rrs*1401 position (Table 3). In *gyrA* gene, mutations at *gyrA*94 position were the most common (15.4%, n = 14), followed by mutation at *gyrA*90 position (5.5%, n = 5) (Tables 3 and S1.6). The *gyrA*95 (Ser-Thr) mutation, previously known not to be associated with FQ resistance, was found in both drug-resistant and sensitive isolates. Two *gyrB* mutations at *gyrB*461 and *gyrB*504 positions were each found in one isolate (Table S1.6).

The gene sequencing for detecting KAN, AMK and CAP resistance showed sensitivity between 61% and 69% and specificity of 100% for all drugs. Overall, sensitivity for detection of SLID resistance was only

57.9% (11/19 SLID resistant isolates identified by DST). For FQ resistance, sensitivity was 88.2% and specificity was 100% (Table 2).

3.9. Multiple locus mutations associated with pre-XDR and XDR

The global analysis of mutations in the 9 studied genes and promoter (*katG*, *inhA* and its promoter, *rpoB*, *rpsL*, *rrs*, *embB*, *gyrA* and *gyrB*) of the 91 quadruple-FLD resistant isolates revealed 78 different mutation patterns, in which 71 were unique and seven were shared by 2–7 isolates/each (average 2.9) (Table S1.1). Finally, out of 91 quadruple-FLD resistant isolates, 21 (23.1%) isolates were pre-XDR and 7 (7.7%) ones were XDR by DNA sequencing. It is worth noting that each pre-XDR or XDR isolate had a unique mutation pattern (Table S1.1).

Among the 14 pre-XDR isolates (out of the 63 quadruple resistant isolates) identified by DST, 10 (71.4%) were genetically confirmed (Table 4). The other four isolates were genetically characterized as resistant to quadruple FLDs (RIF, INH, STR and EMB; 3 isolates) or to only RIF (1 isolate). Among the 11 XDR isolates (out of the 63 quadruple resistant isolates) identified by DST, 4 (45.5%) were genetically confirmed. The other isolates were genetically identified as pre-XDR (Table 4).

Table 3
Frequency of the most frequent mutations in the studied genes and promoter and their distribution according to the *Mycobacterium tuberculosis* families.

Drug(s)	The most frequent mutations	Beijing # (%)	EAI # (%)	T # (%)	Others # (%)	Total # (%)
RIF	<i>rpoB</i> 531 (Ser-Leu)	31 (50)	5 (29.4)	4 (50)	2 (50)	42 (46.2)
	<i>rpoB</i> 526 (His-Asp)	8 (12.9)	6 (35.3)	0	0	14 (15.4)
	<i>rpoB</i> 516 (Asp-Val)	9 (9.9)	2 (11.7)	0	0	11 (12.1)
INH	<i>katG</i> 315 (Ser-Thr)	58 (93.5)	9 (52.9)	5 (62.5)	1 (25)	74 (81.3)
	<i>inhA</i> -15 (C-T)	5 (8.1)	6 (35.3)	2 (25)	0	13 (14.3)
	<i>inhA</i> 94 (Ser-Ala)	3 (4.8)	0	2 (25)	0	5 (5.5)
STR	<i>rpsL</i> 43 (Lys-Arg)	34 (54)	1 (5.9)	4 (50)	1 (25)	40 (44)
	<i>rpsL</i> 88 (Lys-Arg)	16 (25.8)	3 (17.6)	0	0	19 (20.9)
	<i>rrs</i> 514 (A-C)	7 (11.3)	4 (29.4)	0	1 (25)	12 (13.2)
EMB	<i>embB</i> 306 (Met-Val)	23 (37.1)	3 (17.6)	4 (50)	0	30 (33)
	<i>embB</i> 306 (Met-Ile)	13 (21)	8 (47.1)	1 (12.5)	1 (25)	23 (25.3)
FQs	<i>gyrA</i> 94 (Asp-Gly)	6 (9.7)	1 (5.9)	3 (37.5)	0	10 (11)
KAN, AMK and CAP	<i>rrs</i> 1401 (A-G)	9 (14.5)	3 (17.6)	0	0	12 (13.2)

#: Number of quadruple-FLD resistant isolates, Beijing (n = 62), EAI (n = 17), T (n = 8) and Others (n = 4).

Table 4
Mutations associated with resistance to second-line injectable drugs and fluoroquinolones in pre-XDR and XDR isolates.

Phenotype	FQ-resistant mutation	SLID-resistant mutation	Total
Pre-XDR	<i>gyrA94</i> (Asp-Gly)	WT	4
	<i>gyrA94</i> (Asp-Tyr)	WT	1
	<i>gyrB461</i> (Asp-Asn)	WT	1
	WT	<i>rrs1401</i> (A-G)	3
	WT	<i>rrs1484</i> (G-T)	1
	WT	WT	4
XDR	<i>gyrA88</i> (Gly-Ala)	<i>rrs1401</i> (A-G)	1
	<i>gyrA90</i> (Ala-Val)	<i>rrs1401</i> (A-G)	1
	<i>gyrA94</i> (Asp-Gly)	<i>rrs1401</i> (A-G)	3
	<i>gyrA94</i> (Asp-Ala)	WT	2
	<i>gyrA94</i> (Asp-Gly)	WT	1
	<i>gyrB504</i> (Ala-Val)	WT	1
	WT	<i>rrs1401</i> (A-G)	2
	WT	<i>rrs1401</i> (A-G)	2

The sensitivity and specificity for detecting pre-XDR isolates were 71.4% and 100%, respectively, and for detecting XDR isolates were 45.5% and 100%, respectively.

3.10. Mutation patterns, *M. tuberculosis* families and MIRU-VNTR clusters

In the 91 quadruple-FLD resistant isolates, the frequencies of mutations per gene varied according to families and drug resistance (Table S4). For instance, the frequencies of INH and STR resistant mutations were globally significantly different between families ($p = 0.016$ and $p = 0.0012$, respectively) (Table S4). For INH resistance, mutation frequencies in *katG* were significantly different between Beijing and T ($p = 0.0091$) and in *inhA* between EAI and Beijing ($p = 0.017$) (Table S4). For STR resistance, the frequency of *rpsL* mutations was significantly higher in Beijing isolates than in EAI isolates ($p = 0.000017$), whereas frequency of mutations in *rrs-F1* was significantly higher in EAI than in Beijing ($p = 0.00026$) and in T family ($p = 0.0076$) (Table S4). In contrast, for RIF and EMB resistance, the global frequencies of isolates with mutations were not significantly different between the families ($p > 0.05$). Among the pre-XDR and XDR isolates determined by mutations, Beijing family was the most represented (Table S4). Nevertheless, the proportion of isolates with mutations within pre-XDR and XDR samples were not significantly different between the families.

Mutation analysis according to the 24-locus MIRU-VNTR genotypes of the 91 quadruple-FLD resistant isolates showed that the 63 unique MIRU-VNTR genotypes revealed unique patterns of drug resistance-associated gene mutations. Among the 10 clusters including quadruple resistant isolates, only one cluster (cluster 7) contained two Beijing isolates fully phenotypically and genotypically identical (Table S3). Among the 28 pre-XDR or XDR isolates identified by genetic mutations, 19 had unique genotypes. Six clusters contained 9 pre-XDR or XDR isolates and all revealed different mutation patterns (Table S3).

4. Discussion

4.1. SLD resistance in quadruple-FLD resistant isolates assessed by DST

In this study, we found a high proportion of resistance to SLIDs (30.2%) and FQs (27%) among the 63 clinical quadruple-resistant isolates characterized by DST. Thus, the proportion of pre-XDR and XDR isolates was high (22.2% and 17.5%, respectively). It is worth noting that a meta-analysis indicated that, on average, 9.4% (95% CI 7.4–11.6%) of MDR-TB strains have additional resistance to both FQs and SLIDs (Zignol et al., 2012). These data suggest that the risk of XDR is twice as high in quadruple drug resistant isolates in this study. Since these isolates were collected before the PMDT implementation, these findings suggest that the SLD resistance situation was already complicated before the official use of SLDs. The higher SLD resistance found in this study compared to that described in the NTP report collected

during PMDT implementation (17.9% among MDR isolates) (Nguyen et al., 2016) could be explained by the fact that our sample included only isolates with quadruple-FLD resistance. It is worth noting that all isolates resistant to AMK were also resistant to KAN and CAP representing 84.2% of SLID resistant isolates. This is probably due to cross-resistance phenomena as previously described (Jugheli et al., 2009; Zhang and Yew, 2015). Moreover, nearly all FQ resistant isolates were resistant to both OFX and MXF, while no isolate was resistant to LVX and GAT. The use of OFX and LVX in PMDT is thus optimal, with OFX effective in FQ sensitive isolates and LVX in OFX resistant cases.

4.2. Diversity of drug resistance-associated mutation patterns and epistasis

A high diversity of mutations linked to FLD and SLD resistance was detected in quadruple-FLD resistant isolates. Considering all genes and the MIRU-VNTR data, almost each quadruple-FLD resistant isolate (78/91) had a unique genetic and phenotypic pattern. This suggests different evolutionary trajectories towards multiple-drug resistance, certainly in response to various selection pressures (Fonseca et al., 2015; Trauner et al., 2014).

Nevertheless, for all studied genes some mutation positions were predominant, in agreement with previous reports (Campbell et al., 2011; Lipin et al., 2007; Rodwell et al., 2014). As example, in *rpoB* gene, mutations were mainly found at *rpoB531*, *rpoB526* and *rpoB516* positions, with *rpoB531* (Ser-Leu/Trp) and *rpoB526* (His-Arg/Asp/Tyr) previously associated with high level of RIF resistance and low impact on the pathogen fitness (Gagneux et al., 2006b). Similarly, frequent mutations found at *rpsL43*, *katG315*, and *gyrA94* were also previously linked to high resistance level and low fitness cost (Borrell et al., 2013; Bottger et al., 1998; Pym et al., 2002). Furthermore, Spies et al. demonstrated that, the double mutants *rpsL43/katG315*, *rpsL43/rpoB531*, and *rpoB531/katG315* had higher fitness than drug-susceptible strains (Spies et al., 2013). In addition, a recent study showed that *gyrA94* (Asp-Gly) mutation in *Mycobacterium smegmatis* is associated with improved fitness in double *rpoB-gyrA* mutants, irrespective of the *rpoB* mutation, suggesting epistatic interactions (Borrell et al., 2013). In agreement with these studies, 32% of the pre-XDR and XDR isolates of our study harbored the *gyrA94* (Asp-Gly) mutation associated with mutations either *rpoB531* (Ser-Leu) or *rpoB516* (Asp-Val). Moreover, combinations of at least two mutations among *rpoB531*, *katG315*, *rpsL43*, *embB306* and *gyrA94* were often detected. Epistasis could thus explain the specific associations of mutations observed in our study.

4.3. Comparison between genetic and phenotypic data

The comparison of genetic and DST data showed high sensitivity (97–100%) for detecting INH and RIF resistances, less sensitivity (>86%) for detecting STR and EMB resistance, and high specificity (100%) for all drugs, in agreement with previous studies (Campbell et al., 2011; Nhu et al., 2012; Rodwell et al., 2014). For quadruple-drug resistance detection, sensitivity was higher than 79%. Isolates with discordant results suggest additional mechanisms, such as activation of efflux pumps, and/or the involvement of other genes (Fonseca et al., 2015; Smith et al., 2013; Zhang and Yew, 2015). For instance, Okamoto et al. found mutations in *gidB* gene for up to 33% of STR-resistant isolates (Okamoto et al., 2007). However, mutations in *gidB* were often accompanied by mutations in *rpsL* or *rrs* genes and were also found in STR-sensitive isolates, suggesting that the role of *gidB* mutations in STR resistance need to be further investigated (Nhu et al., 2012; Zhang and Yew, 2015).

For the FQ resistance detection, high sensitivity and specificity (>88% and 100%, respectively) were also obtained, in agreement with the published data (Campbell et al., 2011; Nosova et al., 2013; Rodwell et al., 2014). As almost all OFX resistant isolates were also MXF resistant although carrying single mutations, cross-resistance could explain this association, consistently with previous studies (Campbell et al., 2011; Nosova et al., 2013; Rodwell et al., 2014).

Regarding SLID resistance, only 57.9% of the quadruple-FLD resistant isolates resistant to SLIDs carried mutations at one of the two positions (*rrs1401* and *rrs1484*) in the 1400–1500 region of the *rrs* gene. Interestingly, these two mutations were reported linked to cross-resistance to KAN, AMK and CAP in previous studies as observed in the present study (Campbell et al., 2011; Jugheli et al., 2009; Rodwell et al., 2014). The mutation at position *rrs1402* also known to be associated with resistance to KAN and CAP was not detected in our study. The low sensitivity obtained suggests other mechanisms of resistance, such as efflux pump activation or mutations in *eis* gene for KAN resistance, or in *tylA* gene for CAP resistance (Fonseca et al., 2015; Johansen et al., 2006; Zaunbrecher et al., 2009; Zhang and Yew, 2015). Since, only one isolate was mono-SLID resistant to KAN and one isolate was mono-SLID resistant to CAP, we could expect zero to one isolate with mutations in *eis* and *tylA* genes in our sample.

Finally, the genetic analysis revealed that a significant proportion of quadruple-FLD resistant isolates were pre-XDR (23.1%) or XDR (7.7%). Nevertheless, low sensitivity was obtained for the detection of pre-XDR (71.4%) and XDR (45.5%) isolates. These low values can be mainly explained by the low sensitivity obtained for SLID resistance detection in agreement with previous studies (Campbell et al., 2011; Rodwell et al., 2014). Nevertheless, our data show that the quadruple-FLD resistant isolates have a high risk to evolve into pre-XDR and XDR strains.

In terms of molecular diagnostics, our data shows that the GenoType MTBDRplus test (MDR: Hain Lifescience, Nehren, Germany) could detect 90.1% (82/91) of MDR isolates among the quadruple-drug resistant isolates compared to 97.8% (89/91) by Sanger sequencing. Besides, GeneXpert (MTB/RIF resistance: Cepheid, Sunnyvale, USA) could detect 94.5% (86/91) of RIF-resistant isolates among the quadruple-drug resistant samples compared to 100% by Sanger sequencing. In addition, all the mutations that we found in SLID resistant isolates would be detected by the GenoType MTBDRsl V2.0 test (SLD resistance: Hain Lifescience, Nehren, Germany), whereas 2 out of 17 FQ-resistant isolates would not be detected due to mutations not covered by this test in *gyrB* (Brossier et al., 2016).

4.4. Genetic determinants of drug resistance and *M. tuberculosis* families

Mutation type and frequency varied in the different *M. tuberculosis* families. The most frequent mutations were often found in all families, but at different frequencies, especially for INH and STR resistances. Combinations of mutations linked to high level of drug resistance and low fitness cost, such as the mutations at *rpoB531*, *katG315*, *rpsL43*, *embB306* and *gyrA94*, were mainly found in Beijing family isolates, consistent with the high potential of drug resistance and transmission of this family (Casali et al., 2014; Coscolla and Gagneux, 2014; Duong et al., 2009; Merker et al., 2015). Furthermore, the Beijing family was the most represented among the pre-XDR and XDR, in agreement with previous studies (Casali et al., 2014; Hu et al., 2015). However, this finding needs to be confirmed using a sample with a larger number of isolates from the EAI and T families since the difference of mutation frequencies between families were not significant.

5. Conclusion

In summary, this study reveals high proportions of pre-XDR and XDR among quadruple-FLD resistant *M. tuberculosis* isolates in Vietnam. The high diversity of mutations suggests various evolutionary trajectories towards FLD and SLD resistances. Nevertheless, specific mutations, generally known to be associated with high level of resistance and low fitness cost, were predominant. All these data suggest a cumulative effect of mutations in these quadruple-drug resistant isolates and a role for epistasis in the evolution of these isolates. Finally, our study shows that the Beijing family, which is currently invading Vietnam and known to be highly transmissible, was also linked with pre-XDR and XDR forms. Our data suggest that drug resistance might continue

to increase in Vietnam, underline the importance of identifying quadruple-FLD resistant TB cases and the necessity of SLD susceptibility testing to provide the most appropriate treatment and to control the spread of SLD resistance in Vietnam and worldwide.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by grants of the following projects: the PHC Lotus, the GDRI ID-BIO, the KC.10.15/06-10, the JEAI MySA, the LMI DRISA, and an ADB BIO3 USTH project. We would like to thank the Viet Nam National Tuberculosis Programme, National Lung Hospital, Pham Ngoc Thach Hospital and Hue Central Hospital for *M. tuberculosis* sampling. We also thank NIHE, IRD, CNRS and USTH for their support. Nguyen Quang Huy was supported by the project 911 scholarship from the Vietnamese Government. We are grateful to Elisabetta Andermarcher for editing the English.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2017.02.012>.

References

- Borrell, S., Teo, Y., Giardina, F., Streicher, E.M., Klopper, M., Feldmann, J., Muller, B., Victor, T.C., Gagneux, S., 2013. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol. Med. Public Health* 2013, 65–74.
- Bottger, E.C., Springer, B., Pletschette, M., Sander, P., 1998. Fitness of antibiotic-resistant microorganisms and compensatory mutations. *Nat. Med.* 4, 1343–1344.
- Brossier, F., Guindo, D., Pham, A., Reibel, F., Sougakoff, W., Veziris, N., Aubry, A., 2016. Performance of the new version (v2.0) of the GenoType MTBDRsl test for detection of resistance to second-line drugs in multidrug-resistant *Mycobacterium tuberculosis* complex strains. *J. Clin. Microbiol.* 54, 1573–1580.
- Campbell, P.J., Morlock, G.P., Sikes, R.D., Dalton, T.L., Metchock, B., Starks, A.M., Hooks, D.P., Cowan, L.S., Plikaytis, B.B., Posey, J.E., 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 55, 2032–2041.
- Casali, N., Nikolayevskyy, V., Balabanova, Y., Harris, S.R., Ignatyeva, O., Kontsevaya, I., Corander, J., Bryant, J., Parkhill, J., Nejentsev, S., Horstmann, R.D., Brown, T., Drobniewski, F., 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat. Genet.* 46, 279–286.
- Caws, M., Duy, P.M., Tho, D.Q., Lan, N.T., Hoa, D.V., Farrar, J., 2006. Mutations prevalent among rifampin and isoniazid resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam. *J. Clin. Microbiol.* 44, 2333–2337.
- Coscolla, M., Gagneux, S., 2014. Consequences of genomic diversity in *Mycobacterium tuberculosis*. *Semin. Immunol.* 26, 431–444.
- Demay, C., Liens, B., Burguiere, T., Hill, V., Couvin, D., Millet, J., Mokrousov, I., Sola, C., Zozio, T., Rastogi, N., 2012. SITVITWEB—a publicly available international multimer database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 12, 755–766.
- Duong, D.A., Nguyen, T.H., Nguyen, T.N., Dai, V.H., Dang, T.M., Vo, S.K., Do, D.A., Nguyen, V.V., Nguyen, H.D., Dinh, N.S., Farrar, J., Caws, M., 2009. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. *Antimicrob. Agents Chemother.* 53, 4835–4839.
- Fenner, L., Egger, M., Bodmer, T., Altpeter, E., Zwahlen, M., Jaton, K., Pfyffer, G.E., Borrell, S., Dubuis, O., Bruderer, T., Siegrist, H.H., Furrer, H., Calmy, A., Fehr, J., Stalder, J.M., Ninet, B., Bottger, E.C., Gagneux, S., Swiss, H.I.V.C.S., the Swiss Molecular Epidemiology of Tuberculosis Study, G., 2012. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 56, 3047–3053.
- Fonseca, J.D., Knight, G.M., McHugh, T.D., 2015. The complex evolution of antibiotic resistance in *Mycobacterium tuberculosis*. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases* 32, 94–100.
- Ford, C.B., Shah, R.R., Maeda, M.K., Gagneux, S., Murray, M.B., Cohen, T., Johnston, J.C., Gardy, J., Lipsitch, M., Fortune, S.M., 2013. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* 45, 784–790.
- Gagneux, S., Burgos, M.V., DeRiemer, K., Encisco, A., Munoz, S., Hopewell, P.C., Small, P.M., Pym, A.S., 2006a. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. *PLoS Pathog.* 2, e61.

- Gagneux, S., Long, C.D., Small, P.M., Van, T., Schoolnik, G.K., Bohannon, B.J., 2006b. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* 312, 1944–1946.
- Hoa, N.B., Nhung, N.V., Khanh, P.H., Hai, N.V., Quyen, B.T., 2015. Adverse events in the treatment of MDR-TB patients within and outside the NTP in Pham Ngoc Thach hospital, Ho Chi Minh City, Vietnam. *BMC Research Notes* 8, 809.
- Hu, Y., Mathema, B., Zhao, Q., Chen, L., Lu, W., Wang, W., Kreiswirth, B., Xu, B., 2015. Acquisition of second-line drug resistance and extensive drug resistance during recent transmission of *Mycobacterium tuberculosis* in rural China. *Clin. Microbiol. Infect.* 21 (1093) (e1099–1093 e1018).
- Johansen, S.K., Maus, C.E., Plikaytis, B.B., Douthwaite, S., 2006. Capreomycin binds across the ribosomal subunit interface using *tylA*-encoded 2'-O-methylations in 16S and 23S rRNAs. *Mol. Cell* 23, 173–182.
- Jugheli, L., Bzekalava, N., de Rijk, P., Fissette, K., Portaels, F., Rigouts, L., 2009. High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the *rrs* gene. *Antimicrob. Agents Chemother.* 53, 5064–5068.
- Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* 35, 907–914.
- Lee, A.S., Ong, D.C., Wong, J.C., Siu, G.K., Yam, W.C., 2012. High-resolution melting analysis for the rapid detection of fluoroquinolone and streptomycin resistance in *Mycobacterium tuberculosis*. *PLoS One* 7, e31934.
- Lipin, M.Y., Stepanshina, V.N., Shemyakin, I.G., Shinnick, T.M., 2007. Association of specific mutations in *katG*, *rpoB*, *rpsL* and *rrs* genes with spoligotypes of multidrug-resistant *Mycobacterium tuberculosis* isolates in Russia. *Clin. Microbiol. Infect.* 13, 620–626.
- Merker, M., Blin, C., Mona, S., Duforet-Frebourg, N., Lecher, S., Willery, E., Blum, M.G., Rusch-Gerdes, S., Mokrousov, I., Aleksic, E., Allix-Beguec, C., Antierens, A., Augustynowicz-Kopec, E., Ballif, M., Barletta, F., Beck, H.P., Barry 3rd, C.E., Bonnet, M., Borroni, E., Campos-Herrero, I., Cirillo, D., Cox, H., Crowe, S., Crudu, V., Diel, R., Drobniewski, F., Fauville-Dufaux, M., Gagneux, S., Ghebremichael, S., Hanekom, M., Hoffner, S., Jiao, W.W., Kalon, S., Kohl, T.A., Kontsevaya, I., Lillebaek, T., Maeda, S., Nikolayevskyy, V., Rasmussen, M., Rastogi, N., Samper, S., Sanchez-Padilla, E., Savic, B., Shamputa, I.C., Shen, A., Sng, L.H., Stakenas, P., Toit, K., Varaine, F., Vukovic, D., Wahl, C., Warren, R., Supply, P., Niemann, S., Wirth, T., 2015. Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nat. Genet.* 47, 242–249.
- Nguyen, V.A., Nguyen, H.Q., Vu, T.T., Nguyen, N.A., Duong, C.M., Tran, T.H., Nguyen, H.V., Dang, D.A., Banuls, A.L., 2015. Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay. *Clin. Microbiol. Infect.* 21, 1084–1092.
- Nguyen, H.B., Nguyen, N.V., Tran, H.T., Nguyen, H.V., Bui, Q.T., 2016. Prevalence of resistance to second-line tuberculosis drug among multidrug-resistant tuberculosis patients in Viet Nam, 2011. *Western Pac. Surveill. Response J.* 7 (2), 35–40.
- Nhu, N.T., Lan, N.T., Phuong, N.T., Chau, N., Farrar, J., Caws, M., 2012. Association of streptomycin resistance mutations with level of drug resistance and *Mycobacterium tuberculosis* genotypes. *Int. J. Tuberc. Lung Dis.* 16, 527–531.
- Nhung, N.V., Hoa, N.B., Sy, D.N., Hennig, C.M., Dean, A.S., 2015. The fourth national anti-tuberculosis drug resistance survey in Viet Nam. *Int. J. Tuberc. Lung Dis.* 19, 670–675.
- Nosova, E.Y., Bukatina, A.A., Isaeva, Y.D., Makarova, M.V., Galkina, K.Y., Moroz, A.M., 2013. Analysis of mutations in the *gyrA* and *gyrB* genes and their association with the resistance of *Mycobacterium tuberculosis* to levofloxacin, moxifloxacin and gatifloxacin. *J. Med. Microbiol.* 62, 108–113.
- Okamoto, S., Tamaru, A., Nakajima, C., Nishimura, K., Tanaka, Y., Tokuyama, S., Suzuki, Y., Ochi, K., 2007. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol. Microbiol.* 63, 1096–1106.
- Pym, A.S., Saint-Joanis, B., Cole, S.T., 2002. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect. Immun.* 70, 4955–4960.
- Rigouts, L., Nolasco, O., de Rijk, P., Nduwamahoro, E., Van Deun, A., Ramsay, A., Arevalo, J., Portaels, F., 2007. Newly developed primers for comprehensive amplification of the *rpoB* gene and detection of rifampin resistance in *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 45, 252–254.
- Rodwell, T.C., Valafar, F., Douglas, J., Qian, L., Garfein, R.S., Chawla, A., Torres, J., Zadorozhny, V., Kim, M.S., Hoshide, M., Catanzaro, D., Jackson, L., Lin, G., Desmond, E., Rodrigues, C., Eisenach, K., Victor, T.C., Ismail, N., Crudu, V., Gler, M.T., Catanzaro, A., 2014. Predicting extensively drug-resistant *Mycobacterium tuberculosis* phenotypes with genetic mutations. *J. Clin. Microbiol.* 52, 781–789.
- Sandgren, A., Strong, M., Muthukrishnan, P., Weiner, B.K., Church, G.M., Murray, M.B., 2009. Tuberculosis drug resistance mutation database. *PLoS Med.* 6, e2.
- Shi, D., Li, L., Zhao, Y., Jia, Q., Li, H., Coulter, C., Jin, Q., Zhu, G., 2011. Characteristics of *embB* mutations in multidrug-resistant *Mycobacterium tuberculosis* isolates in Henan, China. *J. Antimicrob. Chemother.* 66, 2240–2247.
- Smith, T., Wolff, K.A., Nguyen, L., 2013. Molecular biology of drug resistance in *Mycobacterium tuberculosis*. *Curr. Top. Microbiol. Immunol.* 374, 53–80.
- Spies, F.S., von Groll, A., Ribeiro, A.W., Ramos, D.F., Ribeiro, M.O., Dalla Costa, E.R., Martin, A., Palomino, J.C., Rossetti, M.L., Zaha, A., da Silva, P.E., 2013. Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes. *Tuberculosis (Edinb)* 93, 150–154.
- Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Loch, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 44, 4498–4510.
- Trauner, A., Borrell, S., Reither, K., Gagneux, S., 2014. Evolution of drug resistance in tuberculosis: recent progress and implications for diagnosis and therapy. *Drugs* 74, 1063–1072.
- Vietnam National Tuberculosis Control Programme, N., 2009. Guideline for Management of Multi-drug Resistance Tuberculosis.
- WHO, 2003. Guidelines for surveillance of drug resistance in tuberculosis. WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Geneva 2003 WHO/CDS/CSR/RMD/2003.3, second ed.
- WHO, 2016. Global Tuberculosis Report 2016. WHO/HTM/TB/2016.13. World Health Organization, Geneva, Switzerland.
- Zaunbrecher, M.A., Sikes Jr., R.D., Metchock, B., Shinnick, T.M., Posey, J.E., 2009. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* 106, 20004–20009.
- Zhang, Y., Yew, W.W., 2015. Mechanisms of drug resistance in *Mycobacterium tuberculosis*: update 2015. *Int. J. Tuberc. Lung Dis.* 19, 1276–1289.
- Zignol, M., van Gemert, W., Falzon, D., Sismanidis, C., Glaziou, P., Floyd, K., Ravignone, M., 2012. Surveillance of anti-tuberculosis drug resistance in the world: an updated analysis, 2007–2010. *Bull. World Health Organ.* 90, 111–119D.

	463*	CGG-CTG	Arg-Leu	2	30
	No mutation	WT	WT	7	23
	Total			91	55
<i>inhA</i>	-8	T-C		1	0
	-15	C-T		7	0
	-15 & 21	C-T & ATC-GTC	Ile-Val	1	0
	-15 & 94	C-T & TCG-GCG	Ser-Ala	5	0
	-191*	C-T		0	2
	No mutation	WT	WT	77	53
	Total			91	55

QDR: quadruple-FLD resistant; #: new mutation; WT: wild type.

*: Mutations not associated with drug resistance and thus not considered for calculations of sensitivity and specificity

TABLE S1.3. Mutation positions, nucleotide and amino acid changes in the *rpoB* gene of the studied *M. tuberculosis* isolates.

Mutation position(s)	Nucleotide change(s)	Amino acid change(s)	QDR	Sensitive
146	GTC-TTC	Val-Phe	1	0
511	CTG-CCG	Leu-Pro	1	0
516	GAC-GTC	Asp-Val	9	0
526	CAC-CGC	His-Arg	1	0
526	CAC-GAC	His-Asp	10	0
526	CAC-ACC	His-Thr	1	0
526	CAC-TAC	His-Tyr	5	0

531	TCG-CAG	Ser-Gln	1	0
531	TCG-TTG	Ser-Leu	41	0
531 [#]	TCG-CTG	Ser-Leu	1	0
533	CTG-CCG	Leu-Pro	3	0
572	ATC-TTC	Ile-Phe	4	0
512	Insert CGC	Insert Arg	1	0
518	Deletion AAC	Deletion Asn	1	0
490 & 531	CAG-CGG & TCG-TGG	Gln-Arg & Ser-Trp	1	0
511 & 526	CTG-CCG & CAC-TAC	Leu-Pro & His-Asp	1	0
513 & 753	CAA-CTA & TCC-TTC	Gln-Leu & Ser-Phe	1	0
515 & 516	ATG-ATA & GAC-GTC	Met-Ile & Asp-Val	1	0
516 & 526	GAC-GTC & CAC-GAC	Asp-Val & His-Asp	1	0
526 & 527	CAC-TCC & AAG-CAG	His-Ser & Lys-Gln	1	0
526 & 533	CAC-AAC & CTG-TCG	His-Asn & Leu-Ser	1	0
526 & 533	CAC-GAC & CTG-CCG	His-Asp & Leu-Pro	1	0
526 & 562	CAC-GAC & GAA-GCA	His-Asp & Glu-Ala	1	0
530 & 531	CTG-ATG & TCG-TTC	Leu-Met & Ser-Phe	1	0
531 & 616	TCG-TGG & GAC-GCC	Ser-Trp & Asp-Ala	1	0
517 [*]	CAG-CAA	Gln-Gln	0	1
No mutation	WT	WT	0	54
Total			91	55

QDR: quadruple-FLD resistant; #: new nucleotide substitution; WT: wild type.

*: Mutations not associated with drug resistance and thus not considered for calculations of sensitivity and specificity

TABLE S1.4. Mutation positions, nucleotide and amino acid changes in *rpsL* and *rrs-F1* in the studied *M. tuberculosis* isolates.

Gene	Mutation position(s)	Nucleotide change(s)	Amino acid change(s)	QDR	Sensitive
<i>rpsL</i>	43	AAG-AGG	Lys-Arg	40	0
	43#	AAG-AAT	Lys-Asn	1	0
	88	AAG-AGG	Lys-Arg	19	0
	88	AAG-ATG	Lys-Met	3	0
	88	AAG-ACG	Lys-Thr	1	0
	No mutation	WT	WT	27	55
	Total			91	55
<i>rrs-F1</i>	295*	T-C		0	1
	514	A-C		12	0
	517	C-T		4	0
	878	G-A		2	0
	908	A-C		1	0
	No mutation	WT		72	54
	Total			91	55

#: New allele substitution; WT: wild type; QDR: quadruple-FLD resistant

*: Mutations not associated with drug resistance and thus not considered for calculations of sensitivity and specificity

TABLE S1.5. Mutation positions, nucleotide and amino acid changes in the *embB* gene of the studied *M. tuberculosis* isolates.

Mutation position(s)	Nucleotide change(s)	Amino acid change(s)	QDR	Sensitive
306	ATG-ATA	Met-Ile	7	0
306	ATG-ATC	Met-Ile	4	0
306	ATG-ATT	Met-Ile	1	0
306	ATG-CTG	Met-Leu	1	0
306	ATG-GTG	Met-Val	28	0
330 [#]	TTC-TTA	Phe-Leu	1	0
330	TTC-TCC	Phe-Ser	1	0
360	GTG-ATG	Val-Met	1	0
370 [*]	CTG-CGG	Leu-Arg	0	1
378 [*]	GAG-GCG	Glu-Ala	2	17
406	GGC-AGC	Gly-Ser	2	0
406	GGC-GCC	Gly-Ala	1	0
406	GGC-GAC	Gly-Asp	2	0
450	ATC-CTC	Ile-Leu	1	0
497	CAG-CGG	Gln-Arg	8	0
497	CAG-CCG	Gln-Pro	1	0
534 [*]	GAC-GAT	Asp-Asp	0	1
306 & 378 [*]	ATG-ATA & GAG-GCG	Met-Ile & Glu-Ala	6	0
306 & 378 [*]	ATG-GTG & GAG-GCG	Met-Val & Glu-Ala	1	0
306 & 378 [*]	ATG-ATC & GAG-GCG	Met-Ile & Glu-Ala	1	0
306 & 360	ATG-ATA & GTG-ATG	Met-Ile & Val-Met	1	0
306 & 454 [#]	ATG-ATA & GCG-ACG	Met-Ile & Ala-Thr	1	0
306 & 497	ATG-ATA & CAG-AAG	Met-Ile & Gln-Lys	1	0

328 & 338 [#]	GAT-TAT & GCG-TCG	Asp-Tyr & Ala-Ser	1	0
330 [#] & 378 [*]	TTC-TTA & GAG-GCG	Phe-Leu & Glu-Ala	1	0
354 & 378 [*]	GAC-GCC & GAG-GCG	Asp-Ala & Glu-Ala	1	0
354 & 497	GAC-GCC & CAG-AAG	Asp-Ala & Gln-Lys	1	0
370 [*] & 497	CTG-CGG & CAG-AAG	Leu-Arg & Gln-Lys	1	0
378 [*] & 387 [*]	GAG-GCG & GCG-ACG	Glu-Ala & Ala-Thr	0	1
378 [*] & 406	GAG-GCG & GGC-GAC	Glu-Ala & Gly-Asp	1	0
378 [*] & 438 [*]	GAG-GCG & GCC-ACC	Glu-Ala & Ala-Thr	0	1
306 & 378 [*] & 561 [*]	ATG-GTG & GAG-GCG & AAG-ACG	Met-Val & Glu-Ala & Lys-Thr	1	0
306 & 378 [*] & 387 [*] & 406	ATG-ATA & GAG-GCG & GCG-ACG & GGC-GCC	Met-Ile & Glu-Ala & Ala-Thr & Gly-Ala	1	0
No mutation	WT	WT	11	34
Total			91	55

QDR: quadruple-FLD resistant ; #: new allele substitution; WT: wild type.

*: Mutations not associated with drug resistance and thus not considered for calculations of sensitivity and specificity

TABLE S1.6. Mutation positions, nucleotide and amino acid changes in the *gyrA*, *gyrB* genes and *rrs*-F2 fragment in the *M. tuberculosis* isolates.

Gene	Mutation position(s)	Nucleotide change(s)	Amino acid change(s)	QDR	Sensitive
<i>gyrA</i>	88 & 95*	GGC-GCC & AGC-ACC	Gly-Ala & Ser-Thr	1	0
	90 & 95*	GCG-GTG & AGC-ACC	Ala-Val & Ser-Thr	5	0
	94 & 95*	GAC-GCC & AGC-ACC	Asp-Ala & Ser-Thr	2	0
	94 & 95*	GAC-GGC & AGC-ACC	Asp-Gly & Ser-Thr	10	0
	94 & 95*	GAC-TAC & AGC-ACC	Asp-Tyr & Ser-Thr	2	0
	95*	AGC-ACC	Ser-Thr	70	47
	No mutation	WT	WT	1	8
	Total		91	55	
<i>gyrB</i>	447*	TCC-TCT	Ser-Ser	0	1
	461	GAC-AAC	Asp-Asn	1	0
	504	GCG-GTG	Ala-Val	1	0
	No mutation	WT	WT	89	54
		Total		91	55
<i>rrs</i> -F2	1401	A-G		11	0
	1001& 1401	T-C & A-G		1	0
	1484	G-T		1	0
	No mutation	WT		78	55
		Total		91	55

WT: wild type. QDR: quadruple-FLD resistant.

*: Mutations not associated with drug resistance and thus not considered for calculations of sensitivity and specificity

TABLE S2. Primers used for DNA amplification and sequencing of genes involved in anti-TB drug resistance in *Mycobacterium tuberculosis*

Drug(s)	Gene/gene promoter	Primer sequence	Annealing T°C	Nucleotide position (length)	Target region	Reference
RIF	<i>rpoB</i> -F1	F- <i>rpoB</i> 1: 5'-GTCGACGCTGACCGAAGAAG-3'	62°C	1053 – 2200	RRDR	This study
		R- <i>rpoB</i> 1: 5'-TCTCGCCGTCGTCAGTACAG-3'		(1148 bp)		
	<i>rpoB</i> -F2	F- <i>rpoB</i> 2: 5'- TAGTTGCGTGCGTGAGATCC-3'	60°C	(-132) – 1042	Promoter and codons 1 - 428	This study
		R- <i>rpoB</i> 2: 5'- TGGTCTGACCCTCGTGCAAG-3'		(1174 bp)		
INH	<i>katG</i>	F- <i>katG</i> 1: 5'-CCAACTCCTGGAAGGAATGC-3'	58°C	(-21) – 1147	Full length gene	Lipin et al 2007
		R- <i>katG</i> 1: 5'-AGAGGTCAGTGGCCAGCAT-3'		(1169 bp)		This study
		F- <i>katG</i> 2: 5'-ACGAGTGGGAGCTGACGAA-3'	60°C	1016 – 2244		Lipin et al 2007
		R- <i>katG</i> 2: 5'-AACCCGAATCAGCGCACGT-3'		(1229 bp)		This study
	<i>inhA</i>	F- <i>inhA</i> 1: 5'-GCGACATACCTGCTGCGCAA-3'	60°C	(-220) – 80	Promoter region	This study
		R- <i>inhA</i> 2: 5'-ATCCCCCGGTTTCCTCCGGT-3'		(300 bp)		
		F- <i>inhA</i> 3: 5'-GACACAACACAAGGACGCA-3'	60°C	(-20) – 987	Full length gene	This study
		R- <i>inhA</i> 4: 5'-TGCCATTGATCGGTGATACC-3'		(1008 bp)		
STR	<i>rpsL</i>	F- <i>rpsL</i> : 5'-GCGCCCAAGATAGAAAAG-3'	58°C	(-27) – 424	Full length gene	Lee et al 2012
		R- <i>rpsL</i> : 5'-CAACTGCGATCCGTAGA-3'		(451 bp)		Lee et al 2012

	<i>rrs-F1</i>	F-S1: 5'-GAGAGTTTGATCCTGGCTCAG-3' R-S1: 5'-CCAGGTAAGGTTCTTCGCGTTG-3'	58°C	9 – 981 (973 bp)	Loops 530 & 915	This study Lipin et al 2007
EMB	<i>embB</i>	F-embB: 5'-TGACCGACGCCGTGGTGATA-3 R-embB: 5'-GCCATGAAACCGGCCACGAT-3	62°C	833 – 2144 (1312 bp)	codons 277 - 714	Campbell et al 2011 Shi et al 2011
KAN, AMK & CAP	<i>rrs-F2</i>	F-S2: 5'- GCGCAGATATCAGGAGG-3' R-S2: 5'- CGCCCACTACAGACAAG-3'	58°C	689 – 1606 (918 bp)	1400-1500 region	Lee et al 2012 Lee et al 2012
FQs	<i>gyrA & gyrB</i>	F- <i>gyrAB</i> : 5'-GCAACACCGAGGTCAAATCG-3' R- <i>gyrAB</i> : 5'-CTCAGCATCTCCATCGCCAA-3'	62°C	1127 (<i>gyrB</i>) – 428 (<i>gyrA</i>) (1296bp)	QRDRs of <i>gyrA</i> & <i>gyrB</i>	This study This study

RRDR: rifampicin resistance-determining region; QRDR: Quinone resistance-determining region

Note: The *M. tuberculosis* nucleotide numbering system was used for all the genes

TABLE S3. Phenotype and distribution of drug resistance associated mutations in MIRU-VNTR clusters

Cluster	MIRU-VNTR	N° of isolates	Family	Phenotype	FLD-resistant mutations	SLD-resistant mutations	pre-XDR/XDR
1	263432335444647122334253	8	Beijing	Sensitive, HRSE	katG315Thr rpoB526Asp/rpoB531Leu/rpoB533Pro rpsL43Arg/rpsL88Arg embB306Ile/ embB497Arg	gyrA94Ala	1 pre-XDR
2	271422335444558122134263	3	Beijing	HRSE	katG315Thr/ katG315Thr & inhA15T	rrs1401G,	2 pre-XDR

						rrs1484T	
					rpoB531Leu/ rpoB572Phe rpsL43Arg/rpsL88Arg embB360Met/ embB497Pro		
3	263432335444547122334253	5	Beijing	Sensitive, HRSE	katG315Thr/katG394 & Ala-inhA15T & inhA94Ala/ inhA15T & inhA94Ala rpoB511Pro/rpoB531Leu rrs514C embB306Leu/embB406Ser/embB497Arg	rrs1401G gyrA90Val gyrA94Tyr	1 pre-XDR 1 XDR
4	273452335444688122334253	4	Beijing	HRSE	katG315Thr rpoB146Phe/rpoB516Val/rpoB531Leu rpsL88Arg embB306Val/embB306Ile & embB360Met	gyrA90Val gyrA94Gly	2 pre-XDR
5	273432335444657122334253	4	Beijing	HRSE	katG315Thr rpoB531Leu/rpoB533Pro rpsL43Arg/rpsL88Arg embB306Ile/embB306Val/embB330Ser	gyrA94Gly	1 pre-XDR
6	273432235444658122334254	2	Beijing	HRSE	katG315Thr rpoB516Val/rpoB531Leu rpsL43Arg embB328Tyr & embB338Ser/embB306Val	rrs1401G gyrA94Gly	1 XDR
7*	263432335444637122334253	2	Beijing	HRSE	katG315Thr & katG710Phe rpoB516Val rpsL88Arg embB306Val	WT	

8	273434335444735122334253	3	Beijing	HRSE	katG315Thr rpoB526Tyr/rpoB516Val & rpoB526Asp/rpoB531Leu rpsL43Arg/rpsL88Arg/rrs517T embB306Ile/embB306Val	WT
9	212433512442137112234252	2	T	Sensitive, HRSE	katG315Thr rpoB531Leu rpsL43Arg embB354Ala & embB497Lys	WT
10	623241425247274242333253	2	EAI	Sensitive, HRSE	katG315Thr rpoB526Thr rpsL88Arg	WT
11	273432335344548122334253	2	Beijing	Sensitive	WT	WT
12	623241325247255242333253	2	EAI	Sensitive	WT	WT
13	254232332243533122334252	2	T	Sensitive	WT	WT

* this cluster contains two HRSE resistant isolates fully identical in terms of genotypes and phenotypes

TABLE S4. Fisher's exact test results according to drug resistance, genes and families

Drug	Locus/gene	All families											Fisher's exact test p value	
		EAI			Beijing			T			Others			
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation		Mutation frequency (%)
RIF	<i>rpoB</i>	17	0	100	62	0	100	8	0	100	4	0	100	1
	RRDR of <i>rpoB</i>	17	0	100	57	5	91.9	8	0	100	4	0	100	1
INH	<i>katG</i>	14	3	82.3	60	2	96.8	5	3	62.5	3	1	75	0.0047

	<i>inhA</i> and its promoter	6	11	35.3	6	56	9.7	2	6	25	0	4	0	0.041
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	17	0	100	62	0	100	7	1	87.5	3	1	75	0.016
STR	<i>rpsL</i>	5	12	29.4	53	9	85.5	5	3	62.5	1	3	25	0.0000078
	<i>rrs-F1</i>	10	7	58.8	8	54	12.9	0	8	0	1	3	25	0.00039
	<i>rpsL</i> + <i>rrs-F1</i>	15	2	88.2	60	2	96.8	5	3	62.5	2	2	50	0.0012
EMB	<i>embB</i>	14	3	82.4	55	7	88.7	7	1	87.5	3	1	75	0.57
OFX	<i>gyrA</i>	5	12	29.4	12	50	19.4	3	5	37.5	0	4	0	0.4
	<i>gyrB</i>	0	17	0	1	61	1.6	1	7	12.5	0	4	0	1
	<i>gyrA</i> and <i>gyrB</i>	5	12	26.7	13	49	21	4	4	50	0	4	0	0.2
KAN, AMK & CAP	<i>rrs-F2</i>	3	14	17.7	10	52	16.1	0	8	0	0	4	0	0.75
INH, RIF, STR & EMB	<i>rpoB</i> and <i>katG/inhA</i> and <i>inhA</i> promoter and <i>rpsL</i> and/or <i>rrs-F1</i> and <i>embB</i>	13	4	76.5	53	9	85.5	4	4	50	2	2	50	0.04
pre-XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> or <i>gyrA/gyrB</i>	4	13	23.5	13	49	21	4	4	50	0	4	0	0.24
XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and <i>gyrA/gyrB</i>	2	15	11.8	5	57	8.1	0	8	0	0	4	0	0.87
pre-XDR & XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and /or <i>gyrA/gyrB</i>	6	11	35.3	18	44	29	4	4	50	0	4	0	0.36

Drug	locus/gene	Beijing vs Non-Beijing						Fisher's exact test p value
		Beijing			non-Beijing			
		With mutation	without mutation	frequency (%)	With mutation	without mutation	frequency (%)	
RIF	rpoB	62	0	100	29	0	1	1
INH	katG	60	2	96.8	22	7	75.9	0.0041
	inhA	6	56	9.7	8	21	27.6	0.034
	katG & inhA	62	0	1	27	2	93.1	0.099
STR	rpsL	53	9	85.5	11	18	37.9	0.0000079
	rrs-F1	8	54	12.9	11	18	37.9	0.011
	rpsL & rrs-F1	60	2	96.8	22	7	75.9	0.0041
EMB	embB	55	7	88.7	24	5	82.8	0.51
FQs	gyrA & gyrB	13	49	21	9	20	31	0.43
SLID	rrs-F2	10	52	16.1	3	26	10.3	0.53
INH, RIF, STR & EMB	rpoB, katG/inhA, rpsL/rrs-F1, embB	53	9	85.5	19	10	65.5	0.05
pre-XDR	rpoB, katG/inhA, rpsL/rrs-F1, embB, rrs-F2 or gyrA/gyrB	13	49	21	8	21	27.6	0.59
XDR	rpoB, katG/inhA, rpsL/rrs-F1, embB, rrs-F2 & gyrA/gyrB	5	57	8.1	2	27	6.9	1
pre-XDR & XDR	rpoB, katG/inhA, rpsL/rrs-F1, embB, rrs-F2 & gyrA/gyrB	18	44	29	10	19	34.5	0.63

Drug	Locus/gene	EAI vs Beijing						Fisher's exact test p value
		EAI			Beijing			
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	
RIF	<i>rpoB</i>	17	0	100	62	0	100	1
	RRDR of <i>rpoB</i>	17	0	100	57	5	91.9	0.35
INH	<i>katG</i>	14	3	82.3	60	2	96.8	0.063
	<i>inhA</i> and its promoter	6	11	35.3	6	56	9.7	0.017
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	17	0	100	62	0	100	1
STR	<i>rpsL</i>	5	12	29.4	53	9	85.5	0.000017
	<i>rrs-F1</i>	10	7	58.8	8	54	12.9	0.00026
	<i>rpsL</i> + <i>rrs-F1</i>	15	2	88.2	60	2	96.8	0.2
EMB	<i>embB</i>	14	3	82.4	55	7	88.7	0.68
OFX	<i>gyrA</i>	5	12	29.4	12	50	19.4	0.5
	<i>gyrB</i>	0	17	0	1	61	1.6	1
	<i>gyrA</i> and <i>gyrB</i>	5	12	26.7	13	49	21	0.52
KAN, AMK & CAP	<i>rrs-F2</i>	3	14	17.7	10	52	16.1	1
INH, RIF, STR & EMB	<i>rpoB</i> and <i>katG/inhA</i> and <i>inhA</i> promoter and <i>rpsL</i> and/or <i>rrs-F1</i> and <i>embB</i>	13	4	76.5	53	9	85.5	0.46
pre-XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> or <i>gyrA/gyrB</i>	4	13	23.5	13	49	21	1
XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and <i>gyrA/gyrB</i>	2	15	11.8	5	57	8.1	1
pre-XDR & XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and /or <i>gyrA/gyrB</i>	6	11	35.3	18	44	29	0.76

Drug	Locus/gene	EAI vs T						Fisher's exact test p value
		EAI			T			
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	
RIF	<i>rpoB</i>	17	0	100	8	0	100	1
	RRDR of <i>rpoB</i>	17	0	100	8	0	100	1
INH	<i>katG</i>	14	3	82.3	5	3	62.5	0.34
	<i>inhA</i> and its promoter	6	11	35.3	2	6	25	0.67
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	17	0	100	7	1	87.5	0.32
STR	<i>rpsL</i>	5	12	29.4	5	3	62.5	0.19
	<i>rrs-F1</i>	10	7	58.8	0	8	0	0.0076
	<i>rpsL</i> + <i>rrs-F1</i>	15	2	88.2	5	3	62.5	0.28
EMB	<i>embB</i>	14	3	82.4	7	1	87.5	1
OFX	<i>gyrA</i>	5	12	29.4	3	5	37.5	1
	<i>gyrB</i>	0	17	0	1	7	12.5	1
	<i>gyrA</i> and <i>gyrB</i>	5	12	26.7	4	4	50	0.39
KAN, AMK & CAP	<i>rrs-F2</i>	3	14	17.7	0	8	0	0.52
INH, RIF, STR & EMB	<i>rpoB</i> and <i>katG/inhA</i> and <i>inhA</i> promoter and <i>rpsL</i> and/or <i>rrs-F1</i> and <i>embB</i>	13	4	76.5	4	4	50	0.35
pre-XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> or <i>gyrA/gyrB</i>	4	13	23.5	4	4	50	0.35
XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and <i>gyrA/gyrB</i>	2	15	11.8	0	8	0	0.54
pre-XDR & XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA/inhA</i> promoter and <i>rrs-F2</i> and /or <i>gyrA/gyrB</i>	6	11	35.3	4	4	50	0.66

Drug	Locus/gene	Beijing vs T						Fisher's exact test p value
		Beijing			T			
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	
RIF	<i>rpoB</i>	62	0	100	8	0	100	1
	RRDR of <i>rpoB</i>	57	5	91.9	8	0	100	1
INH	<i>katG</i>	60	2	96.8	5	3	62.5	0.0091
	<i>inhA</i> and its promoter	6	56	9.7	2	6	25	0.22
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	62	0	100	7	1	87.5	0.11
STR	<i>rpsL</i>	53	9	85.5	5	3	62.5	0.13
	<i>rrs</i> -F1	8	54	12.9	0	8	0	0.58
	<i>rpsL</i> + <i>rrs</i> -F1	60	2	96.8	5	3	62.5	0.0091
EMB	<i>embB</i>	55	7	88.7	7	1	87.5	1
OFX	<i>gyrA</i>	12	50	19.4	3	5	37.5	0.35
	<i>gyrB</i>	1	61	1.6	1	7	12.5	0.21
	<i>gyrA</i> and <i>gyrB</i>	13	49	21	4	4	50	0.091
KAN, AMK & CAP	<i>rrs</i> -F2	10	52	16.1	0	8	0	0.35
INH, RIF, STR & EMB	<i>rpoB</i> and <i>katG</i> / <i>inhA</i> and <i>inhA</i> promoter and <i>rpsL</i> and/or <i>rrs</i> -F1 and <i>embB</i>	53	9	85.5	4	4	50	0.034
pre-XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA</i> / <i>inhA</i> promoter and <i>rrs</i> -F2 or <i>gyrA</i> / <i>gyrB</i>	13	49	21	4	4	50	0.091
XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA</i> / <i>inhA</i> promoter and <i>rrs</i> -F2 and <i>gyrA</i> / <i>gyrB</i>	5	57	8.1	0	8	0	1
pre-XDR & XDR	<i>rpoB</i> and <i>katG</i> and/or <i>inhA</i> / <i>inhA</i> promoter and <i>rrs</i> -F2 and /or <i>gyrA</i> / <i>gyrB</i>	18	44	29	4	4	50	0.42

1 **Title:** Molecular analysis of pyrazinamide resistance in *Mycobacterium tuberculosis*
2 in Vietnam highlights the high rate of pyrazinamide resistance-associated mutations in
3 clinical isolates.

4

5 **Running title:** *PncA* mutations and PZA resistance in *M. tuberculosis* in Vietnam.

6

7 **Authors:** Nguyen Quang Huy^{1,2,3,4*}, Lucie Contamin⁴, Tran Thi Thanh Hoa³, Nguyen
8 Van Hung⁵, Nguyen Thi Ngoc Lan⁶, Nguyen Thai Son⁷, Nguyen Viet Nhung⁸, Dang
9 Duc Anh³, Anne-Laure Bañuls^{2,3,4,#} and Nguyen Thi Van Anh^{3#}

10 **Affiliations:**

11 ¹University of Science and Technology of Hanoi, Hanoi 10000, Vietnam

12 ²Institute of Research for Development, Montpellier 34090, France

13 ³National Institute of Hygiene and Epidemiology, Hanoi 10000, Vietnam

14 ⁴LMI Drug Resistance in South East Asia, Hanoi 10000, Vietnam

15 ⁵National Lung Hospital, Hanoi 10000, Vietnam

16 ⁶Pham Ngoc Thach Hospital, Ho Chi Minh 70000, Vietnam

17 ⁷Military Medical University, Hanoi 10000, Vietnam

18 ⁸Vietnam National Tuberculosis Control Program, Hanoi 10000, Vietnam

19 #These senior authors contributed equally to this article.

20 Author's email: Nguyen Quang Huy (huynguyen.biotech@gmail.com), Lucie

21 Contamin (contamin.lucie@gmail.com), Tran Thi Thanh Hoa

22 (hoasinh41@gmail.com), Nguyen Van Hung (hungmtb75@gmail.com), Nguyen Thi
23 Ngoc Lan (ngoclan0456@yahoo.com), Nguyen Thai Son (ntson65@yahoo.com),
24 Nguyen Viet Nhung (viethung@yahoo.com), Dang Duc Anh
25 (dangducanh.nihe@gmail.com), Anne-Laure Bañuls (anne-laure.banuls@ird.fr),
26 Nguyen Thi Van Anh (vananhtdt@gmail.com).

27

28 Address for correspondence: Nguyen Quang Huy, Department of Pharmacological,
29 Medicinal and Agronomical Biotechnology, University of Science and Technology of
30 Hanoi (USTH), Vietnam Academy of Science and Technology (VAST), 18 Hoang
31 Quoc Viet road, Cau Giay district, Hanoi 10000, Vietnam;

32 E-mail: huynguyen.biotech@gmail.com

33

34

35

36

37

38

39

40

41

42

43 **Highlights:** In Vietnam, the high rate of *pncA* mutations detected in clinical
44 *Mycobacterium tuberculosis* samples suggests a high risk of PZA resistance and the
45 future worsening of anti-tuberculosis drug-resistance.

46

47 **Abstract**

48 Pyrazinamide (PZA) is a key antibiotic in current anti-tuberculosis regimens.
49 Although WHO has stressed the urgent need to get data on PZA resistance, in high
50 tuberculosis-burden countries little is known about the level of PZA resistance, the
51 genetic bases of such resistance and its link with *Mycobacterium tuberculosis*
52 families. In this context, this study assessed PZA resistance by molecular analysis in
53 260 Vietnamese *M. tuberculosis* isolates. First-line drug susceptibility testing, *pncA*
54 gene sequencing, spoligotyping and MIRU-VNTR typing and phylogeny were
55 performed. Overall, *pncA* mutation frequency was 38.1% (99/260 isolates), but was
56 higher than 72% (89/123 isolates) in multidrug and quadruple-drug resistant isolates.
57 Many different *pncA* mutations (71 types) were detected, of which 50 types have been
58 previously described as high-confidence PZA-resistance mutations and 14 are likely
59 to be linked to PZA resistance because of their mutations or codon positions.
60 Genotype analysis revealed that PZA resistance can emerge in any *M. tuberculosis*
61 cluster and family, although mutation frequency was highest in Beijing family isolates
62 (47.7%, 62/130 isolates). These data highlight the high rate of PZA resistance-
63 associated mutations and question PZA use for multidrug-resistant tuberculosis.

64 **Key words:** Pyrazinamide resistance, *pncA* mutation, sequencing, multi-drug
65 resistance, spoligotyping, MIRU-VNTR, *Mycobacterium tuberculosis* family.

66

67

68 **INTRODUCTION**

69 Pyrazinamide (PZA) is a crucial first-line drug (FLD) for tuberculosis (TB)
70 treatment because it allows shortening the treatment duration in patients with
71 susceptible, multidrug-resistant (MDR) or extensively drug-resistant (XDR) TB and
72 reduces TB relapse rates ^{1,2}. PZA powerful sterilizing activity is due to its ability to
73 kill persistent tubercle bacilli in macrophages or in the lesion acidic environment that
74 are not eradicated by other anti-TB drugs ³. PZA is the only FLD most likely to be
75 maintained in all new regimens for shortening the treatment course of all TB forms ⁴,
76 ⁵. Indeed, together with fluoroquinolones and second-line injectable drugs, PZA has
77 significantly improved MDR TB treatment success ^{6,7}.

78 Culture-based PZA-susceptibility testing is difficult to perform because of
79 unreliable results due to the need of acidic pH medium that inhibits bacteria growth
80 and of large inoculum volumes that might reduce PZA activity ^{8,9}. Therefore, PZA-
81 susceptibility testing is not routinely performed and little is known about PZA
82 resistance in *M. tuberculosis* populations, especially in high TB burden countries ⁴.
83 Nevertheless, a recent study performed in five high TB and MDR TB burden
84 countries reported PZA resistance rates that ranged from 3.0% to 42.1% ¹⁰.

85 Although several mechanisms have been described, PZA resistance is mostly
86 caused by mutations in the *pncA* gene and its promoter that lead to reduction or loss
87 of pyrazinamidase (PZase) activity ¹¹⁻¹⁵. Many different *pncA* mutations have been
88 described. They are scattered throughout the *pncA* gene and none of them seems to be
89 predominant ^{11,16,17}. The three regions (codons 3-17, 61-85 and 132-142) that contain
90 the PZase active and metal-binding sites show some degree of mutation clustering ¹³,
91 ¹⁸. Mutations at other positions also can modify the protein or disrupt the protein core,

92 leading to loss of PZase activity¹¹⁻¹³. Mutations in the *pncA* promoter also can affect
93 PZase activity by disturbing *pncA* gene translation^{11, 13}. The *pncA* mutation
94 frequency in PZA-resistant *M. tuberculosis* isolates varies between 46% and 97%¹⁸⁻
95 ²³. This variability could be attributed to the unreliable results of PZA-susceptibility
96 testing, as mentioned above. Recent reviews reported that over 80% of PZA-resistant
97 isolates harbor mutations in the *pncA* gene and promoter, whereas up to 10% have no
98 mutation or carry mutations not associated with phenotypic resistance^{16, 17}. *PncA*
99 mutations have also been detected in 9% of PZA-sensitive isolates^{11,17}.

100

101 In Vietnam, a country with high MDR TB burden, the PZA resistance level is
102 unknown and no study has evaluated *pncA* mutations and the link with *M.*
103 *tuberculosis* families in clinical isolates. In the framework of the Vietnamese National
104 TB Control Program, PZA has been used for treating new TB cases and relapses since
105 1990, and is also added in regimens for patients with MDR or XDR TB since 2009²⁴.
106 As PZA-susceptibility testing is not routinely performed in Vietnam, the last national
107 drug resistance survey brought data only on the prevalence of resistance to the four
108 other FLDs (isoniazid, rifampicin, streptomycin and ethambutol)²⁵. The World
109 Health Organization (WHO) has stressed the urgent need to get data on PZA
110 resistance in different settings to limit the risk of using and introducing ineffective TB
111 treatment regimens²⁶.

112

113 Therefore, in this study we assessed PZA resistance in 205 drug-resistant and
114 55 sensitive *M. tuberculosis* clinical isolates collected in Vietnam. To this aim, we
115 determined the rate of *pncA* gene and promoter mutations in these isolates and their
116 distribution according to the *M. tuberculosis* families, mycobacterial interspersed
repetitive units-variable number of tandem repeats (MIRU-VNTR) genotypes and

117 FLD-resistance patterns (isoniazid, rifampicin, streptomycin and ethambutol
118 resistance).

119

120 **MATERIALS AND METHODS**

121 ***M. tuberculosis* isolates and drug susceptibility testing**

122 Isolates were selected from the culture collection of the Laboratory of
123 Tuberculosis, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. *M.*
124 *tuberculosis* samples were collected in three regional TB reference hospitals, the
125 National Lung Hospital (North), Pham Ngoc Thach Hospital (South) and Hue Central
126 Hospital (Centre), between 2005 and 2009. Since the objectives of this study were to
127 evaluate the risk of PZA resistance, isolates were chosen to represent according to
128 their FLD-susceptibility patterns (isoniazid, rifampicin, streptomycin and/or
129 ethambutol resistance) with a special focus on MDR isolates (Table 1) and *M.*
130 *tuberculosis* families. All the available drug-resistance patterns were included. For
131 each FLD-resistance pattern (mono to quadruple resistance), we selected isolates
132 according to their *M. tuberculosis* family, determined by spoligotyping. For each
133 family, when the number of isolates with a specific FLD-resistance pattern was lower
134 than ten, all isolates were tested. Conversely, when more than ten isolates were
135 available, ten or more isolates were randomly selected for analysis. For MDR
136 patterns, a higher number of isolates was selected to investigate PZA resistance in
137 these particularly highly drug resistant isolates. In total, 260 isolates were included in
138 the study (the sample size was not determined by statistical estimation). If we
139 consider the percentage of MDR in 2005 (2.7% in new cases and 19% in retreatment
140 cases) and a capacity of collection of 1300 strains (see last national survey in 2011) ²⁵,
141 over five years, 335 MDR isolates would have been collected in the case of a national

142 survey. In our sample, we characterized 123 MDR isolates that represent 37% of the
143 potential collection and this therefore gives a good representation of the MDR *M.*
144 *tuberculosis* population in Vietnam in this period.

145 FLD (isoniazid, rifampicin, streptomycin and ethambutol) susceptibility
146 testing was performed by using the proportion method, as recommended by the WHO
147 ²⁷, at the Vietnamese TB reference laboratories (National Lung Hospital or Pham
148 Ngoc Thach Hospital), or the proportional agar micro-plate assay developed by
149 Nguyen *et al.* ²⁸.

150 ***Genomic DNA extraction***

151 A full loop of *M. tuberculosis* colonies grown on Löwenstein-Jensen medium
152 was harvested and suspended in 1mL TE buffer (10mM Tris-HCl, 1mM EDTA).
153 After incubation at 95°C for 45 min, bacterial suspensions were centrifuged and
154 DNA-containing supernatants were transferred to new tubes and stored at -20°C until
155 use.

156 **Genotyping methods**

157 Spoligotyping was carried out as previously described ²⁹. Results were
158 compared with the SITVITWEB database for *M. tuberculosis* family identification ³⁰.
159 The 24-locus MIRU-VNTR technique was used to investigate genotype diversity and
160 clustering in the selected *M. tuberculosis* samples ³¹. A Neighbor Joining based
161 phylogenetic tree was built from DSW distances using MIRU-VNTR_{plus}
162 (<http://www.miru-vntrplus.org/>) (Supplementary figure S1). Isolates were classified as
163 “Unknown” when their spoligotypes could not be identified in the SITVITWEB
164 database and when the 24-MIRU-VNTR typing could not classify them in a family.

165 ***PCR amplification and DNA sequencing***

166 A 709bp fragment that included 561bp of the *pncA* coding sequence, 93bp of
167 the promoter and 55bp of the 3' region was amplified and sequenced using the
168 following primers: F-*pncA* (5'-CTTGCGGCGAGCGCTCCA-3') and R-*pncA* (5'-
169 TCGCGATCGTCGCGGCGTC-3') (modified from ¹⁸). Each 25µL of PCR mixture
170 contained 2.5µL of 10X reaction buffer, 5µL of 5X Q solution, 0.5µL of 5mM
171 dNTPs, 0.5µL of each forward and reverse primer (10µM), 0.1µL of 5U/µL HotStar
172 Taq (QIAGEN), 13µL of H₂O and 3µL of DNA template. PCR conditions were as
173 follows: 15min of Taq activation at 95°C, and then 35 cycles of denaturation at 95°C,
174 annealing at 63°C and extension at 72°C for 1min/each step, followed by a final
175 extension at 72°C for 5min. PCR products were examined on 1.5% agarose gels and
176 sequenced bidirectionally by Eurofins MWG Operon (Germany).

177 ***Sequence and Statistical analysis***

178 The *pncA* sequences were aligned to the *M. tuberculosis* H37Rv reference
179 sequence (GenBank accession number NC_000962.3) to identify mutations by using
180 BioEdit version 7.1.10. The two tailed-Fisher's exact test was used to compare the
181 mutation frequencies according to the drug-resistance patterns and in the different *M.*
182 *tuberculosis* families. Odds ratio and 95% confidence interval (95% CI) were
183 calculated to quantify the association of FLD resistance patterns with *pncA* mutation
184 frequency and the association between resistance to each FLD (isoniazid, rifampicin,
185 streptomycin and ethambutol) and *pncA* mutation frequency in our sample. A p-value
186 <0.05 was considered statistically significant.

187

188

189

190 **RESULTS**191 **FLD susceptibility testing and genotyping**

192 Among the 260 *M. tuberculosis* isolates selected for this study, 55 were
193 susceptible and 205 were resistant to at least one FLD (Table 1). Among the 205
194 FLD-resistant isolates, 29.8% (n=61), 12.3% (n=25), 13.7% (n=28) and 44.4% (n=91)
195 were resistant to one, two, three and four drugs respectively, and 60% were MDR.
196 Resistant samples could be classified in 11 distinct FLD resistance patterns (Table 1).

197 Analysis of spoligotyping and 24-locus MIRU-VNTR of 260 isolates revealed
198 209 distinct genotypes of which 188 were unique and 21 were clusters represented by
199 72 isolates (average = 3.4 isolates/cluster) (supplementary figure S1). The biggest
200 cluster consisted of 14 isolates with various FLD-susceptibility patterns, while the
201 smallest clusters (n=14) were composed of only two isolates per each. The 6
202 remaining clusters consisted of 3 to 8 isolates per each.

203 Among the genotypes, the Beijing was the most represented family (n=130),
204 followed by the EAI (n=76), T (n=28), Unknown (n=22), LAM (n=3) and H (n=1)
205 families (Table 1). For comparison of proportion of *pncA* mutation between families,
206 the “Others” group included Unknown spoligotypes, LAM and H families.

207 **Analysis of *pncA* mutations**

208 Among the 260 isolates, 99 (38.1%) carried mutations in *pncA* coding region
209 (32.3%) or its promoter (3.9%), or showed absence of *pncA* amplification (1.9%)
210 (Table 1). Seventy-one different mutations were identified, among which 55 had been
211 previously documented and 16 were new (Supplementary table S1 and Figure 1)^{11, 16,}
212 ^{17, 32, 33}.

213 Mutations in *pncA* coding region were only non-synonymous and were
214 dispersed along the gene. They were found in 50 of the 187 *pncA* codons. No
215 mutation was particularly predominant and the maximum number of isolates with the
216 same mutations was three. The mutation types included single nucleotide
217 substitutions, nucleotide deletions or insertions and double mutations (supplementary
218 table S1). Several single nucleotide substitutions (at codons 103, 108, 119, 164 or
219 181) led to premature stop codons. Nucleotide deletions or insertions led to a shift in
220 the reading frame and resulted in abnormal or early-truncated polypeptides. In
221 addition, *pncA* could not be amplified in five isolates (experiment repeated five
222 times), suggesting gene deletion. Finally, 30.3% (30/91) of mutants carried *pncA*
223 mutations in the three regions described as mutation hot spots (i.e., 3–17, 61–85, and
224 132–142)^{13, 18} (supplementary table S1).

225 Concerning the *pncA* promoter mutations, the most common was a nucleotide
226 substitution at position -11 (seven isolates). Nucleotide substitutions at positions -12, -
227 13 and a 12-nucleotide deletion (from -18 to -7) were also detected (one isolate/each)
228 (supplementary table S1).

229 **Association of FLD-resistance patterns with *pncA* mutations**

230 Among the 205 FLD resistant isolates, 96 (46.8%) had at least one mutation in
231 the *pncA* gene or promoter, while 3 (5.5%) of the 55 FLD sensitive isolates had one
232 mutation in the coding region (Table 1). This difference was statistically significant
233 ($p < 5 \times 10^{-10}$). Similarly, the *pncA* mutation frequency in isolates resistant to a specific
234 FLD (isoniazid, rifampicin, streptomycin and ethambutol) was significantly higher
235 than in drug-sensitive isolates, based on the odds ratio values ($p < 0.0001$) (Table 2).
236 Moreover, when comparing each drug resistance group, Fisher's exact probability test
237 results revealed significant different mutation frequencies ($p < 0.0004$). More

238 specifically, the frequencies of mutations (Table 2) were significantly different
239 between rifampicin, isoniazid and streptomycin drug resistant isolates ($p < 0.015$), and
240 between ethambutol and isoniazid drug resistant isolates ($p < 0.005$).

241 When taking into account the FLD resistance patterns (mono to quadruple
242 resistance), *pncA* mutation frequency was highest in quadruple-resistant isolates
243 (75.8%), followed by triple-resistant (60.7%), double-resistant (32.0%), sensitive
244 (5.5%) and mono-resistant isolates (3.3%) (Table 3). These differences were
245 significant ($p < 10^{-7}$). The odds ratio calculation showed that, except in mono-drug
246 resistant samples, the *pncA* mutation frequency was progressively increasing from
247 double- to quadruple-resistant isolates compared with FLD sensitive isolates (Table
248 3). Nevertheless, the differences were significant only between the mono drug
249 resistant patterns and all the other patterns ($p < 6 \times 10^{-4}$) and between double and
250 quadruple resistant patterns ($p < 8.4 \times 10^{-5}$). In addition, the *pncA* mutation frequency in
251 MDR isolates was significantly higher than in non-MDR isolates (72.4% vs 7.2%;
252 $p < 10^{-7}$) (Table 3).

253 **Association of *pncA* mutations with *M. tuberculosis* families and genotypes**

254 The *pncA* mutation frequency was highest in Beijing isolates (62/130, 47.7%),
255 followed by T (9/28, 32.1%), EAI (23/76, 30.3%) and “Others” isolates (5/26,
256 19.2%). The global Fisher’s exact test was significant supporting a difference between
257 Beijing family and non-Beijing families ($p < 0.002$). The difference was more
258 specifically significant between Beijing and EAI families ($p < 0.018$), and between
259 Beijing and “Others” spoligotypes ($p < 0.008$). Within the Beijing family, *pncA*
260 mutations were found in isolates showing all possible FLD resistance patterns.
261 Among MDR isolates, *pncA* mutation frequency was highest in the T family (8/9,
262 88.9%), followed by Beijing (56/76, 73.7%), EAI (21/30, 70.0%) and “Others” (4/8,

263 50%). Nevertheless, the overall Fisher's exact test revealed no significant differences
264 ($p>0.5$). Due to the low sample size for T, EAI family and "Others" class compared to
265 Beijing isolates, the tests should be done on a larger sample.

266 The 99 *pncA* mutants belonged to 79 different genotypes, including 18
267 clusters (supplementary figure S1). Most of these clusters included isolates showing
268 various FLD resistance patterns and *pncA* mutations. For instance, in the biggest
269 cluster (14 isolates), isolates harbored seven distinct *pncA* mutations and six different
270 FLD patterns. Finally, only two *pncA* mutants had fully similar genetic and
271 phenotypic patterns (supplementary figure S1).³⁴

272

273 **DISCUSSION**

274 **High diversity and frequency of *pncA* mutations in clinical *M. tuberculosis*** 275 **isolates in Vietnam**

276 Our molecular analysis indicates that 38.1% of the clinical *M. tuberculosis*
277 isolates selected in this study carry mutations in the *pncA* gene or its promoter. A
278 study in Northern Vietnam reported that 2.4% of isolates were negative for PZase by
279 PZase assay and were considered to be resistant to PZA³⁵. However, since PZA-
280 resistant isolates are not always PZase negative, it is thus difficult to compare the two
281 studies.

282 In our study, *pncA* mutations were very diverse and distributed along the gene
283 without predominant ones (71 different mutations detected in 50 different codons).
284 This is in agreement with the fact that overall, more than six hundred distinct *pncA*
285 mutations in 171/187 different codons have been identified so far¹⁷. In our study,
286 30.3% of *pncA* mutants carried mutations in the three previously described mutation
287 hot spot regions^{13, 18}. Nevertheless, a recent meta-analysis showed that only 7.0% of

288 PZA-resistant strains carry mutations in these regions ¹⁷. This underlines the great
289 diversity of mutations at different positions that can lead to PZA resistance.
290 Remarkably, for many *pncA* mutations (85.0%), the link with PZA resistance has
291 been experimentally confirmed (high confident mutations) and have been associated
292 with high minimum inhibitory concentrations (MICs) of PZA corresponding to high-
293 level PZA resistance ^{11, 16-18, 22, 23}. Furthermore, Stoffels *et al.* predicted by 3D
294 structural analysis that most of the mutations detected in their PZA-resistant isolates
295 affect PZA protein activity ¹¹.

296 On the other hand, our data indicate that the diversity of mutations in the *pncA*
297 promoter is smaller (4 mutations, 10 mutants), in agreement with the published data
298 (34 mutations globally) ^{11, 16, 17, 32, 33}. These mutations can be associated with low- or
299 high-level PZA resistance.

300 Finally, 55 of the 71 *pncA* mutations detected in our samples were already
301 known, 50 of them have been previously linked to PZA resistance and 5 were found
302 in either PZA-resistant/sensitive isolates or only in PZA-sensitive ones. Among the 16
303 novel mutations, 14 are likely to be linked to PZA resistance because of their
304 mutation types or codon positions^{11, 16, 17, 32, 33}. Altogether, 90.1% (64/71) of the *pncA*
305 mutations identified in our study are likely to be associated with PZA resistance.
306 Thus, out of 99 mutants, 91 (92%) carried high confident *pncA* mutations, 2 carried
307 mutations with unknown link to PZA resistance and 6 are probably not linked to PZA
308 resistance.

309 ***PncA* mutation frequency and FLD resistance patterns**

310 In our study, three FLD sensitive isolates carried *pncA* mutations located in
311 the PZase active or metal-binding site. These isolates could be mono-PZA resistant,
312 especially the two carrying mutations at codons 49 and 138, which have been linked

313 to high-level PZA resistance ^{11, 12, 18}. In agreement, a recent study reported a
314 significant proportion of phenotypic mono-PZA resistance (4.2%) in clinical isolates
315 ²². Nevertheless, as expected, *pncA* mutation frequency was significantly higher in
316 FLD-resistant than in FLD-sensitive isolates.

317 Although a recent study showed an association between *pncA* mutation
318 frequency and rifampicin resistance, the link between *pncA* mutations and resistance
319 to other FLD has not yet been reported ¹⁰. In our study, we found that the frequency of
320 *pncA* mutations was significantly different between each drug resistance group
321 (isoniazid, rifampicin, streptomycin and ethambutol resistance) and the corresponding
322 sensitive isolates, suggesting an association between PZA resistance and each FLD
323 drug resistance.

324 Analyses according to the FLD resistance patterns suggest that the frequency
325 of *pncA* gene mutations progressively increases with the FLD resistance (mono,
326 double, triple and quadruple resistance). Quadruple-resistant isolates showed the
327 highest *pncA* mutation rate (75.8%). Similarly, *pncA* mutation frequency was very
328 high also in MDR isolates (72.4%), consistent with many other studies worldwide ^{11,}
329 ^{22, 23, 36}, suggesting a cumulative effect of drug resistance mutation. Another study
330 showed that the proportion of PZA-resistant strains is higher in pre-XDR and XDR
331 than in MDR isolates ³⁷. Altogether, these data suggest a higher risk to detect PZA
332 resistance in more severely drug-resistant *M. tuberculosis* isolates. This is very
333 worrying because PZA resistance in MDR and XDR TB is generally associated with
334 poor treatment outcome ^{6, 7}. Thus, PZA-susceptibility profile prior to treatment is a
335 priority especially in patients with MDR and XDR TB.

336 ***PncA* mutation frequency is highest in the Beijing family**

337 The genotyping analysis did not highlight any association between specific
338 *pncA* mutations and the different *M. tuberculosis* families or MIRU-VNTR clusters,
339 in agreement with other studies ^{11, 36, 38}. Nevertheless, the mutation frequency was
340 globally higher in Beijing isolates compared with other families or genotypes,
341 suggesting a greater association of this family with PZA resistance. Furthermore,
342 *pncA* mutations in this family were found in FLD susceptible to quadruple-resistant
343 isolates. Conversely, in the other families, mutations were detected particularly in
344 triple- and quadruple-resistant isolates. We thus hypothesize that the Beijing family
345 might be more susceptible to acquire *pncA* mutations, whatever the resistance profile.
346 This should be experimentally demonstrated. Although the situation differs according
347 to the country ^{22, 36, 38}, the Beijing family seems to be strongly linked to PZA
348 resistance in Vietnamese samples. This is all the more worrying that the Beijing
349 family is currently spreading in Vietnam ³⁹.

350 **Evolution of PZA resistance in *M. tuberculosis* population**

351 The *pncA* mutation diversity and frequency are very high in our study. This
352 can be caused by several factors: high rate of PZA-resistance acquisition (10^{-5} bacilli
353 in vitro) ¹¹; the non-necessity of *pncA* (*M. tuberculosis* can survive and grow without
354 this gene) ⁴⁰ and the absence of loss of bacterial fitness in the presence of *pncA*
355 mutations ¹¹. However, the *pncA* mutations may also bear high fitness costs impaired
356 with transmission since lack of clusters of *pncA* mutants in clinical isolates was
357 largely observed worldwide ⁴¹. In fact, the spread of some MDR and XDR clones
358 carrying few specific *pncA* mutations has been reported ^{34, 42} leading to a hypothesis
359 that some *pncA* mutations have no reduction of transmission fitness of the bacteria.
360 This peculiar evolution of the *pncA* gene might lead to the emergence of PZA
361 resistance at high frequency, thus challenging the use of PZA as a key drug in current

362 and future treatment regimens, despite its powerful sterilizing capacities. Studying the
363 PZA resistance mechanisms and following its evolution are crucial for TB control.

364

365 **CONCLUSION**

366 This study shows that a high proportion of Vietnamese clinical *M. tuberculosis*
367 isolates carry *pncA* mutations, particularly MDR, quadruple-FLD resistant and
368 Beijing family isolates. Nevertheless, the proportion of PZA resistance in these
369 samples might be under estimated since it is well known that a proportion of PZA-
370 resistant isolates without *pncA* mutations is existed. This finding highlights the high
371 risk of PZA resistance and challenges the use of PZA in treatment regimens without
372 prior PZA-susceptibility information of the TB patients to be treated.

373

374 **Acknowledgements**

375 This work was supported by the following grants: PHC Lotus, GDRI ID-BIO,
376 KC.10.15/06-10, JEAJ MySA, LMI DRISA, and ADB-BIO3 USTH project. We
377 would like to thank the Viet Nam National Tuberculosis Program, National Lung
378 Hospital, Pham Ngoc Thach Hospital and Hue Central Hospital for *M. tuberculosis*
379 sampling. We also thank NIHE, IRD, CNRS and USTH for their support. Nguyen
380 Quang Huy was supported by the project 911 - doctoral scholarship from the
381 Vietnamese Government. We also thank Elisabetta Andermacher for assistance in
382 preparing and editing the manuscript.

383

384 **Disclaimers:** The authors declare no competing interests.

385

386

387 Supplementary information accompanies the manuscript on the *Emerging Microbes &*
 388 *Infections* website <http://www.nature.com/emi>

389

390 **References**

391 1. Zumla A, Chakaya J, Centis R, D'Ambrosio L, Mwaba P, Bates M, *et al.* Tuberculosis
 392 treatment and management--an update on treatment regimens, trials, new drugs,
 393 and adjunct therapies. *Lancet Respir Med* 2015 Mar; **3**(3): 220-234.

394

395 2. Steele MA, Des Prez RM. The role of pyrazinamide in tuberculosis chemotherapy.
 396 *Chest* 1988 Oct; **94**(4): 845-850.

397

398 3. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity in vitro against
 399 semidormant *Mycobacterium tuberculosis* bacterial populations. *Am Rev Respir Dis*
 400 1992 May; **145**(5): 1223-1225.

401

402 4. WHO. Global tuberculosis report 2016. *WHO/HTM/TB/201613 World Health*
 403 *Organization, Geneva, Switzerland* 2016.

404

405 5. Organization WH. WHO treatment guidelines for drug-resistant tuberculosis: 2016
 406 update. *WHO Handbook* 2016; ISBN 978 92 4 154963 9([www.who.int/tb/areas-of-](http://www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/resources/)
 407 [work/drug-resistant-tb/treatment/resources/](http://www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/resources/)).

408

409 6. Chang KC, Leung CC, Yew WW, Leung EC, Leung WM, Tam CM, *et al.* Pyrazinamide
 410 may improve fluoroquinolone-based treatment of multidrug-resistant tuberculosis.
 411 *Antimicrob Agents Chemother* 2012 Nov; **56**(11): 5465-5475.

412

413 7. Zhang Y, Chiu Chang K, Leung CC, Wai Yew W, Gicquel B, Fallows D, *et al.* 'Z(S)-MDR-

414 TB' versus 'Z(R)-MDR-TB': improving treatment of MDR-TB by identifying
 415 pyrazinamide susceptibility. *Emerg Microbes Infect* 2012 Jul; **1**(7): e5.

416

417 8. Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility
 418 testing of *Mycobacterium tuberculosis* to pyrazinamide. *J Med Microbiol* 2002 Jan;
 419 **51**(1): 42-49.

420

421 9. Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F. Potential for erroneous
 422 results indicating resistance when using the Bactec MGIT 960 system for testing
 423 susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *J Clin Microbiol* 2010
 424 Jan; **48**(1): 300-301.

425

426 10. Zignol M, Dean AS, Alikhanova N, Andres S, Cabibbe AM, Cirillo DM, *et al.*
 427 Population-based resistance of *Mycobacterium tuberculosis* isolates to pyrazinamide

- 428 and fluoroquinolones: results from a multicountry surveillance project. *Lancet Infect*
429 *Dis* 2016 Oct; **16**(10): 1185-1192.
- 430
- 431 11. Stoffels K, Mathys V, Fauville-Dufaux M, Wintjens R, Bifani P. Systematic analysis of
432 pyrazinamide-resistant spontaneous mutants and clinical isolates of *Mycobacterium*
433 *tuberculosis*. *Antimicrob Agents Chemother* 2012 Oct; **56**(10): 5186-5193.
- 434
- 435 12. Petrella S, Gelus-Ziental N, Maudry A, Laurans C, Boudjelloul R, Sougakoff W. Crystal
436 structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural
437 and acquired resistance to pyrazinamide. *PLoS One* 2011; **6**(1): e15785.
- 438
- 439 13. Zhang Y, Shi W, Zhang W, Mitchison D. Mechanisms of Pyrazinamide Action and
440 Resistance. *Microbiol Spectr* 2014 Aug; **2**(4): MGM2-0023-2013.
- 441
- 442 14. Zhang S, Chen J, Shi W, Liu W, Zhang W, Zhang Y. Mutations in *panD* encoding
443 aspartate decarboxylase are associated with pyrazinamide resistance in
444 *Mycobacterium tuberculosis*. *Emerg Microbes Infect* 2013 Jun; **2**(6): e34.
- 445
- 446 15. Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE, 3rd, *et al*. Pyrazinamide inhibits
447 trans-translation in *Mycobacterium tuberculosis*. *Science* 2011 Sep 16; **333**(6049):
448 1630-1632.
- 449
- 450 16. Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniowski F, Rodionova Y, *et al*.
451 *Mycobacterium tuberculosis* pyrazinamide resistance determinants: a multicenter
452 study. *MBio* 2014; **5**(5): e01819-01814.
- 453
- 454 17. Ramirez-Busby SM, Valafar F. Systematic review of mutations in pyrazinamidase
455 associated with pyrazinamide resistance in *Mycobacterium tuberculosis* clinical
456 isolates. *Antimicrob Agents Chemother* 2015 Sep; **59**(9): 5267-5277.
- 457
- 458 18. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, *et al*.
459 Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium*
460 *tuberculosis*. *Antimicrob Agents Chemother* 1997 Mar; **41**(3): 540-543.
- 461
- 462 19. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major
463 mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tuber Lung*
464 *Dis* 1997; **78**(2): 117-122.
- 465
- 466 20. Bhuju S, Fonseca Lde S, Marsico AG, de Oliveira Vieira GB, Sobral LF, Stehr M, *et al*.
467 *Mycobacterium tuberculosis* isolates from Rio de Janeiro reveal unusually low
468 correlation between pyrazinamide resistance and mutations in the *pncA* gene. *Infect*
469 *Genet Evol* 2013 Oct; **19**: 1-6.
- 470

- 471 21. Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, *et al.* Role of *pncA* and *rpsA* gene
472 sequencing in detection of pyrazinamide resistance in *Mycobacterium tuberculosis*
473 isolates from southern China. *J Clin Microbiol* 2014 Jan; **52**(1): 291-297.
- 474
- 475 22. Li D, Hu Y, Werngren J, Mansjo M, Zheng X, Drobniewski F, *et al.* Multicenter Study
476 of the Emergence and Genetic Characteristics of Pyrazinamide-Resistant
477 Tuberculosis in China. *Antimicrob Agents Chemother* 2016 Sep; **60**(9): 5159-5166.
- 478
- 479 23. Gu Y, Yu X, Jiang G, Wang X, Ma Y, Li Y, *et al.* Pyrazinamide resistance among
480 multidrug-resistant tuberculosis clinical isolates in a national referral center of China
481 and its correlations with *pncA*, *rpsA*, and *panD* gene mutations. *Diagn Microbiol*
482 *Infect Dis* 2016 Mar; **84**(3): 207-211.
- 483
- 484 24. Vietnam national tuberculosis control programme N. Guidline for management of
485 multi-drug resistance tuberculosis. 2009.
- 486
- 487 25. Nhung NV, Hoa NB, Sy DN, Hennig CM, Dean AS. The fourth national anti-
488 tuberculosis drug resistance survey in Viet Nam. *Int J Tuberc Lung Dis* 2015 Jun;
489 **19**(6): 670-675.
- 490
- 491 26. Organization WH. New survey investigates levels of resistance to essential TB drugs.
492 Geneva: WHO, 2016 2016; Available from:
493 http://www.who.int/tb/features_archive/TB_resistance_survey_2016/en/
494 (accessed 11 July 2016).
- 495
- 496 27. WHO. Guidelines ofr surveillance of drug resistance in tuberculosis. Second Edition.
497 WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance
498 Geneva 2003 2003; WHO/CDS/CSR/RMD/2003.3.
- 499
- 500 28. Nguyen VA, Nguyen HQ, Vu TT, Nguyen NA, Duong CM, Tran TH, *et al.* Reduced turn-
501 around time for *Mycobacterium tuberculosis* drug susceptibility testing with a
502 proportional agar microplate assay. *Clin Microbiol Infect* 2015 Dec; **21**(12): 1084-
503 1092.
- 504
- 505 29. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al.*
506 Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for
507 diagnosis and epidemiology. *J Clin Microbiol* 1997 Apr; **35**(4): 907-914.
- 508
- 509 30. Demay C, Liens B, Burguiere T, Hill V, Couvin D, Millet J, *et al.* SITVITWEB--a publicly
510 available international multimarker database for studying *Mycobacterium*
511 *tuberculosis* genetic diversity and molecular epidemiology. *Infect Genet Evol* 2012
512 Jun; **12**(4): 755-766.
- 513

- 514 31. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al.*
 515 Proposal for standardization of optimized mycobacterial interspersed repetitive unit-
 516 variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin*
 517 *Microbiol* 2006 Dec; **44**(12): 4498-4510.
- 518
 519 32. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB.
 520 Tuberculosis drug resistance mutation database. *PLoS Med* 2009 Feb 10; **6**(2): e2.
- 521
 522 33. Whitfield MG, Soeters HM, Warren RM, York T, Sampson SL, Streicher EM, *et al.* A
 523 Global Perspective on Pyrazinamide Resistance: Systematic Review and Meta-
 524 Analysis. *PLoS One* 2015; **10**(7): e0133869.
- 525
 526 34. Cohen KA, Abeel T, Manson McGuire A, Desjardins CA, Munsamy V, Shea TP, *et al.*
 527 Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole
 528 Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates
 529 from KwaZulu-Natal. *PLoS Med* 2015 Sep; **12**(9): e1001880.
- 530
 531 35. Hung NV, Ando H, Thuy TT, Kuwahara T, Hang NT, Sakurada S, *et al.* Clonal expansion
 532 of *Mycobacterium tuberculosis* isolates and coexisting drug resistance in patients
 533 newly diagnosed with pulmonary tuberculosis in Hanoi, Vietnam. *BMC Res Notes*
 534 2013; **6**: 444.
- 535
 536 36. Chiu YC, Huang SF, Yu KW, Lee YC, Feng JY, Su WJ. Characteristics of *pncA* mutations
 537 in multidrug-resistant tuberculosis in Taiwan. *BMC Infect Dis* 2011; **11**: 240.
- 538
 539 37. Xia Q, Zhao LL, Li F, Fan YM, Chen YY, Wu BB, *et al.* Phenotypic and genotypic
 540 characterization of pyrazinamide resistance among multidrug-resistant
 541 *Mycobacterium tuberculosis* isolates in Zhejiang, China. *Antimicrob Agents*
 542 *Chemother* 2015 Mar; **59**(3): 1690-1695.
- 543
 544 38. Doustdar F, Khosravi AD, Farnia P. *Mycobacterium tuberculosis* genotypic diversity in
 545 pyrazinamide-resistant isolates of Iran. *Microb Drug Resist* 2009 Dec; **15**(4): 251-256.
- 546
 547 39. Nguyen VA, Banuls AL, Tran TH, Pham KL, Nguyen TS, Nguyen HV, *et al.*
 548 *Mycobacterium tuberculosis* lineages and anti-tuberculosis drug resistance in
 549 reference hospitals across Viet Nam. *BMC Microbiol* 2016; **16**(1): 167.
- 550
 551 40. Martinez E, Holmes N, Jelfs P, Sintchenko V. Genome sequencing reveals novel
 552 deletions associated with secondary resistance to pyrazinamide in MDR
 553 *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2015 Sep; **70**(9): 2511-2514.
- 554
 555 41. den Hertog AL, Sengstake S, Anthony RM. Pyrazinamide resistance in
 556 *Mycobacterium tuberculosis* fails to bite? *Pathog Dis* 2015 Aug; **73**(6): ftv037.

557
 558 42. Muller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, *et al.*
 559 Programmatically selected multidrug-resistant strains drive the emergence of
 560 extensively drug-resistant tuberculosis in South Africa. *PLoS One* 2013; **8**(8): e70919.

561

562 **Table 1** Distribution of first-line drug susceptibility patterns according to the

563 *Mycobacterium tuberculosis* families and their relative frequency of *pncA* mutations

Type	Drug-susceptibility patterns	<i>M. tuberculosis</i> families				Total	<i>pncA</i> mutation n (%)
		EAI	Beijing	T	“Others”		
FLD sensitive	Sensitive	20	14	11	10	55	3 (5.5)
Mono-resistant	H	12	11	2	2	27	2 (7.4)
	R	0	0	0	2	2	0 (0)
	S	11	14	4	2	31	0 (0)
	E	0	1	0	0	1	0 (0)
Double-resistant	HS	3	12	2	2	19	5 (26.3)
	HR	4	1	0	1	6	3 (50)
Triple-resistant	HRS	5	11	1	3	20	12 (60)

	HRE	4	2	0	0	6	5 (83.3)
	HSE	0	1	0	0	1	0 (0)
	RSE	0	1	0	0	1	0 (0)
Quadruple-resistant	HRSE	17	62	8	4	91	69 (75.8)
	Total	76	130	28	26	260	99 (38.1)

564 H: Resistant to isoniazid, R: Resistant to rifampicin, S: Resistant to streptomycin, E:

565 Resistant to ethambutol.

566 FLD sensitive: sensitive to all four first-line drugs (FLDs) (isoniazid, rifampicin,
567 streptomycin and ethambutol).

568 “Others”: Include 1 H, 3 LAM and 22 Unknown genotype isolates.

569 Mono-resistant (isolates resistant to H or R, S, E); double-resistant (isolates resistant
570 to two of the four FLDs), triple-resistant (isolates resistant to three FLDs) and
571 quadruple-resistant (isolates resistant to all four FLDs).

572

573 **Table 2** Comparison of the *pncA* mutation frequencies of isoniazid, rifampin,
574 streptomycin or ethambutol resistant isolates and of the corresponding sensitive
575 isolates.

Resistant/ sensitive pattern	N° of isolates		Related frequency of <i>pncA</i> mutation	Odds ratio, 95% CI	p-value
	With <i>pncA</i>	Without <i>pncA</i>			

	mutation	mutation			
isoniazid-resistant	96	74	56.5%		
				37.6, 11.4-123.7	p<0.0001
isoniazid-sensitive	3	87	3.3%		
rifampicin-resistant	89	37	70.6%		
				29.8, 14.1-63.1	p<0.0001
rifampicin-sensitive	10	124	7.5%		
streptomycin-resistant	86	77	52.8%		
				7.2, 3.7-14.0	p<0.0001
streptomycin-sensitive	13	84	13.4%		
ethambutol-resistant	74	26	74%		
				15.4, 8.3-28.5	p<0.0001
ethambutol-sensitive	25	135	15.6%		

576

577 **Table 3** Comparison of the *pncA* mutation frequencies of isolates with different drug
578 resistant patterns (sensitive, mono-, double-, triple- and quadruple- resistant) and first-
579 line drug sensitive isolates.

Type of isolates	N° of isolates		Related frequency of <i>pncA</i> mutation	Odds ratio, 95% CI	p-value
	With mutation	Without mutation			

Sensitive	3	52	5.5%		
Mono-resistant	2	59	3.3%	0.6, 0.1-3.7	p>0.5
Double-resistant	8	17	32%	8.2, 1.9-34.3	p<0.005
Triple-resistant	17	11	60.7%	26.8, 6.7-107.5	p<0.001
Quadruple-resistant	69	22	75.8%	54.4, 15.4-191.4	p<0.001
MDR	89	34	72.4%	45.4, 13.3-155.1	p<0.0001
Non MDR	10	127	7.9%	1.4, 0.4-5.2	p>0.5

580

581

582

583

584 **Supplementary table S1** Mutations found in *pncA* promoter and coding region in the selected Vietnamese *Mycobacterium tuberculosis* isolates.

585 The table shows the diversity of *pncA* mutations (71 different types), the mutation positions and their nucleotide/amino acid change(s) and

586 characteristics of the mutations (reported/unreported in the literature). The table also presents that the mutations were found in PZA-resistant

587 and/or PZA-susceptible isolates and were associated with PZA resistant/sensitive according to the literature.

No	DST pattern	No of isolate(s)	Nucleotide position(s)	Codon position(s)	Nucleotide substitution(s)	Amino acid change(s)	Mutations reported/unreported in the literature	PZA Resistant/sensitive isolates	Linked to PZA resistant/sensitive	Reference
1	HS	7	-11	NA	A-G	NA	Yes	R & S	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
2	HRSE	1	-12	NA	T-G	NA	Yes	R	R	Whitfield et al.2015
3	H	1	-13	NA	G-T	NA	Yes	R & S	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
4	HRS	1	(-18)-(-7)	NA	Deletion CGAACGTATGGT	NA	Novel deletion	U	R	New
5	HRSE	1	2	1	ATG-AGG	Met-Arg (frameshift)	New amino acid replacement	U	R	New
6	HRS	1	4	2	CGG-TGG	Arg-Trp	New amino acid replacement	U	U	New
7	HRE	1	16	6	ATC-CTC	Ile-Leu	Yes	R & S	S	Mittio et al. 2014 & Ramirez-Busby et al. 2015
8	HRS	1	17	6	ATC-ACC	Ile-Thr	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
9	HRS	1	19	7	GTC-TTC	Val-Phe	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
10	HRS	1	29	10	CAG-CCG	Gln-Pro	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
11	HRSE	1	29	10	CAG-CGG	Gln-Arg	Yes	R & S	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
12	HR	1	40	14	TGC-CGC	Cys-Arg	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015

13	HRSE	2	55	19	del C	frameshift	Novel deletion	U	R	New
14	HRSE	1	104	35	CTG-CCG	Leu-Pro	Yes	R	R	Mittio 2014
15	HRSE	1	139	47	ACC-GCC	Thr-Ala	Yes	R & S	S	Mittio 2014
16	HRE	1	140	47	ACC-ATC	Thr-Ile	New amino acid replacement	U	U	New
17	HRSE	1	146	49	GAC-GCC	Asp-Ala	Yes	R	R	Mittio 2014
18	Sensitive, HRSE	2	147	49	GAC-GAG	Asp-Glu	Yes	S	S	Bhujy et al. 2013
19	HRSE	1	151	51	CAC-TAC	His-Tyr	Yes	R	R	Mittio 2014
20	HRSE	1	152	51	CAC-CGC	His-Arg	Yes	R	R	Mittio 2014
21	HRSE	1	161	54	CCG-CAG	Pro-Gln	Yes	R	R	Mittio 2014
22	HRSE	1	161	54	CCG-CTG	Pro-Leu	Yes	R	R	Mittio 2014
23	HRSE	1	169	57	CAC-TAC	His-Tyr	Yes	R	R	Mittio 2014
24	HRSE	1	170	57	CAC-CCC	His-Pro	Yes	R	R	Mittio 2014
25	HRSE	1	172	58	TTC-GTC	Phe-Val	New amino acid replacement	U	R	New
26	HRSE	2	173	58	TTC-TCC	Phe-Ser	Yes	R	R	Ramirez-Busby et al. 2015
27	HRSE	1	176	59	TCC-TTC	Ser-Phe	Yes	S	S	Campbel et al. 2011, Xia et al. 2015
28	HRS	1	181	61	ACA-CCA	Thr-Pro	Yes	R	R	Ramirez-Busby et al. 2015
29	HRSE	2	184	62	CCG-TCG	Pro-Ser	Yes	R	R	Ali et al. 2015
30	HRSE	1	185	62	CCG-CTG	Pro-Leu	Yes	R	R	Mittio 2014
31	HR	1	188	63	GAC-GGC	Asp-Gly	Yes	R	R	Mittio 2014
32	HRSE	1	188	63	GAC-GCC	Asp-Ala	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
33	HRSE	2	202	68	TGG-CGG	Trp-Arg	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
34	HRE	2	226	76	ACT-CCT	Thr-Pro	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
35	HRSE	1	246	82	CAT-CAA	His-Gln	New amino acid replacement	U	R	New
36	HRSE	3	286	96	AAG-GAG	Lys-Glu	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015

37	HRS	1	287	96	AAG-ACG	Lys-Thr	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
38	HRSE	1	305	102	GCG-GTG	Ala-Val	Yes	R & S	S	Mittio et al. 2014 & Ramirez-Busby et al. 2015
39	HR	2	308	103	TAC-TCC	Tyr-Ser	Yes	R	R	Ramirez-Busby et al. 2015
40	HRSE	1	309	103	TAC-TAG	Tyr-Stop	Yes	R	R	Mittio 2014
41	HRSE	2	312	104	AGC-AGA	Ser-Arg	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
42	HRSE	1	322	108	GGA-TGA	Gly-Stop	Yes	R	R	Ramirez-Busby et al. 2015
43	HRSE	1	347	116	CTG-CCG	Leu-Pro	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
44	HS	1	357	119	TGG-TGT	Trp-Cys	Yes	R	R	Mittio 2014
45	HRSE	1	357	119	TGG-TGA	Trp-Stop	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2015
46	HRE	1	359	120	CTG-CCG	Leu-Pro	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2016
47	HS	1	383	128	GTC-GGC	Val-Gly	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2017
48	HRS	2	389	130	GTG-GGG	Val-Gly	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2018
49	HRSE	1	401	134	GCC-GTC	Ala-Val	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2019
50	HRSE	1	410	137	CAT-CGT	His-Arg	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2020
51	HRSE	1	412	138	TGT-CGT	Cys-Arg	Yes	R	R	Mittio et al. 2014 & Ramirez-Busby et al. 2021
52	Sensitive, HRSE	2	412	138	TGT-AGT	Cys-Ser	Yes	R	R	Ramirez-Busby et al. 2015
53	HRSE	2	422	141	CAG-CCG	Gln-Pro	Yes	R	R	Mittio 2014, Buszy et al 2015
54	HS	2	424	142	ACG-GCG	Thr-Ala	Yes	R	R	Mittio 2014, Buszy et al 2015
55	HRSE	1	425	142	ACG-AGG	Thr-Arg	New amino acid replacement	U	R	New
56	HRSE	1	436	146	GCG-CCG	Ala-Pro	Yes	R	R	Ramirez-Busby et al. 2015
57	H	1	446	149	AAT-AGT	Asn-Ser	New amino acid replacement	U	R	New
58	HRSE	1	460	154	AGG-GGG	Arg-Gly	Yes	R	R	Mittio 2014, Buszy et al 2015
59	HR	3	464	155	GTG-GGG	Val-Gly	Yes	R	R	Mittio 2014, Buszy et al

										2015
60	HRSE	1	467	156	CTG-CCG	Leu-Pro	Yes	R	R	Mittio 2014, Buszy et al 2015
61	HRSE	1	491	164	TCG-TAG	Ser-Stop	New amino acid replacement	U	R	New
62	HRSE	1	541	181	GAG-TAG	Glu-Stop	New amino acid replacement	U	R	New
63	HRSE	1	187-188 & 319	63 & 107	Insertion ACTATTCCTC & GAA-AAA	frameshift and Glu-Lys	New insertion and new mutation pattern	U	R	New
64	HRSE	1	231-232	78	Insertion G	frameshift	Yes	R	R	Mittio 2014, Buszy et al 2015
65	HRSE	1	290 & 548	97 & 183	GGT-GAT & deletion T	Gly-Asp & frameshift	New deletion and new mutation pattern	U	R	New
66	HRSE	2	389-390	130	insertion GG	frameshift	Yes	R	R	Whitfield et al.2015
67	HRSE	2	482-483	161	Insertion G	frameshift	New insertion	U	R	New
68	HRSE	1	491-492	164	Insertion C	frameshift	New insertion	U	R	New
69	HS	1	82 & 418-419	28 & 140	GCC-ACC & deletion G	Ala-Thr & frameshift	New deletion and new mutation pattern	U	R	New
70	HRE	5	Whole pncA deletion	Whole pncA deletion	Whole pncA deletion	Whole pncA deletion	Yes	R	R	Aono et al. 2014, Martinez et al. 2015
71	Susceptible	1	307	103	TAC-GAC	Tyr-Asp	Yes	R	R	Mittio 2014, Buszy et al 2015

588

589 H: resistant to isoniazid; R: resistant to rifampin; S: resistant to streptomycin; E: resistant to ethambutol; NA: mutation in *pncA* promoter region;
590 #: references [1-5](#).

591

592

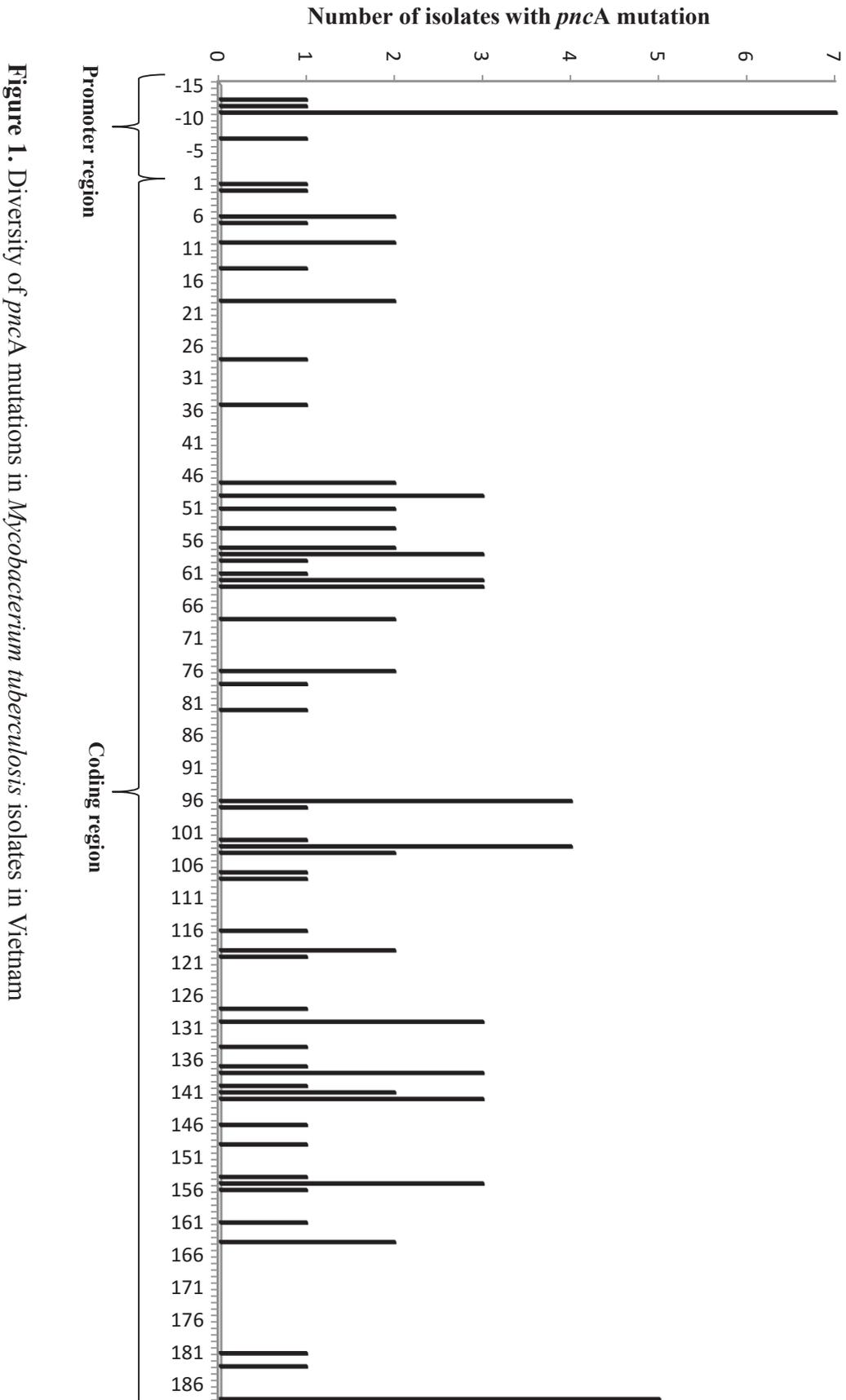


Figure 1. Diversity of *pncA* mutations in *Mycobacterium tuberculosis* isolates in Vietnam

3.4 Evolution of drug resistance in *Mycobacterium tuberculosis* in Vietnam

3.4.1 Objectives

As described in the introduction, Vietnam showed a high proportion of TB cases resistant to any FLD (32.7% and 54.2% among new and among previously treated TB cases, respectively) (316). The proportion of MDR-TB in between 2005 – 2011 was increased from 2.7% to 4.0% and from 19.0% to 23.3% among new and among previously treated TB cases, respectively (316). Furthermore, the proportion of MDR-TB cases resistant to any SLDs was 17.9% in 2011 (319). Nevertheless, the data remain limited and fragmented and do not give a global view of FLD and SLD resistance evolution at the country scale. To progress in knowledge of this critical domain in terms of public health, we performed a genetic and phenotypic analysis of clinical *M. tuberculosis* isolates collected in Vietnam to study the FLD and SLD resistance and to investigate how *M. tuberculosis* evolved from sensitive to XDR in this country. The materials and methods as well as the 260 isolates used in this part are described in the chapter 2.

3.4.2 Results

A. FLD susceptibility testing

Globally, among the 260 samples, 12 different phenotypic FLD-susceptibility patterns (except PZA resistance) were detected including FLD-sensitive (21.2%), mono-FLD resistant (23.5%, 4 types), double-FLD resistant (9.6%, 2 types), triple-FLD resistant (10.8%, 4 types) and quadruple-FLD resistant (35%) (**Error! Reference source not found.**). Regarding each drug, 65.4% of FLD-resistant isolates were INH resistant, 48.5% were RIF resistant, 38.5% were EMB resistant and 62.7% were STR resistant. Finally, among the samples, 47.3% were MDR.

Table 3.1. Distribution of the 260 isolates according to the FLD-resistance patterns and the *M. tuberculosis* families

Resistance type	Drug resistance	<i>M. tuberculosis</i> families				Total
		EAI	Beijing	T	Others	
Sensitive	Sensitive	20	14	11	10	55
Mono resistance	H	12	11	2	2	27
	R	0	0	0	2	2
	S	11	14	4	2	31
	E	0	1	0	0	1
Double resistance	HS	3	12	2	2	19
	HR	4	1	0	1	6
Triple resistance	HRS	5	11	1	3	20
	HRE	4	2	0	0	6
	RSE	0	1	0	0	1
	HSE	0	1	0	0	1
Quadruple resistance	HRSE	17	62	8	4	91
	Total	76	130	28	26	260

Sensitive: isolates susceptible to all the four FLDs, isoniazid, rifampicin, streptomycin and ethambutol; H: isoniazid, R: rifampicin, S: streptomycin, E: ethambutol.

B. SLD susceptibility testing

Among the 125 isolates tested, 29 (23.2%) isolates were resistant to at least one SLD in which 21 were resistant to at least one SLID, 19 were resistant to at least one FQ and 11 were resistant to both SLD classes (**Error! Reference source not found.**). Considering the FLD and SLD data together, sixteen (12.8%) and 11 (8.8%) isolates met criteria for pre-XDR and XDR, respectively.

20 different FLD and SLD-susceptibility patterns were identified (**Error! Reference source not found.**). The most complex drug-resistant pattern was the combination of 9 different drug resistances (4 FLDs and 5 SLDs) (9 isolates, 7.2%).

Table 3.2. FLD and SLD susceptibility testing profiles of the 125 isolates according to the *M. tuberculosis* families

Type of SLD resistance	Drug resistant patterns	<i>M. tuberculosis</i> families				Total
		EAI	Beijing	T	Others	
Sensitive	Pan-susceptible	5	8	4	3	20
Mono-FLD	H	5	2	0	1	8
	R	0	0	0	2	2
	S	6	4	0	2	12
Double-FLD	HS	0	3	0	0	3
Triple-FLD	HRS	0	8	0	2	10
	HRE	2	1	0	0	3
Quadruple-FLD	HRSE	7	28	2	1	38
Mono-SLID	SKAC	0	1	0	0	1
Mono-FQ	HOMG	0	1	0	0	1
Pre-XDR	HROM	0	0	0	1	1
	HRSKAC	1	0	0	0	1
	HRSEK	0	1	0	0	1
	HRSEC	0	1	0	0	1
	HRSEKA	0	0	0	1	1
	HRSEKAC	0	5	0	0	5
	HRSEOM	0	4	2	0	6
XDR	HRSEKOM	0	0	1	0	1
	HRSEKACO	1	0	0	0	1
	HRSEKACOM	2	6	1	0	9
	Total	29	73	10	13	125

Pan-susceptible: isolates susceptible to all the tested FLDs and SLDs; H: isoniazid resistant, R: rifampicin resistant, S: streptomycin resistant, E: ethambutol resistant; K: kanamycin resistant; A: amikacin resistant; C: capreomycin resistant; O: ofloxacin resistant; M: moxifloxacin resistant; G: gatifloxacin resistant.

C. Genotyping

Since the data were already used in paper 4, they are briefly described below:

A total of 101 different spoligotypes were identified (see Table A2 in appendix for details). Spoligotype data allowed assigning the majority of samples to

families using SITVITWEB. In addition, the 24-MIRU-VNTR typing could characterize 9 unknown spoligotypes as EAI, called here EAI-like. In total, 130 isolates were Beijing (50%), 76 isolates were EAI and EAI-like (29.2%), 28 isolates were T (10.8%). Besides 2 isolates were LAM, 1 isolate was H and 23 isolates were unknown, accounting in total for 10% (these isolates were classified in “Others” class). The distribution of each FLD and SLD resistant pattern according to the *M. tuberculosis* families is described in **Tables 3.1** and **3.2**.

The 24-locus MIRU-VNTR typing revealed 192 distinct genotypes including 163 unique patterns and 29 clusters represented by 97 isolates (containing 2 – 15 isolates/each, average=3.3) (see Table A2 in appendix for details). In Beijing family, 80 different genotypes were identified including 62 unique patterns and 18 clusters containing 2 – 15 isolates/each (average=3.8). In EAI family, 64 different genotypes were identified including 57 unique patterns and 7 clusters containing 2 – 5 isolates/each (average=2.7). For T family, 23 different genotypes were identified including 20 unique patterns and 3 clusters containing 2-3 isolates/each (average =2.7). In “Others” group, 25 different genotypes were identified including 24 unique patterns and one cluster containing 2 isolates.

D. Mutations associated with resistance to FLDs

The distribution of drug resistance-associated mutations according to the drug resistance patterns, as well as the specificity and sensitivity are described in the Error! Reference source not found..

Table 3.3. Number of isolates with and without genetic mutations according to drugs and sensitivity and specificity data between phenotypic and genotypic data

Drug	Locus/gene	Drug-resistant isolates		Drug-susceptible isolates		Comparison of phenotypic and genotypic data*	
		With mutation	Without mutation	With mutation	Without mutation	Sensitivity (%)	Specificity (%)
RIF	<i>rpoB</i>	125	1	2	132	99.2	98.5
	RRDR of <i>rpoB</i>	120	6	2	132	95.2	98.5
INH	<i>katG</i>	146	24	0	90	85.9	100
	<i>inhA</i> and its promoter	30	140	1	89	17.6	98.9
	<i>katG</i> , <i>inhA</i> and <i>inhA</i> promoter	165	5	1	89	97.0	98.9
STR	<i>rpsL</i>	101	62	0	97	62.0	100

	<i>rrs-F1</i>	34	129	0	97	20.9	100
	<i>rpsL</i> and <i>rrs-F1</i>	132	31	0	97	81.0	100
EMB	<i>embB</i>	87	13	0	160	87.0	100
OFX	<i>gyrA</i>	15	4	0	106	79.0	100
	<i>gyrB</i>	2	17	0	106	10.5	100
	<i>gyrA</i> and <i>gyrB</i>	17	2	0	106	89.5	100
KAN	<i>rrs-F2</i>	13	7	0	105	65.0	100
AMK	<i>rrs-F2</i>	13	5	0	107	72.2	100
CAP	<i>rrs-F2</i>	13	5	0	107	72.2	100
Double		19	6	0	55	76	100
Triple		22	6	0	55	78.6	100
Quadruple		72	19	0	55	79.1	100
MDR		120	3	0	55	97.6	100
Pre-XDR		12	4	0	20	75	100
XDR		5	6	0	20	45.5	100

*: The natural polymorphisms (*katG463*, *embB378* and *gyrA95*) and the mutations not associated with drug resistance were excluded.

It is worth noting that the comparison of phenotypic and genotypic data showed 81 to 99% of sensitivity and 98.5 to 100% of specificity, for detecting FLD-resistant isolates (**Error! Reference source not found.**).

❖ *INH resistance and mutations in katG and inhA genes*

A total of 165/170 (97.1%) INH-resistant isolates revealed INH resistance-associated mutations in either *katG* and/or *inhA* (**Error! Reference source not found.**). The INH resistance-associated mutations in *katG*, *inhA* and *inhA* promoter are described in the **Error! Reference source not found.**. The most frequent mutations were at *katG315* position (Asp-Asn (3.5%) and Asp-Thr (76.5%), total=80%) and at *inhA-15* (C-T, 15.9%), and the combination of the two positions covered 93.5% of INH-resistant isolates. Five out of 90 INH-sensitive isolates had either mutations in *katG* or in *inhA* gene, in which only *inhA21* (Ile-Val) mutation was previously associated with low-level of INH resistance (see Table A3 in appendix for details).

Table 3.4. The INH resistance-associated mutations found in *katG* and *inhA* genes and *inhA* promoter of the 170 INH-resistant isolates and their distribution according to the *Mycobacterium tuberculosis* families

Gene	Position(s)	Nucleotide change(s)	Amino acid change(s)	Family (number of isolates)				Total
				Beijing	EAI	T	Others	
<i>katG</i>	31	GAG-GAT	Glu-Asp	0	1	0	0	1
	112	ACC-ATC	Thr-Ile	0	1	0	0	1
	127	CAG-CCG	Gln-Pro	0	0	0	1	1
	169	GGC-AGC	Gly-Ser	0	1	0	0	1
	232	CCG-GCG	Pro-Ala	0	0	0	1	1
	257	ATG-ATA	Met-Ile	0	1	0	0	1
	315	AGC-AAC	Ser-Asn	2	2	0	2	6
	315	AGC-ACC	Ser-Thr	87	24	8	6	125
	325	CCG-CTG	Pro-Leu	0	1	0	0	1
	394	ACG-GCG	Thr-Ala	1	0	0	0	1
	439	CAG-CCG	Gln-Pro	0	1	0	0	1
	480*	Deletion GCG	Deletion Ala	0	0	0	1	1
	33 & 315	GGC-GAC & AGC-ACC	Gly-Asp & Ser-Thr	0	1	0	0	1
	315 & 430	AGC-ACC & CTG-CCG	Ser-Thr & Leu-Pro	1	0	0	0	1
	315 & 710	AGC-ACC & GTC-TTC	Ser-Thr & Val-Phe	3	0	0	0	3
No mutation	None	None	7	7	5	5	24	
TOTAL							170	
<i>inhA</i>	-8	T-C		1	1	0	0	2
	-15	C-T		6	9	1	3	19
	-15 & 21	C-T & ATC-GTC	Ile-Val	0	2	0	0	2
	-15 & 94	C-T & TCG-GCG	Ser-Ala	3	0	2	0	5
	-15 & 194	C-T &	Ile-Thr	1	0	0	0	1
	95	ATT-GTT	Ile-Val	0	1	0	0	1
	No mutation	None	None	90	26	10	14	140
	TOTAL							170

❖ *RIF resistance and mutations in the rpoB gene*

In total 125/126 (99.2%) RIF-resistant isolates harbored mutations in *rpoB* gene (**Error! Reference source not found.**). The RIF resistance-associated mutations in *rpoB* are described in the **Error! Reference source not found.** 120 (95.2%) out of the 126 RIF-resistant isolates had at least one mutation in the RRDR, 3.2% (4 isolates) had a mutation in cluster II and 0.8% (1 isolate) had a mutation in the N-terminal region of *rpoB*. No mutation was found in cluster III of this gene. The three most common mutations were located at codon *rpoB*531 (50%, 63 isolates), codon *rpoB*526 (24.6%, 31 isolates), codon *rpoB*516 (13.5%, 17 isolates), accounting for 87.3% of RIF resistant isolates (110 isolates). Mutations at position *rpoB*526 were the most variable with 7 different amino acid replacements (His-Asp/Tyr/Arg/Asn/Leu/Thr/Ser), in which His526Asp accounted for 12.7% of the RIF-resistant isolates. The *rpoB*531 mutations showed 4 different amino acid replacements (Ser-Leu/Trp/Phe/Gln), in which Ser531Leu was detected in 46.0% of the RIF-resistant isolates. Regarding position *rpoB*516, only two amino acid replacements (Ser-Val/Tyr) were detected and the Ser516Val was the most common, accounted for 12.7% of RIF-resistant isolates. Other mutations in the RRDR were detected at low frequency (total = 7.9%). The *rpoB*572 (Ile-Phe) mutation was the only mutation found in Cluster II, and the *rpoB*146 (Val-Phe) mutation was the only one found in the N-terminal region. Two (1.5%) out of the 134 RIF-sensitive isolates revealed the mutations Ser516Tyr or His526Asn previously linked to low-level of RIF resistance (see Table A3 in appendix for details).

Table 3.5. Mutations detected in the *rpoB* gene of the 126 RIF-resistant isolates and their distribution according to *M. tuberculosis* families

Position(s)	Nucleotide change(s)	Amino acid change(s)	Family (number of isolates)				Total
			Beijing	EAI	T	Others	
146	GTC-TTC	Val-Phe	1	0	0	0	1
511	CTG-CCG	Leu-Pro	1	1	0	0	2
513	CAA-CCA	Gln-Pro	0	1	0	0	1
516	GAC-GTC	Asp-Val	9	2	0	2	14
526	CAC-CGC	His-Arg	0	2	0	0	2
526	CAC-AAC	His-Asn	0	1	0	0	1
526	CAC-GAC	His-Asp	4	7	1	0	12

526	CAC-ACC	His-Thr	0	1	0	0	1
526	CAC-TAC	His-Tyr	4	1	1	1	7
526	CAC-CTC	His-Leu	0	0	0	1	1
531	TCG-CAG	Ser-Gln	0	2	0	0	2
531	TCG-TTG	Ser-Leu	38	8	4	5	55
531*	TCG-CTG	Ser-Leu	1	0	0	0	1
533	CTG-CCG	Leu-Pro	4	0	0	0	4
572	ATC-TTC	Ile-Phe	4	0	0	0	4
512	Insertion CGC	Insertion Arg	0	0	1	0	1
518	Deletion AAC	Deletion Asn	0	0	1	0	1
490 & 531	CAG-CGG & TCG-TGG	Gln-Arg & Ser-Trp	0	0	1	0	1
511 & 526	CTG-CCG & CAC-TAC	Leu-Pro & His-Asp	1	0	0	0	1
511 & 531	CTG-CCG & TCG-TTG	Leu-Pro & Ser-Leu	0	1	0	0	1
513 & 753	CAA-CTA & TCC-TTC	Gln-Leu & Ser-Phe	0	0	0	1	1
515 & 516	ATG-ATA & GAC-GTC	Met-Ile & Asp-Val	1	0	0	0	1
516 & 757	GAC-GTC & ACT-GCT	Asp-Val & Thr-Ala	1	0	0	0	1
516 & 518	GAC-TAC & AAC-CAC	Asp-Tyr & Asn-His	0	1	0	0	1
516 & 526	GAC-GTC & CAC-GAC	Asp-Val & His-Asp	1	0	0	0	1
522 & 531	TCG-TTG & TCG-TTG	Ser-Leu & Ser-Leu	1	0	0	0	1
526 & 527	CAC-TCC & AAG-CAG	His-Ser & Lys-Gln	1	0	0	0	1
526 & 533	CAC-AAC & CTG-TCG	His-Asn & Leu-Ser	1	0	0	0	1
526 & 533	CAC-GAC & CTG-CCG	His-Asp & Leu-Pro	1	0	0	0	1
526 & 562	CAC-GAC & GAA-GCA	His-Asp & Glu-Ala	1	0	0	0	1
530 & 531	CTG-ATG & TCG-TTC	Leu-Met & Ser-Phe	1	0	0	0	1
531 & 616	TCG-TGG & GAC-GCC	Ser-Trp & Asp-Ala	0	0	0	1	1
No mutation	None	None	0	0	0	1	1
TOTAL							126

❖ *EMB resistance and mutations in embB gene*

Eighty-eight (88%) out of the 100 EMB-resistant isolates revealed non-synonymous mutations in *embB*, among which 87% carried mutations previously associated with EMB resistance (**Error! Reference source not found.** and **Error! Reference source not found.**). The EMB resistance-associated mutations in *embB* are described in the **Table 3.6**. The most common mutation was found at *embB306*

position (60%, 60 isolates), followed by *embB497* (14%, 14 isolates) and *embB406* (8%, 8 isolates). Mutations at these positions covered 80% of EMB-resistant isolates. Other mutations were detected at low frequency (total = 7%). Five out of the 160 EMB-sensitive isolates revealed *embB* mutations never associated with EMB resistance (see Table A3 in appendix for details).

Table 3.6. Mutations found in the *embB* gene of the 100 EMB-resistant isolates and their distribution according to the *M. tuberculosis* families

Position(s)	Nucleotide change(s)	Amino acid change(s)	Family (number of isolates)				Total
			Beijing	EAI	T	Others	
306	ATG-ATA	Met-Ile	6	6	1	0	13
306	ATG-ATC	Met-Ile	2	1	0	1	4
306	ATG-ATT	Met-Ile	0	0	0	1	1
306	ATG-CTG	Met-Leu	1	0	0	0	1
306	ATG-GTG	Met-Val	27	2	4	0	33
330	TTC-TTA	Phe-Leu	1	1	0	0	2
330	TTC-TCC	Phe-Ser	1	0	0	0	1
354	GAC-GCC	Asp-Ala	0	1	0	0	1
360	GTG-ATG	Val-Met	1	0	0	0	1
406	GGC-AGC	Gly-Ser	1	1	1	0	3
406	GGC-GCC	Gly-Ala	1	0	0	0	1
406	GGC-GAC	Gly-Asp	1	1	0	1	3
450	ATC-CTC	Ile-Leu	1	0	0	0	1
497	CAG-CGG	Gln-Arg	7	1	0	0	8
497	CAG-CCG	Gln-Pro	1	0	0	0	1
306 & 360	ATG-ATA & GTG-ATG	Met-Ile & Val-Met	1	0	0	0	1
306 & 454	ATG-ATA & GCG-ACG	Met-Ile & Ala-Thr	1	0	0	0	1
306 & 497	ATG-ATA & CAG-AAG	Met-Ile & Gln-Lys	1	0	0	0	1
306 & 557	ATG-ATC & ATG-ATC	Met-Ile & Met-Ile	3	0	0	0	3
306 & 561	ATG-GTG & AAG-ACG	Met-Val & Lys-Thr	0	1	0	0	1
328 & 338	GAT-TAT & GCG-TCG	Asp-Tyr & Ala-Ser	1	0	0	0	1
354 & 497	GAC-GCC & CAG-AAG	Asp-Ala & Gln-Lys	0	0	1	0	1
354 & 497	GAC-GCC & CAG-CCG	Asp-Ala & Gln-Pro	0	0	0	1	1
370 & 497	CTG-CGG & CAG-AAG	Leu-Arg & Gln-Lys	1	0	0	0	1

497 & 557	CAG-CGG & ATG-ATC	Gln-Arg & Met-Ile	1	0	0	0	1
306 & 387 & 406	ATG-ATA & GCG-ACG & GGC-GCC	Met-Ile & Ala-Thr & Gly-Ala	0	0	0	1	1
No mutation	None	None	7	4	1	1	13
TOTAL							100

❖ *STR resistance and mutations in rpsL and rrs genes*

A total of 132 (81%) out of 163 STR-resistant isolates revealed mutations in *rpsL* and/or *rrs* (**Error! Reference source not found.**). The STR resistance-associated mutations in *rpsL* and *rrs* genes are described in the **Table 3.7**. In *rpsL* gene, mutations were only detected in two positions *rpsL*43 (39.3%, 64 isolates) and *rpsL*88 (22.7%, 37 isolates). In *rrs* gene, mutations were mainly detected in two positions, *rrs*514 (10.4%, 17 isolates) and *rrs*517 (5.5%, 9 isolates). Six other *rrs* mutations were also detected at low frequency (total = 4.5%). None of the 137 STR-sensitive isolates had mutations in *rpsL* gene, while one isolate carried a mutation never associated with SM resistance in *rrs* gene (see Table A3 in appendix for details).

Table 3.7. Mutations found in the *rpsL* and *rrs* genes of the 163 STR-resistant and their distribution according to the *M. tuberculosis* families

Gene	Position(s)	Nucleotide change(s)	Amino acid change(s)	Family (number of isolates)				Total
				Beijing	EAI	T	Others	
<i>rpsL</i>	43	AAG-AGG	Lys-Arg	54	3	5	1	63
	43	AAG-AAT	Lys-Asn	1	0	0	0	1
	88	AAG-AGG	Lys-Arg	28	3	0	1	32
	88	AAG-ATG	Lys-Met	2	1	1	0	4
	88	AAG-ACG	Lys-Thr	0	1	0	0	1
	No mutation	None	None	16	25	9	12	62
TOTAL							163	
<i>rrs</i> -F1	151	C-G		1	0	0	0	1
	239	C-T		0	1	0	0	1
	513	C-T		0	0	1	0	1
	514	A-C		11	4	1	1	17

517	C-T	1	6	0	2	9
878	G-A	0	2	0	0	2
905	C-G	0	1	0	0	1
908	A-C	1	1	0	0	2
No mutation	None	87	18	13	11	129
TOTAL						163

E. Mutations associated with resistance to SLDs and pre-XDR and XDR detection

❖ SLID resistance associated mutations

In total, 16 isolates carried SLID resistance-associated mutations of which 13 were resistant to KAN, AMK and CAP by DST and three did not have DST data (**Table 3.8**). The mutations were detected in the 1400-1500 region of *rrs* gene (*rrs*-F2 fragment), in which *rrs*1401(A-C) mutation was the most common (**Table 3.9**). The *rrs*1484 (A-T) mutation was detected in one SLID-resistant isolate and the *rrs*1001 mutation in one isolate in combination with *rrs*1401.

Table 3.8. Distribution of SLD resistant mutations among drug resistance patterns and mutation frequency

Drug resistance pattern	Gene	With mutation	Without mutation	Total	Mutation frequency (%)
KAN	<i>rrs</i> -F2	0	2	2	0
CAP		0	1	1	0
KAN & AMK		0	1	1	0
KAN, AMK & CAP		13	4	17	76.5
No DST		3	132	135	2.2
Sensitive		0	104	104	0
Total		16	244	260	6.2
OFX	<i>gyrA</i> & <i>gyrB</i>	1	0	1	100
OFX & MFX		15	2	17	88.2
OFX, MFX & GFX		1	0	1	100

No DST	8	127	135	5.9
Sensitive	0	106	106	0
Total	25	235	260	9.6

Table 3.9. Mutations detected in *gyrA* and *gyrB* genes and *rrs*-F2 fragment and distribution within the *M. tuberculosis* families

Drug	Gene	Mutation position(s)	Nucleotide change(s)	Amino acid change(s)	Family (number of isolates)				Total
					Beijing	EAI	T	Others	
SLIDs	<i>rrs</i> -F2	1001& 1401	T-C & A-G		1	0	0	0	1
		1401	A-G		9	5	0	0	14 ^a
		1484	G-T		1	0	0	0	1
FQs	<i>gyrA</i>	88	GGC-GCC	Gly-Ala	0	1	0	0	1
		90	GCG-GTG	Ala-Val	2	2	0	1	5 ^b
		94	GAC-GCC	Asp-Ala	2	0	0	0	2
		94	GAC-GGC	Asp-Gly	6	2	3	0	11 ^c
		94	GAC-TAC	Asp-Tyr	3	0	0	1	4 ^d
	<i>gyrB</i>	461	GAC-AAC	Asp-Asn	1	0	0	0	1
		504	GCG-GTG	Ala-Val	0	0	1	0	1

^a: included 3 isolates without DST results

^b: included 4 isolates without DST results

^c: included 3 isolates without DST results

^d: included 1 isolate without DST results

❖ FQ resistance associated mutations

In total 25 isolates out of 260 carried mutations associated with resistance to FQs, of which 17 were resistant by DST either to OFX or to OFX and MFX or to OFX, MFX and GFX and eight did not have any DST (**Table 3.8**). None of them was resistant to LFX. Twenty-three isolates carried mutation in *gyrA* gene while two had mutations in *gyrB* gene. The mutations associated with FQ resistance in *gyrA* and *gyrB* genes are described in the **Table 3.9**. In *gyrA* gene, mutations at position *gyrA*94 were the most frequent and the most polymorphic (3 different amino acid changes). Two other *gyrA* mutations at positions *gyrA*88 and *gyrA*90 were also

detected at low frequency. In *gyrB* gene, two mutations *gyrB461* and *gyrB504* were observed.

Globally, based on mutation detection, a total of 33 (12.7%) isolates were resistant to at least one of the SLDs in which 8 carried both FQ and SLID mutations. Thus, 2 isolates were genetically identified as mono-SLID or mono-FQ resistant, 23 as pre-XDR and 8 as XDR (**Table 3.10**).

Table 3.10. SLD resistances, pre-XDR and XDR identified by genetic mutations

Genotype	SLID-resistant mutation	FQ-resistant mutation	Total
Mono-SLID resistance	<i>rrs1401</i> (A-G)	WT	1
Mono-FQ resistance	WT	<i>gyrA94</i> (Asp-Tyr)	1
Pre-XDR	1001 (T-C) & 1401 (A-G)	WT	1
	<i>rrs1401</i> (A-G)	WT	5 ^a
	<i>rrs1484</i> (G-T)	WT	1
	WT	<i>gyrA90</i> (Ala-Val)	4
	WT	<i>gyrA94</i> (Asp-Val)	2 ^b
	WT	<i>gyrA94</i> (Asp-Gly)	6 ^c
	WT	<i>gyrA94</i> (Asp-Tyr)	2
	WT	<i>gyrB461</i> (Asp-Asn)	1
	WT	<i>gyrB504</i> (Ala-Val)	1 ^d
Total			23
XDR	<i>rrs1401</i> (A-G)	<i>gyrA88</i> (Gly-Ala)	1
	<i>rrs1401</i> (A-G)	<i>gyrA90</i> (Ala-Val)	1
	<i>rrs1401</i> (A-G)	<i>gyrA94</i> (Asp-Gly)	5
	<i>rrs1401</i> (A-G)	<i>gyrA94</i> (Asp-Tyr)	1
Total			8

^a: included 2 isolates identified as XDR by DST

^b: included 2 isolates identified as XDR by DST

^c: included 1 isolate identified as XDR by DST

^d: included 1 isolate identified as XDR by DST

The comparison of phenotypic and genotypic data showed more than 65% of sensitivity and 100% of specificity for detecting isolates resistant to at least one of the three SLIDs, and 76.5% of sensitivity and 100% of specificity for detecting isolates resistant to all the three SLIDs (**Table 3.3**). The sensitivity was 89.5% and specificity

was 100% for detecting FQ-resistant isolates (**Table 3.3**). Of the 16 pre-XDR isolates defined by DST, 12 carried mutations associated with resistance to SLIDs or to FQs. Among the 11 XDR isolates defined by DST, 5 were confirmed by genetic mutations, while the remaining 6 isolates were characterized as pre-XDR. Sensitivity between phenotypic and genotypic data for detecting pre-XDR and XDR were 75% and 45.5%, respectively and 100% specificity for both patterns (**Table 3.3**).

F. Link between drug resistant patterns and mutations

Regarding the INH-resistant patterns, frequency of *katG* mutations was significantly higher than frequency of *inhA* mutations ($p < 0.01$) (**Table 3.11**). Frequencies of *katG* and *katG315* mutations were higher in quadruple-drug resistant isolates, followed by triple-drug, double-drug and mono-drug resistant patterns, but these differences were not significant ($p > 0.05$) (**Table 3.11**). On the contrary, frequencies of *inhA* and *inhA-15* mutations were higher in mono-resistant isolates, followed by double, triple and quadruple-resistant isolates, but with no significant difference ($p > 0.05$) (**Table 3.11**). Altogether, the frequencies of INH resistance-associated mutations in both genes were not significantly different among INH-resistant patterns, and between MDR and non-MDR isolates (**Table 3.11**).

Table 3.11. Frequencies of specific mutations according to FLD drug resistance patterns (mono, double, triple and quadruple resistance, MDR and non-MDR)

Drug resistance	Gene/frequent mutation	Drug resistance patterns					
		Mono	Double	Triple	Quadruple	Non-MDR	MDR
INH	<i>katG</i>	77.8	80	85.2	90.1	83	87
	<i>katG315</i> (Ser-Thr)	70.4	72	81.5	84.6	78.7	80.5
	<i>inhA</i>	22.2	20	18.5	15.4	14.9	18.7
	<i>inhA-15</i> (C-T)	22.2	16	14.8	14.3	14.9	16.3
	<i>katG</i> & <i>inhA</i>	96.3	92	100	97.8	95.7	97.6
STR	<i>rpsL</i>	41.9	63.2	54.5	70.3	51.9	66.7
	<i>rpsL44</i> (Lys-Arg)	25.8	42.1	31.8	44	32.7	41.4
	<i>rpsL88</i> (Lys-Arg)	12.9	21.1	22.7	20.9	17.3	20.7
	<i>rrs</i>	19.4	15.8	27.3	20.9	17.3	22.5
	<i>rrs514</i> (A-C)	6.5	5.3	9.1	13.2	5.8	12.6
	<i>rpsL</i> & <i>rrs</i>	58.1	78.9	77.3	90.1	67.3	87.4

RIF	<i>rpoB</i>	100	83.3	100	100	100	99.2
	<i>rpoB531</i> (Ser-Leu)	50	50	44.4	46.2	33.3	46.3
EMB	<i>embB</i>	100	NA	87.5	87	100	87.6
	<i>embB306</i> (Met-Val)	100	NA	37.5	33	66.7	33
	<i>embB306</i> (Met-Ile)	0	NA	25	25.3	33.3	24.7

Mono: resistant only to one FLD (either isoniazid, rifampicin, streptomycin or ethambutol) among the 4 FLDs, double: resistant to two drugs, triple: resistant to 3 drugs; quadruple: resistant to 4 drugs.

The frequencies of mutations in *rpsL* or *rrs* or in both genes were not significantly different among the resistant patterns ($p>0.05$), except between quadruple-FLD resistant isolates and mono resistant isolates ($p<0.01$) (**Table 3.11**). Furthermore, the frequencies of mutations in both genes were significantly higher in MDR isolates than in non-MDR ones ($p<0.01$) (**Table 3.11**).

Mono and double resistant patterns (non MDR) were rarely associated with EMB or RIF resistance; in our sample only 9 isolates were detected. Almost all RIF-resistant or EMB-resistant isolates were MDR (**Table 3.11**). Thus, globally the frequencies of mutations associated with RIF or EMB resistance between the patterns were not significantly different ($p>0.05$) (**Table 3.11**).

The frequency of SLD resistance-associated mutations was significantly higher in quadruple-FLD resistance, compared to all other FLD-resistant patterns ($p<0.007$) and was significantly different between MDR and non-MDR isolates ($p<0.02$) (**Table 3.12**).

Table 3.12. Frequencies of SLD-resistant mutations according to FLD-resistant patterns (sensitive, mono, double, triple and quadruple resistance and Odds Ratio estimation).

FLD resistant pattern	N° of isolates		Frequency of mutations	Odds ratio, 95% CI	p-value
	With mutation	Without mutation			
Sensitive	0	55	0%		
Mono resistance	2	59	3.3%	4.7, 0.2 to 99.3	$p>0.3$
Double resistance	2	23	8%	11.8, 0.5 to 255.5	$p>0.1$
Triple resistance	1	27	3.6%	6.1, 0.2 to 153.5	$p>0.2$
Quadruple resistance	28	63	30.8%	49.8, 2.8 to 835.1	$p<0.007$

MDR	31	92	25.2%	37.8, 2.3 to 630.1	p<0.02
Non MDR	2	135	1.5%	3.4, 0.2 to 73.2	p>0.4

G. Mutations and Mycobacterium tuberculosis families

The frequency of *katG* mutations was significantly higher in Beijing (94%) than in EAI (80%), “Others” (66.7%) and T (61.5%) families (p<0.02) (**Table 3.13**) as well as the frequency of *katG*315 (Ser-Thr) mutation (p<0.01) (**Table 3.14**). Conversely, the frequency of *inhA* mutations was higher in EAI (33.3%) than in T (23.1%), “Others” (16.7%) and Beijing (10%), but a significant difference was obtained only between EAI and Beijing (p<0.01) (**Table 3.13**). The frequency of *inhA*-15 (C-T) mutations was significantly different only between EAI (28.9%) and Beijing (9%) families (p<0.005) (**Table 3.14**). Overall, the frequency of INH resistance-associated mutations in both genes was highest in Beijing family (100%), and was significantly different compared to T family and “Others” class (p<0.02) (**Table 3.13**).

The frequencies of *rpoB* mutations were not significantly different between the families (**Table 3.13**) (p>0.05), as well as the frequencies of the three most common mutations *rpoB*531 (Ser-Leu), *rpoB*526 (His-Asp) and *rpoB*516 (Asp-Val) (**Table 3.14**). Nevertheless, *rpoB*531 (Ser-Leu) was mainly found in T and Beijing, *rpoB*526 (His-Asp) was mainly found in EAI, and *rpoB*516 (Asp-Val) was mainly detected in “Others” class (**Table 3.5**). The RIF resistance-associated mutations outside the RRDR of *rpoB* were only found in Beijing family (**Table 3.5**).

Table 3.13. Frequency of mutations according to *M. tuberculosis* families

Drug	Locus/gene	<i>M. tuberculosis</i> families											
		EAI			Beijing			T			Others		
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)
RIF	<i>rpoB</i>	30	0	100	77	0	100	9	0	100	9	1	90
	RRDR of <i>rpoB</i>	30	0	100	72	5	93.5	9	0	100	9	1	90
INH	<i>katG</i>	36	9	80	94	6	94	8	5	61.5	8	4	66.7
	<i>inhA</i> and its promoter	15	30	33.3	10	90	10	3	10	23.1	2	10	16.7
	<i>katG</i> + <i>inhA</i> and <i>inhA</i> promoter	44	1	97.8	100	0	100	11	2	84.6	10	2	83.3
STR	<i>rpsL</i>	8	28	22.2	85	16	84.2	6	9	40	2	9	18.2
	<i>rrs</i> -F1	17	19	47.2	14	87	13.9	2	13	13.3	1	10	90.1
	<i>rpsL</i> + <i>rrs</i> -F1	24	12	66.7	97	4	96	8	7	53.3	3	8	27.3
EMB	<i>embB</i>	17	4	81	60	7	89.6	7	1	87.5	3	1	75
OFX	<i>gyrA</i>	2	1	66.7	9	2	81.8	3	1	75	1	0	100
	<i>gyrB</i>	0	3	0	1	10	9.1	1	3	25	0	1	0
	<i>gyrA</i> and <i>gyrB</i>	2	1	66.7	10	1	90.1	4	0	100	1	0	100
KAN, AMK, CAP	<i>rrs</i> -F2	4	0	100	9	5	64.3	0	2	0	0	1	0

Table 3.14. Distribution of the most frequent mutations associated with FLD resistance according to the *M. tuberculosis* families

Drug	Frequent mutations	<i>M. tuberculosis</i> families											Total with mutation (%)	
		EAI			Beijing			T			Others			
		With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation	Mutation frequency (%)	With mutation	Without mutation		Mutation frequency (%)
RIF	<i>rpoB</i> 531 (Ser-Leu)	10	20	33.3	40	37	51.9	4	5	44.4	4	6	40	58 (46)
	<i>rpoB</i> 526 (His-Asp)	7	23	23.3	8	69	10.4	1	8	11.1	0	10	0	16 (12.7)
	<i>rpoB</i> 516 (Asp-Val)	2	28	66.7	12	65	15.6	0	9	0	2	8	20	16 (12.7)
INH	<i>katG</i> 315 (Ser-Thr)	27	18	60	91	9	91	8	5	61.5	4	8	33.3	130 (76.5)
	<i>inhA</i> -15 (C-T)	13	32	28.9	9	91	9	3	10	23.1	2	10	16.7	27 (15.9)
STR	<i>rpsL</i> 43 (Lys-Arg)	3	33	8.3	54	47	53.5	5	10	33.3	1	10	9.1	63 (38.7)
	<i>rpsL</i> 88 (Lys-Arg)	3	33	8.3	28	73	27.7	0	15	0	1	10	9.1	32 (19.6)
	<i>rrs</i> 514 (A-C)	5	31	13.9	11	90	10.9	1	14	6.7	1	10	9.1	18 (11)
EMB	<i>embB</i> 306 (Met-Val)	3	18	14.3	27	40	40.3	4	4	50	0	4	0	34 (34)
	<i>embB</i> 306 (Met-Ile)	9	12	42.9	14	53	20.9	1	7	12.5	1	3	33.3	25 (25)
OFX	<i>gyrA</i> 94 (Asp-Gly)	1	2	33.3	4	7	36.4	3	1	75	0	1	0	8 (42.1)
KAN, AMK, CAP	<i>rrs</i> 1401 (A-G)	4	0	100	8	6	57.1	0	2	0	0	1	0	12 (60)

The frequency of *rpsL* mutations was significantly higher in Beijing isolates than in all other families ($p < 0.01$) (**Table 3.13**). Nevertheless, the frequency of *rpsL43* (Lys-Arg) mutation was significantly higher in Beijing than in EAI and “Others” isolates ($p < 0.03$) (**Table 3.14**). Conversely, the frequency of *rrs* mutations was significantly higher in EAI family than in all other families ($p < 0.03$) (**Table 3.13**), although no mutation was significantly more represented (**Table 3.14**). Overall, the frequency of STR resistance-associated mutations in both genes was significantly higher in Beijing isolates ($p < 0.02$) (**Table 3.13**).

The frequencies of *embB* mutations were not significantly different between the families ($p > 0.05$) (**Table 3.13**) as well as the frequencies of mutations at the three main positions *embB306*, *embB406* and *embB497*. Nevertheless, *embB306* (Met-Ile) mutation was mainly detected in EAI and “Others” class, whereas *embB306* (Met-Val) was mainly detected in Beijing and T families (**Table 3.6**).

The *rrs* mutations associated with resistance to SLIDs were only found in Beijing and EAI families (**Table 3.13**). Regarding FQ resistance, the frequencies of FQ resistance-associated mutations were not significantly different between the families (**Table 3.14**). For the prevalent mutations, no significant difference was obtained between the families.

Among the 27 pre-XDR and XDR isolates identified by DST, the most dominant family was Beijing (17/27), followed by EAI (4/27), T (4/27) and “Others” families (2/27) (see Table A3). In Beijing, 8/11 pre-XDR isolates had mutations associated with resistance either to SLIDs or to FQs, while 3/6 XDR isolates had mutations in both genes associated to FQs and to SLIDs. In EAI family, mutation detection allowed confirming the pre-XDR isolate defined by DST, while 2/3 XDR isolates determined by DST were genetically confirmed with the remaining one genetically characterized as pre-XDR. For T isolates, 2/2 pre-XDR determined by DST were confirmed by mutations, but the 2 XDR isolates defined by DST were genetically characterized as pre-XDR. In “Others” class, only 1/2 pre-XDR isolates was confirmed by mutation detection.

3.3.2.8. Compensatory mutations in *rpoA* and *rpoC* gene

Four out of the 126 RIF-resistant isolates had 4 different mutations in *rpoA* gene (Table 3.15). These isolates carried *rpoB* mutations at codon 531 (Ser-Leu, n=3) and codon 572 (Ile-Phe, n=1). All these isolates were quadruple drug resistant and one was characterized as pre-XDR by both DST and mutation detection.

Table 3.15. Compensatory mutations found in *rpoA* and *rpoC* genes in RIF-resistant isolates and distribution according to the *M. tuberculosis* families

Gene	Mutation position	Nucleotide change	Amino acid change	<i>M. tuberculosis</i> families				Total
				Beijing	EAI	T	U	
<i>rpoA</i>	181	ACC-GCC	Thr-Ala	1	0	0	0	1
	183	GTC-GCC	Val-Ala	1	0	0	0	1
	187	ACC-GCC	Thr-Ala	1	0	0	0	1
	202	ATC-ACC	Ile-Thr	1	0	0	0	1
<i>rpoC</i>	416	AAC-AGC	Asn-Ser	2	0	0	0	2
	448	GCG-GAG	Ala-Glu	2	0	1	0	3
	452	TTC-TCC	Phe-Ser	1	0	0	0	1
	483	GTG-GGG	Val-Gly	4	0	0	0	4
	483	GTG-GCG	Val-Ala	1	0	0	0	1
	485	GAT-AAT	Asp-Asn	1	0	0	0	1
	491	ATC-GTC	Ile-Val	0	1	0	0	1
	525	CAC-AAC	His-Asn	1	0	0	0	1
	542	GCC-GCG	Ala-Ala	0	0	0	1	1
	566	CTG-GTG	Leu-Val	0	1	0	0	1
	WT			61	26	8	11	106
Total			77	28	9	12	126	

Regarding *rpoC* gene, 16/126 RIF-resistant isolates carried 10 different mutations (Table 3.15). The most common mutations were detected at codons 483 (n=5) and 448 (n=3). Of these 16 isolates, 15 had *rpoB* mutations, mainly at codon 531 (Ser-Leu, n=11), followed by codon 526 (His-Tyr, n=2; His-Arg, n=1) and 146 (Ile-Val, n=1). Nevertheless, other drug resistance-associated mutations were also detected including *katG*315 (n=15), *rpsL*43 (n=7), *rpsL*88 (n=5), *embB*306 (n=10),

pncA (n=10), *rrs1401* (n=3), *rrs1484* (n=1), *gyrA90* (n=1) and *gyrA94* (n=1). All the 16 isolates carrying compensatory mutations in *rpoC* gene were MDR, in which 7 were quadruple drug resistant and 6 were pre-XDR or XDR by DST.

In brief, compensatory mutations in either *rpoA* or *rpoC* were detected in 20/126 (15.9%) of RIF-resistant isolates. These mutations were found in all MDR isolates, in which 7 were pre-XDR or XDR by DST, mutation detection or both. The compensatory mutations were mainly found in isolates carrying mutations in the RRDR of *rpoB*, but also detected in isolates with mutations in the Cluster II and N-terminal region of this gene. Of the RIF-resistant mutants carrying compensatory mutations, a high proportion of *rpoB531* mutants (14/20, 53.8%) was detected.

Among these 20 mutants, the majority was Beijing (16/20), followed by EAI (n=2), T (n=1) and “Others” class (**Table 3.15**).

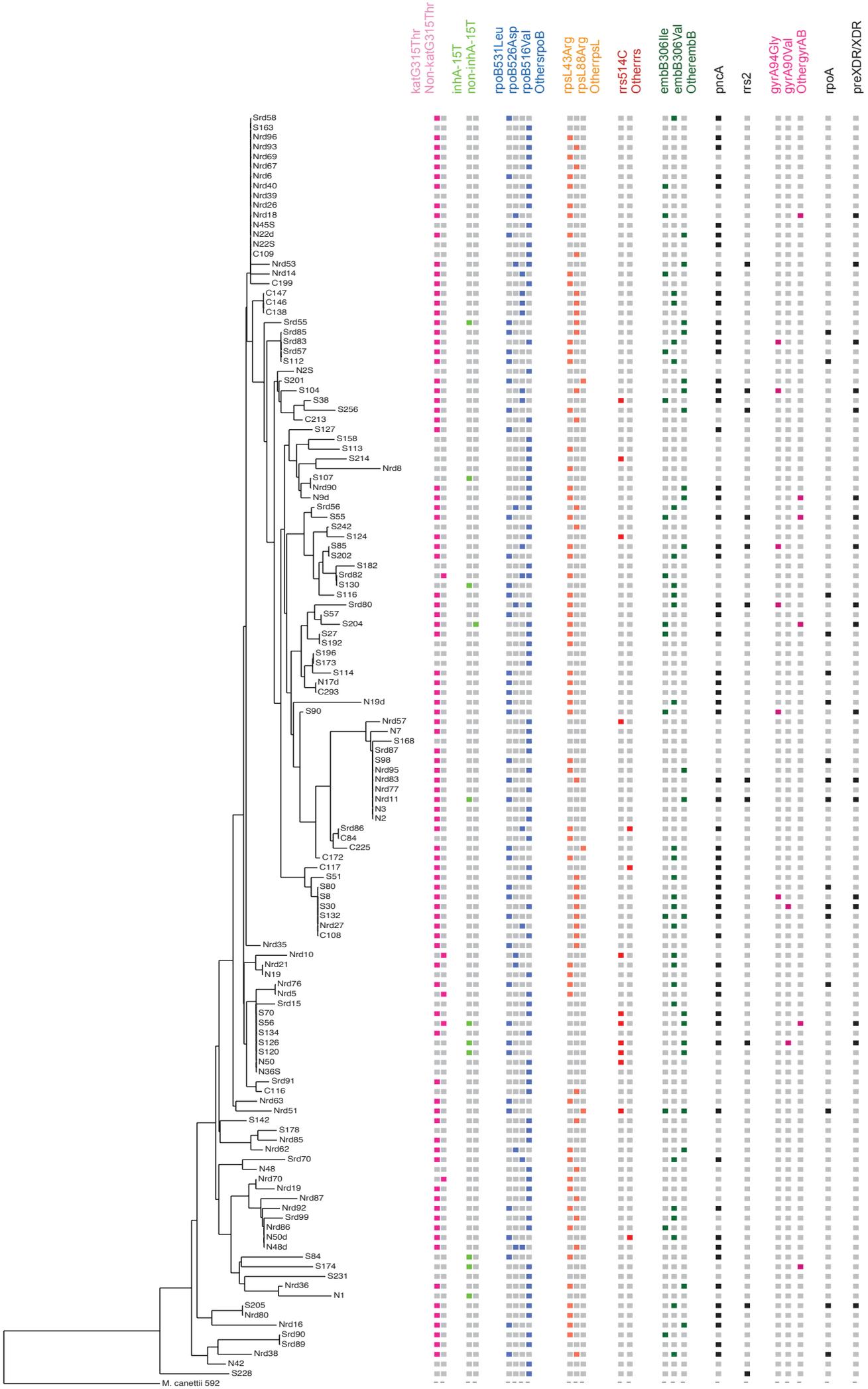
H. Compensatory mutations in *ahpC* gene of INH-resistant isolates

Only 2/170 INH-resistant isolates carried an *ahpC* mutation (C-51T) in promoter region (see Table A3). Nevertheless, only one isolate was mono-INH resistance and the remaining ones was INH-sensitive, suggesting this mutation may not act as compensatory mutation for INH-resistant mutants.

I. MIRU-VNTR polymorphism and drug resistance patterns

❖ Association of genotypes and drug-resistant phenotypes

As expected, phylogenetic tree based on MIRU-VNTR data clearly showed three main clades corresponding to the three main *M. tuberculosis* lineages including lineage 1 (mainly EAI family), lineage 2 (Beijing family) and lineage 4 (T, LAM and Unknown families) (**Figure 3.1**). In each MIRU-VNTR clades, sub-clades and clusters various patterns of resistance from sensitive to XDR were observed.



❖ MIRU-VNTR clusters and phenotypic and genotypic drug resistant data

Mapping drug resistance-associated mutations on phylogenetic tree globally showed that the FLD and SLD mutations were distributed in the MIRU-VNTR tree without specific structuring. Moreover, the combinations of mutations were diverse within clades (see phylogenetic tree, **Figure 3.1**) and the most common combinations of mutations such as *katG315Thr-rpoB531Leu* were dispersed along the phylogenetic tree. These data suggest multiple events of drug resistant acquisition of mutations even in a same cluster (see phylogenetic tree **Figure 3.1** and Table A3 for details).

The high diversity of mutation patterns and the combination of mutations within each family, each MIRU-VNTR clades and clusters suggest various evolutionary trajectories towards FLD and SLD resistances as observed in QDR isolates (see paper 3). It is worth noting that the 20 RIF-resistant mutants (mainly belonging to Beijing family) carrying fitness-compensatory mutations were found in 15 different genotypes. Of these genotypes, 12 were unique patterns and 3 were clusters containing 2-3 isolates/each (8 isolates). Moreover, all these 20 isolates revealed unique drug-resistance mutation patterns.

J. PhyloTypes and drug resistance-associated mutations

Although our sample revealed high diversity of mutation patterns, the PhyloType analysis revealed some clades sharing specific mutation patterns (phylotypes) (**Table 3.16**). Nevertheless, the majority of these phylotypes consisted of only 2 isolates. The biggest phylotype (*rpsL43Arg*, STR resistance) consisted of 32 Beijing isolates. This STR resistant phylotype was composed of different patterns of resistance (single, double, triple, quadruple resistant, pre-XDR and XDR) and various Beijing genotypes. These data suggest that a Beijing phylotype associated with high level of STR resistance are spreading in Vietnam. The other most represented phylotypes were characterized by the following mutations: *rpsL88Arg*, *katG315Thr* & *rpsL43Arg*, *katG315Thr* & *rpsL88Arg*, *katG315Thr* & *embB306Val*. These mutations are known to be associated to high level of drug resistance and low fitness costs. Nevertheless, because of the high diversity of MIRU-VNTR genotypes, the high diversity of mutations and the high diversity of drug resistant patterns, the sample size appeared too small to detect and validate these phylotypes.

Table 3.16. Phylotypes linked to specific drug resistance-associated mutations. Each phylotype is characterized by the number of isolates, the drug resistance phenotype and the *Mycobacterium tuberculosis* family.

Type of resistance	Drug	Drug-resistant mutations	No of isolates	Phenotype	Family
Single	INH	katG315Thr & katG710Phe	3	HRS, HRSE	Beijing
		inhA-15T & inhA21Val	2	HRSE	EAI
		inhA-15T & inhA21Val	2	HR, HRSE	EAI
		inhA-15T & inhA94Ala	2	HRSE	Beijing
		inhA-15T & inhA94Ala	2	HRSE	T
	RIF	rpoB516Val	3	HRS, HRSE	Beijing
	STR	rpsL43Arg	32	S, HS, HRS, HRSE	Beijing
		rpsL88Arg	11	HRS, HRSE	Beijing
		rrs514C	5	S, HRSE	Beijing
		rrs878A	2	HRSE	EAI
	EMB	No	No	No	No
	PZA	pncA96Glu	2	HRSE	EAI
		pncA161 insertion(G)	2	HRSE	T
		pncA115Gly	2	HR, HRSE	EAI
SLIDs	rrs1401G	2	HR, HRSE	EAI	
FQs	gyrA94Gly	2	HRSE	T	
Double	HS	inhA-15T & inhA94Ala & rpsL43Arg	2	HRSE	T
		katG315Asn	2	HRS	U
		katG315Asn & rrs878C	2	HRSE	EAI
		katG315Thr & rrs517T	2	HRS, HRSE	EAI
		katG315Thr & katG710Phe & rpsL88Arg	3	HRS, HRSE	Beijing

		katG315Thr & rpsL43Arg	9	HS, HRS, HRSE	Beijing
		katG315Thr & rpsL88Arg	7	HRS, HRSE	Beijing
HR		katG315Thr & rrs517T	2	HRS, HRSE	EAI
		inhA-15T & inhA21Val & rpoB531Leu	2	HR, HRSE	EAI
		inhA-15T & inhA94Ala & rpoB531Leu	2	HRSE	T
		katG315Asn & rpoB516Val	2	HRS	U
		katG315Asn & rpoB516Val	2	HRSE	EAI
		katG315Thr & katG710Phe & rpoB516Val	3	HRS, HRSE	Beijing
HE		katG315Thr & embB306Val	3	HSE, HRSE	Beijing
		katG315Thr & embB306Val	7	HRSE	Beijing
RE		rpoB531Leu & embB306Val	2	HRSE	T
		rpoB531Leu & embB306Val	2	HRSE	Beijing
SR		rpoB516Val	2	HRS	U
		rpsL88Arg & rpoB516Val	3	HRS, HRSE	Beijing
		rpsL88Arg & rpoB531Leu	3	HRSE	Beijing
		rrs878A & rpoB516Val	2	HRSE	EAI
SE		rpsL88Arg & embB306Val	3	HRSE	Beijing
Triple	HRS	inhA-15T & inhA94Ala & rpsL43Arg & rpoB531Leu	2	HRSE	T
		katG315Asn & rpoB516Val	2	HRS	U
		katG315Asn & rrs878C & rpoB516Val	2	HRSE	EAI
		katG315Thr & katG710Phe & rpsL88Arg & rpoB516Val	3	HRS, HRSE	Beijing
		katG315Thr & rpsL88Arg & rpoB531Leu	3	HRSE	Beijing
HRE		inhA-15T & inhA94Ala & rpoB531Leu & embB306Val	2	HRSE	T
HSE		inhA-15T & inhA94Ala & rpsL43Arg & embB306Val	2	HRSE	T
		katG315Thr & rpsL88Arg & embB306Val	3	HRSE	Beijing
RSE		rpsL43Arg & embB306Val & rpoB531Leu	2	HRSE	T

		rpsL43Arg & embB306Val & rpoB531Leu	2	HRSE	Beijing
Quadruple	HRSE	inhA-15T & inhA94Ala & rpoB531Leu & rpsL43Arg & embB306Val	2	HRSE	T
pre-XDR/XDR		inhA-15T & inhA21Val & rpoB531Leu & rrs1401G & gyrA94Gly	2	HR, HRSE	EAI

3.4.3 Discussion

Mutation detection, FLD and SLD resistance towards pre-XDR and XDR

In this study, we detected high frequencies (81-99%) of mutations responsible for resistance to FLDs, with the highest for RIF resistance and the lowest for STR resistance. The sensitivity (97-99%) and specificity (>98.5%) were very high for INH and RIF resistances, consistent with many previous studies supporting the usefulness of molecular-based diagnostic tools for rapid detection of RIF-resistance and MDR (183, 207, 345-347). It is worth noting that about 10% of the drug resistant isolates from our sample could not be detected by the two diagnostic kits endorsed by WHO, the MTBDRplus LPA and Xpert MTB/RIF. Indeed these isolates did not carry the targeted “mutations” (1, 24, 324). The drug resistant isolates without drug resistance-associated mutations suggest other mechanisms such as drug efflux pumps or the involvement of other resistance genes (87, 95).

Concerning the SLD resistance, we found 9.6% of FQ resistant isolates with mutations and 6.1% of SLID resistant ones in the whole sample. Indeed, a high frequency of FQ-resistant isolates carried mutations in *gyrA* or *gyrB* genes, consistent with previous studies (6, 183, 227, 228, 346, 347). The mutations in these genes have been reported to be often associated with high level of OFX resistance (6, 227, 228). Nevertheless, these mutations are generally associated with low level of resistance to the last generation of FQs such as LVX, MFX and GFX (227, 228). Conversely, in previous studies, the frequency of mutations in SLID resistant isolates was lower than for the other drug resistances and showed cross-resistance between SLIDs (183, 207, 345-347). Nevertheless, the cross-resistance was incomplete since we also detected mono and double SLID resistance TB cases. The mutations conferring high level of SLID resistance were detected only in isolates resistant to all the three SLIDs (KAN, AMK and CAP). Finally, our study showed high proportion of pre-XDR and XDR by both phenotypic DST and mutation detection. Nevertheless, because of a low sensitivity for SLID resistance detection (<76%), the genetic characterization allowed to identify only 50% of XDR isolates. This finding underlines the necessity to improve knowledge on the genetic bases of SLID resistance and thus XDR.

It is worth noting that 100% specificity was obtained for all SLDs supporting the LPA test (GenoType MTBDRsl) as initial test for rapid detection of SLD resistance in high TB burden settings (27, 348). Finally, since some resistant isolates did not carry any mutation, additional resistance mechanisms might be involved and thus need to be further investigated in order to improve molecular diagnosis of pre-XDR and XDR resistant strains (87, 183, 203, 213, 346).

Evolutionary factors and drug resistance patterns

❖ Diversity of mutations and cumulative effect of drug resistant mutations

Despite the high diversity of mutations observed in our sample, the ones commonly described worldwide (87, 88) were also detected at high frequency. Furthermore, our study clearly showed a link between the phenotypic drug-resistant patterns and the type of mutations. The highly drug-resistant isolates (quadruple resistant isolates, pre-XDR and XDR) were generally associated with the most common mutations and linked to high level of drug resistance, as well as for the MDR isolates compared to the non MDR isolates. As example, the frequency of *katG/katG315* mutation was the highest in the quadruple-FLD resistant isolates, while the frequency of *inhA/inhA-15* mutation was more represented in the mono-INH resistant isolates. Furthermore, the data showed that the quadruple FLD resistant isolates carried higher proportion of mutations associated to SLD resistance than the other FLD drug resistance patterns. This phenomenon has been previously described as a cumulative effect of drug resistant mutations (277, 280).

❖ Epistasis

Despite the high variability of mutations, our data on the whole sample as well as the results described in paper 3 showed specific associations of mutations. This is in agreement with studies that showed high frequencies of specific combinations of mutations (such as *rpsL43/katG315*, *rpsL43/rpoB531*, and *rpoB531/katG315*, or *gyrA94* with one *rpoB* mutation). These mutations were generally associated with high level of drug resistance and low fitness cost (232, 264). From these works, authors suggest the existence of epistatic interactions between drug resistance associated genes or between specific mutations. In our study, 32% of the pre-XDR and XDR isolates harbored the *gyrA94* (Asp-Gly) mutation associated either with the

mutation *rpoB531* (Ser-Leu) or *rpoB516* (Asp-Val). Moreover, the combinations of at least two frequent mutations including *rpoB531*, *katG315*, *rpsL43*, *embB306* and *gyrA94* were often detected. These findings support the existence of epistasis phenomena in agreement with the data obtained in QDR study (see paper 3).

❖ **Fitness cost and high level of drug resistance**

As mentioned in the literature, the acquisition of drug resistance generally leads to fitness cost and low transmission rate. Nevertheless, in *M. tuberculosis*, the common mutations are usually associated with low fitness cost, high level of drug resistance, and successful transmission in human population (83, 120-123, 191, 207, 230, 231, 281, 346). For instance, over 85% of INH-resistant isolates carried *katG315* (Ser-Thr) mutation that was not associated with reduced virulence and fitness cost (83, 120, 121, 281, 282, 323). A recent study showed that INH-resistant isolates carrying *katG315* mutation were spreading in Vietnam (323) in agreement with our study. In addition, the main mutations in our sample are detected at positions *rpoB531*, *rpsL43*, *gyrA94* and *rrs1401* and these mutations were previously associated with high level of RIF, STR, FQ and SLID resistances (6, 130, 131, 190, 228, 346) and low or no fitness cost (122, 230, 296). Since, these mutations were more specifically represented in MDR, quadruple-FLD resistant, pre-XDR and XDR isolates, this suggests that these mutations are favored and fixed in drug-resistant *M. tuberculosis* population in Vietnam.

❖ **Compensatory mutations**

Besides the low fitness cost characteristics of certain mutations, some other mutations called compensatory mutations are able to restore the strain fitness in *M. tuberculosis* (296, 349). In the clinical samples collected in Vietnam, we found high proportion (15.9%) of MDR isolates carrying fitness-compensatory mutations associated with RIF resistance. In these MDR isolates carrying compensatory mutations, high proportion (35%) was pre-XDR or XDR. These data are in agreement with the study of Comas *et al.* in which 27.1% of MDR samples isolated during an epidemic in South Africa having a mutation in the RRDR of *rpoB* also carried a fitness-compensatory mutation in *rpoA* or *rpoC*. Subsequent studies from South Africa, Russia and China have also found high proportion of compensatory mutations

in MDR and XDR samples (83, 307, 311). These findings underline that fitness-compensatory mutations are associated with high transmission of MDR and XDR in high MDR-TB burden settings. It is worth noting that, in our sample the compensatory mutations were acquired independently and do not belong to the same epidemics (15 different genotypes). This situation may suggest that the acquisition of compensatory mutations is also favored by epistatic interactions. This is in agreement with several studies that showed that epistasis phenomena also occur between compensatory mutations and drug resistance-associated mutations (311, 315). The high proportion of compensatory mutations found in our samples underlines the likelihood of successful transmission of MDR, pre-XDR and XDR in Vietnam. Nevertheless, no compensatory mutations associated with INH resistance was detected. This result can be explained by the fact that the majority of INH resistance-associated mutations found in our samples were *katG315* and *inhA-15* known to produce no fitness cost (123, 350).

❖ Genetic background

The analyses of gene sequences revealed that the distribution of mutations depends on *M. tuberculosis* families. More specifically, the mutations known to be associated with high level of drug resistance and low fitness cost appeared more specifically associated with Beijing family as described in the literature (6, 83, 190, 191, 280, 281). The mutations such as *katG315* (INH resistance) or *rpsL43* (STR resistance) were strongly associated with Beijing family, compared to other families. On the contrary, mutations associated with low level of drug resistance such as *inhA/inhA-15* (INH resistance) and *rrs* (STR resistance) mutations were strongly associated with EAI family, also consistent with previous studies (118, 280). Furthermore, Beijing family was strongly associated with each drug resistance patterns and particularly MDR, quadruple-resistant, pre-XDR and XDR, consistent with global reports (77, 83, 207, 280, 345, 351). Previous studies showed that Beijing strains have a higher global mutation rate compared to other families that could lead to a greater capacity of drug resistance-associated mutation acquisition (65, 352). In addition, a recent study showed that compensatory mutations of RIF resistance are strongly associated with transmission of MDR Beijing strains (311).

❖ **Various evolutionary pathways toward highly drug-resistant**

The genotypic diversity linked to the diversity of DST patterns and of mutation patterns suggests that the *M. tuberculosis* isolates collected in Vietnam can follow various evolutionary pathways to become highly drug resistant (quadruple, pre-XDR and XDR), as described in paper 3 focused on quadruple drug resistance isolates. Nevertheless, it appears that for all drugs, the mutations associated with high level of drug resistance and low fitness cost or compensatory mutations were mainly selected and fixed in the population. This can explain why specific mutations such as *katG315*, *rpoB531*, *rpsL43*, *rrs1401* and *gyrA94* as well as the compensatory mutations were principally detected in the highly drug resistant isolates. These worrying combinations are the results of multiple evolutionary factors, such as the cumulative effect of drug resistance mutations and epistasis.

Chapter 4. GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

4.1 General discussion

In this study, several specific aspects were investigated including the level of drug resistance, the genetic determinants associated with resistance to FLD and SLD, the genetic processes responsible for drug resistance and the evolutionary pathways that follow the isolates from sensitive status to become MDR, quadruple drug resistant, pre-XDR or XDR. Finally, the data permitted to explore the epidemiological issues of drug resistance and to evaluate the risk of drug resistance increase in Vietnam. Here, the major findings are discussed as well as the potential implications that could have these findings for diagnosis, detection of drug resistance and TB control in general.

4.1.1 PZA and SLD resistance, and drug-resistant TB control

This study, for the first time estimated the level of PZA resistance in clinical *M. tuberculosis* samples in Vietnam. Molecular analysis revealed high proportion (38%) of drug resistant isolates carrying highly confident PZA resistance-associated mutations, more particularly detected in MDR and quadruple-FLD resistant isolates (>72%). This finding is consistent with several studies in high MDR-TB burden countries (353-355). This situation critically challenges the use of this key-drug in TB treatment without drug resistance testing. In addition, this study showed high levels of SLD resistance and more specifically high proportion of pre-XDR and XDR by both phenotypic DST and mutation detection. It is worth noting that since these samples were collected before the implementation of the PMDT in Vietnam, this finding suggests, first that the SLD resistance situation was already complicated before the application of the official guidelines for the use of SLDs in MDR-TB treatment. Second that the SLD were used before the PMDT without any guidelines (318). For FQ resistance, the mutations detected were known to be associated with high level of OFX resistance but with low level of resistance to the last generation of FQs such as LVX, MFX and GFX (227, 228, 233, 356). This suggests that even if we have a high

frequency of mutations linked to FQ resistance, the use of last generation of FQs is optimal as recommended in the PMDT. Indeed, OFX will be effective on FQ sensitive isolates and LVX/MFX on OFX resistant cases. Regarding SLID resistance, we detected 16 isolates (7.8% of the drug resistant isolates) with mutations conferring high level of resistance to all the three SLIDs under study (KAN, AMK and CAP). In this case, the SLIDs should be replaced by drugs from groups 4 or 5 (see Table 1) in order to provide the most efficient treatment regimen. All these data showed the urgent need to perform cheap and rapid diagnostic tests for PZA and SLD resistance detection in routine in Vietnam but also in all high TB burden countries.

4.1.2 Mutations in drug resistance-associated genes

The sequencing of the genes involved in drug resistance showed different level of mutation diversity. For example *pncA*, *rpoB* and *embB* genes showed high diversity of drug resistance mutation patterns compared to *katG*, *inhA*, *rpsL*, *rrs*, *gyrA* and *gyrB* genes. However, whatever the drug resistance genes and the mutation diversity (except for *pncA* gene), only few mutations were prevalent in the drug-resistant clinical isolates, consistent with previous studies (83, 153, 183, 207, 345, 346). These data suggest that these prevailing mutations are strongly selected in the population (297, 298). It is interesting to note that these mutations are generally associated with high level of drug resistance and low fitness cost. The pattern is different for *pncA* gene, we observed a high diversity without preminent mutations. Since this gene is nonessential for bacteria survival (153, 357), its evolution is completely different compared to the other essential genes such as *rpoB*, *katG*, *gyrA* and *rpsL*. The mutation rate is higher (10^{-4} mutation/bacterium/cell division) and almost all the mutations detected in *pncA* could produce PZA resistance. Thus we are facing a high risk of PZA resistance in Vietnam.

4.1.3 Evolution of drug resistance in *M. tuberculosis*

To explain the emergence of MDR- and XDR-TB cases, several extrinsic factors were identified such as the lack of drug resistance detection, inadequate treatment regimens, interruption of drug supply, poor drug quality, insufficient

adherence and observance to the treatment, etc. (16, 265). However, many intrinsic factors are also known to contribute to the emergence of highly drug resistant isolates (232, 265, 268, 306, 358). In this study, we showed that the genetic background of the bacteria, cross-resistance, the fitness cost of mutations and the epistatic interactions between drug resistance mutations and between drug resistance mutations and compensatory mutations could play a role in the evolution of drug resistance towards quadruple-FLD resistance, pre-XDR and XDR in the Vietnamese drug resistant samples. These characteristics and processes led to highly drug resistant isolates with a low fitness cost and thus a high selective advantage. Besides these highly resistant isolates carrying mutations with a low fitness cost, an important proportion of MDR isolates carried also fitness-compensatory mutations associated with RIF resistance. These MDR strains carrying fitness-compensatory mutations can be positively selected and can be highly transmitted without efficient drug resistant TB control.

Furthermore, all the factors mentioned above were specifically linked to Beijing family, which is known to be associated with high level of drug resistance, high intrinsic mutation rate, high transmissibility and higher virulence compared to other families (77, 270, 352). Therefore, our finding suggests a more worrying situation of drug resistance in the future since the Beijing strains are currently invading Vietnam (6, 8, 325, 326). These results underline the importance of identifying severe FLD resistant TB cases to provide the most appropriate treatment and to control the spread of SLD resistance.

4.1.4 Towards new diagnostic tool for drug resistance detection

Besides all the epidemiological and evolutionary information that we obtained from this study, the sequencing analysis allowed to build the first database of mutations linked to FLD and SLD resistance in Vietnam. This database completed with the published data is being used for the development of a diagnostic kit based on DNA chip technology for the rapid detection of drug resistant TB in South-East Asia. The database will be completed by data collected in Laos and Cambodia that are currently in process in collaboration. All the mutations detected in this work plus the main mutations described in the literature were chosen for the development of a prototype that will be available in few months. More than 200 probes including the

selected mutations and the corresponding wild types (H37Rv) were designed. The objective here is to get an efficient, rapid and cheap test to help a better and wider detection of drug resistance. This kit should detect the drug resistant isolates that are not detected by LPA and Xpert system and should also evaluate the risk of drug resistance to the other FLD and SLD drugs. Besides, several pipelines were developed for detection of drug resistant mutations in whole genome sequencing data. This technique is very powerful and promising but still not affordable for low-income countries.

4.2 Conclusion

This work allowed acquiring unique knowledge on FLD and SLD resistance in *M. tuberculosis* clinical isolates in Vietnam as well as on the processes, which favor the emergence of highly drug resistance isolates. Molecular analysis gave information on the genetic determinants associated with these resistances and showed a high sensitivity between DST data and mutation detection for RIF and INH resistance, moderate to high for EMB, STR and FQ resistance, but low for SLID resistance. Conversely, the specificity was high for all drug resistances. Our data confirm that the current molecular tests (LPAs, Xpert) are efficient for the detection of RIF resistance or RIF/INH resistance (although 4.8% of RIF resistant isolates or 10% of the RIF-INH resistant isolates could not be detected) but not optimal for the other drugs. These data showed that several drug resistance mechanisms such as the resistance to SLIDs need to be further investigated and that molecular tests still need to be improved to obtain a maximal efficiency for drug resistance detection. The data collected in this work allowed building a drug resistance mutation database that could be used, with the data published in the literature, to develop a diagnostic tool based on DNA chip technology. This project is ongoing.

The high diversity of mutations is in agreement with the large variety of drug resistance patterns and with the important genetic diversity in terms of families and MIRU-VNTR genotypes. This study demonstrates a clear effect of the genetic background on the frequencies and distribution of the mutations. Furthermore, the specific combination of mutations associated with high level of drug resistance, low fitness cost and compensatory mutations suggest that intrinsic factors such as

cumulative effect of drug resistance-associated mutations and epistatic interactions also drive the evolution of drug resistance in *M. tuberculosis* in Vietnam.

On an epidemiological and clinical point of view, these data are of high concern in terms of public health. First, the study highlights high risk of PZA resistance in these clinical samples. As described in paper 4, this drug is a key drug for the current TB and MDR-TB treatments but also in the treatments under development. Furthermore, the *pncA* mutations associated with PZA resistance detected in our sample were strongly associated with highly FLD resistance profiles. This finding challenges the use of PZA in the current and future regimens for treating drug-resistant TB particularly MDR, highly drug resistant, pre-XDR and XDR-TB.

Second, this study shows a high risk of evolution of highly drug resistant isolates into pre-XDR and XDR isolates. Moreover, the detection of combination of mutations characterized by a high level of drug resistance and low/no fitness costs and the compensatory mutations, is also worrying in terms of evolution of drug resistance in Vietnam. Indeed, highly drug resistant isolates with a high fitness seem to be selected and transmitted in the community. The last point concerns the specific association of these highly resistant and fit isolates with the Beijing family, which is currently invading Vietnam. All these aspects strongly underline the urgent need to improve the detection and the control of TB and MDR-TB in Vietnam but also worldwide.

4.3 Perspectives

The perspectives of this work are multiple. First, since these isolates were collected between 2005 and 2009, we will compare the present data with data collected on recent isolates. This comparison will allow evaluating if the PMDT help control the evolution of drug resistance in Vietnam. Second we are currently developing a diagnostic tool based on DNA chip technology to improve the drug resistance detection. Third, it is essential to currently estimate the PZA resistance level to help the implementation of the best treatment strategies. Fourth, since some specific combinations of FLD resistance mutations are specifically linked to highly drug resistant including pre-XDR and XDR, it could be very useful in terms of patient care to be able to specifically detect these mutations in order to prescribe more quickly the best treatment regimen. Fifth, the drug resistant isolates (especially the pre-XDR and XDR isolates) missing some drug resistance-associated mutations will be further studied by whole genome sequencing analysis to determine the peculiar mechanisms of resistance in these samples.

Reference

1. WHO. Global tuberculosis report 2016. WHO/HTM/TB/201613 World Health Organization, Geneva, Switzerland. 2016.
2. Dawson R, Diacon AH, Everitt D, van Niekerk C, Donald PR, Burger DA, et al. Efficiency and safety of the combination of moxifloxacin, pretomanid (PA-824), and pyrazinamide during the first 8 weeks of antituberculosis treatment: a phase 2b, open-label, partly randomised trial in patients with drug-susceptible or drug-resistant pulmonary tuberculosis. *Lancet*. 2015 May 2;385(9979):1738-47.
3. Zumla A, Chakaya J, Centis R, D'Ambrosio L, Mwaba P, Bates M, et al. Tuberculosis treatment and management--an update on treatment regimens, trials, new drugs, and adjunct therapies. *The Lancet Respiratory medicine*. 2015 Mar;3(3):220-34.
4. Organization WH. WHO treatment guidelines for drug-resistant tuberculosis 2016 update. WHO/HTM/TB/201604. 2016;World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.
5. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, et al. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. *Int J Tuberc Lung Dis*. 2009 Jul;13(7):900-6.
6. Duong DA, Nguyen TH, Nguyen TN, Dai VH, Dang TM, Vo SK, et al. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. *Antimicrob Agents Chemother*. 2009 Nov;53(11):4835-9.
7. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. *Emerging infectious diseases*. 2003 Dec;9(12):1633-5.
8. Nguyen VA, Banuls AL, Tran TH, Pham KL, Nguyen TS, Nguyen HV, et al. *Mycobacterium tuberculosis* lineages and anti-tuberculosis drug resistance in reference hospitals across Viet Nam. *BMC microbiology*. 2016;16(1):167.
9. Huyen MN, Buu TN, Tiemersma E, Lan NT, Dung NH, Kremer K, et al. Tuberculosis relapse in Vietnam is significantly associated with *Mycobacterium tuberculosis* Beijing genotype infections. *The Journal of infectious diseases*. 2013 May 15;207(10):1516-24.
10. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, et al. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nature genetics*. 2013 Oct;45(10):1176-82.
11. Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. *The Lancet infectious diseases*. 2007 May;7(5):328-37.
12. (WHO) TWHO. Global Tuberculosis Report 2015. WHO/HTM/TB/201522. 2015;Geneva, Switzerland.
13. Tola HH, Tol A, Shojaeizadeh D, Garmaroudi G. Tuberculosis Treatment Non-Adherence and Lost to Follow Up among TB Patients with or without HIV in Developing Countries: A Systematic Review. *Iranian journal of public health*. 2015 Jan;44(1):1-11.
14. Tesfahuneygn G, Medhin G, Legesse M. Adherence to Anti-tuberculosis treatment and treatment outcomes among tuberculosis patients in Alamata District, northeast Ethiopia. *BMC research notes*. 2015 Sep 29;8:503.
15. Marahatta SB. Multi-drug resistant tuberculosis burden and risk factors: an update. *Kathmandu University medical journal*. 2010 Jan-Mar;8(29):116-25.
16. Nachega JB, Chaisson RE. Tuberculosis drug resistance: a global threat. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2003 Jan 15;36(Suppl 1):S24-30.
17. Matteelli A, Roggi A, Carvalho AC. Extensively drug-resistant tuberculosis: epidemiology and management. *Clinical epidemiology*. 2014;6:111-8.

18. Niemann S, Diel R, Khechinashvili G, Gegia M, Mdivani N, Tang YW. Mycobacterium tuberculosis Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia. *J Clin Microbiol*. 2010 Oct;48(10):3544-50.
19. Pang Y, Song Y, Xia H, Zhou Y, Zhao B, Zhao Y. Risk factors and clinical phenotypes of Beijing genotype strains in tuberculosis patients in China. *BMC infectious diseases*. 2012 Dec 17;12:354.
20. (WHO) WHO. IMPLEMENTING TUBERCULOSIS DIAGNOSTICS. Policy framework. WHO/HTM/TB/201511. 2015;ISBN 978 92 4 150861 2. WHO, Geneva, Switzerland.
21. WHO. Tuberculosis Laboratory Biosafety Manual. Geneva: World Health Organization. 2012.
22. (WHO) WHO. Guidelines for surveillance of drug resistance in tuberculosis. 5th Edition. Geneva, Switzerland ISBN 978 92 4 154913 4. 2015.
23. Angeby K, Jureen P, Kahlmeter G, Hoffner SE, Schon T. Challenging a dogma: antimicrobial susceptibility testing breakpoints for *Mycobacterium tuberculosis*. *Bulletin of the World Health Organization*. 2012 Sep 1;90(9):693-8.
24. (WHO) WHO. Xpert MTB/RIF implementation manual Technical and operational 'how-to': practical considerations. Geneva, Switzerland 2014;ISBN: 978 92 4 150670 0(http://apps.who.int/iris/bitstream/10665/112469/1/9789241506700_eng.pdf).
25. (WHO) WHO. THE USE OF MOLECULAR LINE PROBE ASSAY FOR THE DETECTION OF RESISTANCE TO SECOND-LINE ANTI-TUBERCULOSIS DRUGS EXPERT GROUP MEETING REPORT Geneva, Switzerland. 2013;WHO/HTM/TB/2013.01.
26. Tomacicchio M, Theron G, Pietersen E, Streicher E, Stanley-Josephs D, van Helden P, et al. The diagnostic accuracy of the MTBDRplus and MTBDRsl assays for drug-resistant TB detection when performed on sputum and culture isolates. *Scientific reports*. 2016;6:17850.
27. WHO. The use of molecular line probe assays for the detection of resistance to second-line anti-tuberculosis drugs. Policy guidance. WHO/HTM/TB/201607 World Health Organization, Geneva, Switzerland. 2016.
28. Sanchez-Padilla E, Merker M, Beckert P, Jochims F, Dlamini T, Kahn P, et al. Detection of drug-resistant tuberculosis by Xpert MTB/RIF in Swaziland. *The New England journal of medicine*. 2015 Mar 19;372(12):1181-2.
29. Bayer R, Wilkinson D. Directly observed therapy for tuberculosis: history of an idea. *Lancet*. 1995 Jun 17;345(8964):1545-8.
30. Diacon AH, Dawson R, von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, et al. 14-day bactericidal activity of PA-824, bedaquiline, pyrazinamide, and moxifloxacin combinations: a randomised trial. *Lancet*. 2012 Sep 15;380(9846):986-93.
31. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *The New England journal of medicine*. 2014 Oct 23;371(17):1577-87.
32. Sellier PO, Clevenbergh P, Bergmann JF. Shorter moxifloxacin-based regimens for drug-sensitive tuberculosis. *The New England journal of medicine*. 2015 Feb 5;372(6):577.
33. Merle CS, Fielding K, Sow OB, Gninafon M, Lo MB, Mthiyane T, et al. A four-month gatifloxacin-containing regimen for treating tuberculosis. *The New England journal of medicine*. 2014 Oct 23;371(17):1588-98.
34. Wallis RS, Maeurer M, Mwaba P, Chakaya J, Rustomjee R, Migliori GB, et al. Tuberculosis-advances in development of new drugs, treatment regimens, host-directed therapies, and biomarkers. *The Lancet Infectious diseases*. 2016 Apr;16(4):e34-46.
35. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. *Tubercle*. 1985 Sep;66(3):219-25.
36. Grosset J. Mechanisms of action of antituberculosis drugs in standard and short-course chemotherapy regimens. *Revista de igiena, bacteriologie, virusologie, parazitologie, epidemiologie, pneumoftiziologie Pneumoftiziologia*. 1985 Apr-Jun;34(2):108-15.

37. EA/BMRC. Controlled clinical trial of short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. *Lancet*. 1972 May 20;1(7760):1079-85.
38. WHO. Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis. WHO/HTM/TB/201411 Geneva, World Health Organization, 2014. 2014.
39. Williams K, Minkowski A, Amoabeng O, Peloquin CA, Taylor D, Andries K, et al. Sterilizing activities of novel combinations lacking first- and second-line drugs in a murine model of tuberculosis. *Antimicrob Agents Chemother*. 2012 Jun;56(6):3114-20.
40. Tasneen R, Li SY, Peloquin CA, Taylor D, Williams KN, Andries K, et al. Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. *Antimicrobial agents and chemotherapy*. 2011 Dec;55(12):5485-92.
41. Alffenaar JW, Gumbo T, Aarnoutse R. Shorter moxifloxacin-based regimens for drug-sensitive tuberculosis. *The New England journal of medicine*. 2015 Feb 5;372(6):576.
42. Gillespie SH, Mendel CM, Consortium RE. Shorter moxifloxacin-based regimens for drug-sensitive tuberculosis. *The New England journal of medicine*. 2015 Feb 5;372(6):577.
43. Andersen P, Doherty TM. The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nature reviews Microbiology*. 2005 Aug;3(8):656-62.
44. Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against *Mycobacterium tuberculosis* infection in children: systematic review and meta-analysis. *Bmj*. 2014;349:g4643.
45. Hong DN, Huyen MN, Lan NT, Duong NH, Ngo VV, Ngoc DT, et al. Rifampin-resistant *Mycobacterium bovis* BCG-induced disease in HIV-infected infant, Vietnam. *Emerg Infect Dis*. 2013 Jul;19(7):1168-70.
46. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*. 2006 Feb 21;103(8):2869-73.
47. Koch R. Die Aetiologie der Tuberculose (Nach einemin der physiologischen Gesellschaft zu Berlin am 24.Maärzgehaltenem Vortrage). *Berliner klin Wochenschr* 19: 221–230. 1882.
48. Gengenbacher M, Kaufmann SH. *Mycobacterium tuberculosis*: success through dormancy. *FEMS microbiology reviews*. 2012 May;36(3):514-32.
49. Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. *J Clin Microbiol*. 1999 Nov;37(11):3578-82.
50. Somoskovi A, Magyar P. Comparison of the mycobacteria growth indicator tube with MB redox, Lowenstein-Jensen, and Middlebrook 7H11 media for recovery of mycobacteria in clinical specimens. *J Clin Microbiol*. 1999 May;37(5):1366-9.
51. Naveen G, Peerapur BV. Comparison of the Lowenstein-Jensen Medium, the Middlebrook 7H10 Medium and MB/BacT for the Isolation of *Mycobacterium Tuberculosis* (MTB) from Clinical Specimens. *Journal of clinical and diagnostic research : JCDR*. 2012 Dec;6(10):1704-9.
52. Adler H, Straub C, Frei R. Comparison of BacT/ALERT 3D, Lowenstein-Jensen medium and Middlebrook 7H10/7H11 biplate for recovering mycobacteria from clinical specimens. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*. 2005 Jul;24(7):499-500.
53. Kaur D, Guerin ME, Skovierova H, Brennan PJ, Jackson M. Chapter 2: Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. *Advances in applied microbiology*. 2009;69:23-78.

54. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*. 1998 Jun 11;393(6685):537-44.
55. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al. Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *The Lancet infectious diseases*. 2013 Feb;13(2):137-46.
56. Fleischmann RD, Alland D, Eisen JA, Carpenter L, White O, Peterson J, et al. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *Journal of bacteriology*. 2002 Oct;184(19):5479-90.
57. Merker M, Kohl TA, Roetzer A, Truebe L, Richter E, Rusch-Gerdes S, et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant *Mycobacterium tuberculosis* Beijing strains in patients. *PloS one*. 2013;8(12):e82551.
58. Casali N, Nikolayevskyy V, Balabanova Y, Ignatyeva O, Kontsevaya I, Harris SR, et al. Microevolution of extensively drug-resistant tuberculosis in Russia. *Genome research*. 2012 Apr;22(4):735-45.
59. Iliina EN, Shitikov EA, Ikryannikova LN, Alekseev DG, Kamashev DE, Malakhova MV, et al. Comparative genomic analysis of *Mycobacterium tuberculosis* drug resistant strains from Russia. *PloS one*. 2013;8(2):e56577.
60. Zhang H, Li D, Zhao L, Fleming J, Lin N, Wang T, et al. Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. *Nature genetics*. 2013 Oct;45(10):1255-60.
61. Dos Vultos T, Mestre O, Rauzier J, Golec M, Rastogi N, Rasolofo V, et al. Evolution and diversity of clonal bacteria: the paradigm of *Mycobacterium tuberculosis*. *PloS one*. 2008;3(2):e1538.
62. Coscolla M, Gagneux S. Consequences of genomic diversity in *Mycobacterium tuberculosis*. *Seminars in immunology*. 2014 Dec;26(6):431-44.
63. David HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Applied microbiology*. 1970 Nov;20(5):810-4.
64. Gumbo T. Biological variability and the emergence of multidrug-resistant tuberculosis. *Nature genetics*. 2013 Jul;45(7):720-1.
65. Ford CB, Lin PL, Chase MR, Shah RR, Iartchouk O, Galagan J, et al. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nature genetics*. 2011 May;43(5):482-6.
66. Roetzer A, Diel R, Kohl TA, Ruckert C, Nubel U, Blom J, et al. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. *PLoS medicine*. 2013;10(2):e1001387.
67. Gutierrez MC, Brisse S, Brosch R, Fabre M, Omais B, Marmiesse M, et al. Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS pathogens*. 2005 Sep;1(1):e5.
68. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, et al. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. *Emerg Infect Dis*. 2013 Mar;19(3):460-3.
69. Tessema B, Beer J, Merker M, Emmrich F, Sack U, Rodloff AC, et al. Molecular epidemiology and transmission dynamics of *Mycobacterium tuberculosis* in Northwest Ethiopia: new phylogenetic lineages found in Northwest Ethiopia. *BMC infectious diseases*. 2013;13:131.
70. Comas I, Hailu E, Kiros T, Bekele S, Mekonnen W, Gumi B, et al. Population Genomics of *Mycobacterium tuberculosis* in Ethiopia Contradicts the Virgin Soil Hypothesis for Human Tuberculosis in Sub-Saharan Africa. *Current biology : CB*. 2015 Dec 21;25(24):3260-6.

71. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Sciences of the United States of America*. 2002 Mar 19;99(6):3684-9.
72. Galagan JE. Genomic insights into tuberculosis. *Nature reviews Genetics*. 2014 May;15(5):307-20.
73. Supply P, Warren RM, Banuls AL, Lesjean S, Van Der Spuy GD, Lewis LA, et al. Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. *Molecular microbiology*. 2003 Jan;47(2):529-38.
74. Banuls AL, Sanou A, Anh NT, Godreuil S. *Mycobacterium tuberculosis*: ecology and evolution of a human bacterium. *J Med Microbiol*. 2015 Nov;64(11):1261-9.
75. Demay C, Liens B, Burguiere T, Hill V, Couvin D, Millet J, et al. SITVITWEB--a publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2012 Jun;12(4):755-66.
76. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC microbiology*. 2006;6:23.
77. Merker M, Blin C, Mona S, Duforet-Freboung N, Lecher S, Willery E, et al. Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nature genetics*. 2015 Mar;47(3):242-9.
78. Qian L, Van Embden JD, Van Der Zanden AG, Weltevreden EF, Duanmu H, Douglas JT. Retrospective analysis of the Beijing family of *Mycobacterium tuberculosis* in preserved lung tissues. *J Clin Microbiol*. 1999 Feb;37(2):471-4.
79. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *J Clin Microbiol*. 1995 Dec;33(12):3234-8.
80. Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M, et al. Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. *mBio*. 2013;4(4).
81. Brites D, Gagneux S. Co-evolution of *Mycobacterium tuberculosis* and *Homo sapiens*. *Immunological reviews*. 2015 Mar;264(1):6-24.
82. Gagneux S. Host-pathogen coevolution in human tuberculosis. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2012 Mar 19;367(1590):850-9.
83. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nature genetics*. 2014 Mar;46(3):279-86.
84. Ribeiro SC, Gomes LL, Amaral EP, Andrade MR, Almeida FM, Rezende AL, et al. *Mycobacterium tuberculosis* strains of the modern sublineage of the Beijing family are more likely to display increased virulence than strains of the ancient sublineage. *J Clin Microbiol*. 2014 Jul;52(7):2615-24.
85. Klopper M, Warren RM, Hayes C, Gey van Pittius NC, Streicher EM, Muller B, et al. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. *Emerg Infect Dis*. 2013 Mar;19(3):449-55.
86. Caminero JA, Sotgiu G, Zumla A, Migliori GB. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *The Lancet infectious diseases*. 2010 Sep;10(9):621-9.

87. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*: update 2015. *Int J Tuberc Lung Dis*. 2015 Nov;19(11):1276-89.
88. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. *PLoS medicine*. 2009 Feb 10;6(2):e2.
89. Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, et al. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. *PLoS medicine*. 2006 Nov;3(11):e466.
90. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature*. 2000 Jun 22;405(6789):962-6.
91. Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, et al. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science*. 2005 Jan 14;307(5707):223-7.
92. Hartkoorn RC, Uplekar S, Cole ST. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2014 May;58(5):2979-81.
93. Bloemberg GV, Keller PM, Stucki D, Trauner A, Borrell S, Latshang T, et al. Acquired Resistance to Bedaquiline and Delamanid in Therapy for Tuberculosis. *The New England journal of medicine*. 2015 Nov 12;373(20):1986-8.
94. Pedro Eduardo Almeida da Silva AVG, Anandi Martin & Juan Carlos Palomino. Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. *FEMS immunology and medical microbiology*. 2011;63:1-9.
95. Smith T, Wolff KA, Nguyen L. Molecular biology of drug resistance in *Mycobacterium tuberculosis*. *Current topics in microbiology and immunology*. 2013;374:53-80.
96. Sarathy JP, Dartois V, Lee EJ. The role of transport mechanisms in mycobacterium tuberculosis drug resistance and tolerance. *Pharmaceuticals*. 2012;5(11):1210-35.
97. Karakousis PC, Bishai WR, Dorman SE. *Mycobacterium tuberculosis* cell envelope lipids and the host immune response. *Cellular microbiology*. 2004 Feb;6(2):105-16.
98. Brennan PJ. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis*. 2003;83(1-3):91-7.
99. Pasipanodya JG, Gumbo T. A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. *Current opinion in pharmacology*. 2011 Oct;11(5):457-63.
100. De Rossi E, Arrigo P, Bellinzoni M, Silva PA, Martin C, Ainsa JA, et al. The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. *Molecular medicine*. 2002 Nov;8(11):714-24.
101. Viveiros M, Portugal I, Bettencourt R, Victor TC, Jordaan AM, Leandro C, et al. Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2002 Sep;46(9):2804-10.
102. Louw GE, Warren RM, Gey van Pittius NC, McEvoy CR, Van Helden PD, Victor TC. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Chemother*. 2009 Aug;53(8):3181-9.
103. Palomino JC, Ramos DF, da Silva PA. New anti-tuberculosis drugs: strategies, sources and new molecules. *Current medicinal chemistry*. 2009;16(15):1898-904.
104. Grossman TH, Shoen CM, Jones SM, Jones PL, Cynamon MH, Locher CP. The efflux pump inhibitor timcodar improves the potency of antimycobacterial agents. *Antimicrob Agents Chemother*. 2015 Mar;59(3):1534-41.
105. Pule CM, Sampson SL, Warren RM, Black PA, van Helden PD, Victor TC, et al. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *The Journal of antimicrobial chemotherapy*. 2016 Jan;71(1):17-26.

106. Song L, Wu X. Development of efflux pump inhibitors in antituberculosis therapy. *Int J Antimicrob Agents*. 2016 Jun;47(6):421-9.
107. Adams KN, Szumowski JD, Ramakrishnan L. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *The Journal of infectious diseases*. 2014 Aug 1;210(3):456-66.
108. Li G, Zhang J, Guo Q, Wei J, Jiang Y, Zhao X, et al. Study of efflux pump gene expression in rifampicin-monoresistant *Mycobacterium tuberculosis* clinical isolates. *The Journal of antibiotics*. 2015 Jul;68(7):431-5.
109. Martin JCPaA. Drug Resistance Mechanisms in *Mycobacterium tuberculosis*. *Antibiotics*. 2014;3:317-40.
110. McGrath M, Gey van Pittius NC, van Helden PD, Warren RM, Warner DF. Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. *The Journal of antimicrobial chemotherapy*. 2014 Feb;69(2):292-302.
111. Getahun H, Matteelli A, Abubakar I, Aziz MA, Baddeley A, Barreira D, et al. Management of latent *Mycobacterium tuberculosis* infection: WHO guidelines for low tuberculosis burden countries. *The European respiratory journal*. 2015 Dec;46(6):1563-76.
112. Getahun H, Chaisson RE, Raviglione M. Latent *Mycobacterium tuberculosis* Infection. *The New England journal of medicine*. 2015 Sep 17;373(12):1179-80.
113. Paramasivan CN, Sulochana S, Kubendiran G, Venkatesan P, Mitchison DA. Bactericidal action of gatifloxacin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2005 Feb;49(2):627-31.
114. Xie Z, Siddiqi N, Rubin EJ. Differential antibiotic susceptibilities of starved *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother*. 2005 Nov;49(11):4778-80.
115. Jindani A, Dore CJ, Mitchison DA. Bactericidal and sterilizing activities of antituberculosis drugs during the first 14 days. *American journal of respiratory and critical care medicine*. 2003 May 15;167(10):1348-54.
116. Hazbon MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2006 Aug;50(8):2640-9.
117. Vilcheze C, Jacobs WR, Jr. Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis*: Genes, Mutations, and Causalities. *Microbiology spectrum*. 2014 Aug;2(4):MGM2-0014-2013.
118. Gagneux S, Burgos MV, DeRiemer K, Encisco A, Munoz S, Hopewell PC, et al. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. *PLoS pathogens*. 2006 Jun;2(6):e61.
119. Muller B, Streicher EM, Hoek KG, Tait M, Trollip A, Bosman ME, et al. inhA promoter mutations: a gateway to extensively drug-resistant tuberculosis in South Africa? *Int J Tuberc Lung Dis*. 2011 Mar;15(3):344-51.
120. van Soolingen D, de Haas PE, van Doorn HR, Kuijper E, Rinder H, Borgdorff MW. Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *The Journal of infectious diseases*. 2000 Dec;182(6):1788-90.
121. Mokrousov I, Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. High prevalence of *KatG* Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. *Antimicrob Agents Chemother*. 2002 May;46(5):1417-24.

122. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannon BJ. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science*. 2006 Jun 30;312(5782):1944-6.
123. Pym AS, Saint-Joanis B, Cole ST. Effect of katG mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infection and immunity*. 2002 Sep;70(9):4955-60.
124. Bergval IL, Schuitema AR, Klatser PR, Anthony RM. Resistant mutants of *Mycobacterium tuberculosis* selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. *The Journal of antimicrobial chemotherapy*. 2009 Sep;64(3):515-23.
125. Machado D, Perdigo J, Ramos J, Couto I, Portugal I, Ritter C, et al. High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. *The Journal of antimicrobial chemotherapy*. 2013 Aug;68(8):1728-32.
126. Guo H, Seet Q, Denkin S, Parsons L, Zhang Y. Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. *J Med Microbiol*. 2006 Nov;55(Pt 11):1527-31.
127. Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. Genetic mutations associated with isoniazid resistance in *Mycobacterium tuberculosis*: a systematic review. *PLoS one*. 2015;10(3):e0119628.
128. Lipsitch M, Levin BR. The population dynamics of antimicrobial chemotherapy. *Antimicrob Agents Chemother*. 1997 Feb;41(2):363-73.
129. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, et al. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell*. 2001 Mar 23;104(6):901-12.
130. Koch A, Mizrahi V, Warner DF. The impact of drug resistance on *Mycobacterium tuberculosis* physiology: what can we learn from rifampicin? *Emerging microbes & infections*. 2014 Mar;3(3):e17.
131. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*. 1993 Mar 13;341(8846):647-50.
132. Li GL, Zhao DF, Xie T, Ju HF, Mu C, Zhao H, et al. Molecular characterization of drug-resistant Beijing family isolates of *Mycobacterium tuberculosis* from Tianjin, China. *Biomed Environ Sci*. Jun;23(3):188-93.
133. Kapur V, Li LL, Iordanescu S, Hamrick MR, Wanger A, Kreiswirth BN, et al. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J Clin Microbiol*. 1994 Apr;32(4):1095-8.
134. Yuen LK, Leslie D, Coloe PJ. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia. *J Clin Microbiol*. 1999 Dec;37(12):3844-50.
135. Pozzi G, Meloni M, Iona E, Orru G, Thoresen OF, Ricci ML, et al. *rpoB* mutations in multidrug-resistant strains of *Mycobacterium tuberculosis* isolated in Italy. *J Clin Microbiol*. 1999 Apr;37(4):1197-9.
136. Valim AR, Rossetti ML, Ribeiro MO, Zaha A. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from Brazil. *J Clin Microbiol*. 2000 Aug;38(8):3119-22.
137. Heep M, Brandstatter B, Rieger U, Lehn N, Richter E, Rusch-Gerdes S, et al. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *J Clin Microbiol*. 2001 Jan;39(1):107-10.
138. Siu GK, Zhang Y, Lau TC, Lau RW, Ho PL, Yew WW, et al. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in

- Mycobacterium tuberculosis*. The Journal of antimicrobial chemotherapy. 2011 Apr;66(4):730-3.
139. Barnes PF, Bloch AB, Davidson PT, Snider DE, Jr. Tuberculosis in patients with human immunodeficiency virus infection. The New England journal of medicine. 1991 Jun 6;324(23):1644-50.
140. Hillemann D, Rusch-Gerdes S, Richter E. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol. 2007 Aug;45(8):2635-40.
141. Van Deun A, Barrera L, Bastian I, Fattorini L, Hoffmann H, Kam KM, et al. *Mycobacterium tuberculosis* strains with highly discordant rifampin susceptibility test results. J Clin Microbiol. 2009 Nov;47(11):3501-6.
142. Rigouts L, Gumusboga M, de Rijk WB, Nduwamahoro E, Uwizeye C, de Jong B, et al. Rifampin resistance missed in automated liquid culture system for *Mycobacterium tuberculosis* isolates with specific *rpoB* mutations. J Clin Microbiol. 2013 Aug;51(8):2641-5.
143. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, et al. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol. 2013 Aug;51(8):2633-40.
144. Steele MA, Des Prez RM. The role of pyrazinamide in tuberculosis chemotherapy. Chest. 1988 Oct;94(4):845-50.
145. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* bacterial populations. The American review of respiratory disease. 1992 May;145(5):1223-5.
146. Zhang Y, Shi W, Zhang W, Mitchison D. Mechanisms of Pyrazinamide Action and Resistance. Microbiology spectrum. 2014 Aug;2(4):MGM2-0023-2013.
147. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrobial agents and chemotherapy. 1997 Mar;41(3):540-3.
148. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tubercle and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 1997;78(2):117-22.
149. Ramirez-Busby SM, Valafar F. Systematic review of mutations in pyrazinamidase associated with pyrazinamide resistance in *Mycobacterium tuberculosis* clinical isolates. Antimicrobial agents and chemotherapy. 2015 Sep;59(9):5267-77.
150. Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniowski F, Rodionova Y, et al. *Mycobacterium tuberculosis* pyrazinamide resistance determinants: a multicenter study. mBio. 2014;5(5):e01819-14.
151. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2003 Jan;7(1):6-21.
152. Petrella S, Gelus-Ziental N, Maudry A, Laurans C, Boudjelloul R, Sougakoff W. Crystal structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural and acquired resistance to pyrazinamide. PloS one. 2011;6(1):e15785.
153. Stoffels K, Mathys V, Fauville-Dufaux M, Wintjens R, Bifani P. Systematic analysis of pyrazinamide-resistant spontaneous mutants and clinical isolates of *Mycobacterium tuberculosis*. Antimicrobial agents and chemotherapy. 2012 Oct;56(10):5186-93.
154. Miotto P, Cirillo DM, Migliori GB. Drug resistance in *Mycobacterium tuberculosis*: molecular mechanisms challenging fluoroquinolones and pyrazinamide effectiveness. Chest. 2015 Apr;147(4):1135-43.

155. Sheen P, Lozano K, Gilman RH, Valencia HJ, Loli S, Fuentes P, et al. *pncA* gene expression and prediction factors on pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tuberculosis*. 2013 Sep;93(5):515-22.
156. Bhujju S, Fonseca Lde S, Marsico AG, de Oliveira Vieira GB, Sobral LF, Stehr M, et al. *Mycobacterium tuberculosis* isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the *pncA* gene. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2013 Oct;19:1-6.
157. Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F. Potential for erroneous results indicating resistance when using the Bactec MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *Journal of clinical microbiology*. 2010 Jan;48(1):300-1.
158. Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE, 3rd, et al. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. *Science*. 2011 Sep 16;333(6049):1630-2.
159. Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, et al. Role of *pncA* and *rpsA* gene sequencing in detection of pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from southern China. *Journal of clinical microbiology*. 2014 Jan;52(1):291-7.
160. Gu Y, Yu X, Jiang G, Wang X, Ma Y, Li Y, et al. Pyrazinamide resistance among multidrug-resistant tuberculosis clinical isolates in a national referral center of China and its correlations with *pncA*, *rpsA*, and *panD* gene mutations. *Diagnostic microbiology and infectious disease*. 2016 Mar;84(3):207-11.
161. Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. *Journal of clinical microbiology*. 2012 Nov;50(11):3726-8.
162. Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soolingen D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. *J Clin Microbiol*. 2013 Jan;51(1):382.
163. Xia Q, Zhao LL, Li F, Fan YM, Chen YY, Wu BB, et al. Phenotypic and genotypic characterization of pyrazinamide resistance among multidrug-resistant *Mycobacterium tuberculosis* isolates in Zhejiang, China. *Antimicrobial agents and chemotherapy*. 2015 Mar;59(3):1690-5.
164. Zhang S, Chen J, Shi W, Liu W, Zhang W, Zhang Y. Mutations in *panD* encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*. *Emerging microbes & infections*. 2013 Jun;2(6):e34.
165. Shi W, Chen J, Feng J, Cui P, Zhang S, Weng X, et al. Aspartate decarboxylase (PanD) as a new target of pyrazinamide in *Mycobacterium tuberculosis*. *Emerging microbes & infections*. 2014 Aug;3(8):e58.
166. Controlled clinical trial of 4 short-course regimens of chemotherapy (three 6-month and one 8-month) for pulmonary tuberculosis: final report. East and Central African/British Medical Research Council Fifth Collaborative Study. *Tubercle*. 1986 Mar;67(1):5-15.
167. Shen X, Shen GM, Wu J, Gui XH, Li X, Mei J, et al. Association between *embB* codon 306 mutations and drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2007 Jul;51(7):2618-20.
168. Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR, Jr., et al. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. *Antimicrob Agents Chemother*. 1997 Aug;41(8):1677-81.
169. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, et al. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-beta-D-arabinose biosynthetic and utilization pathway genes. *Nature genetics*. 2013 Oct;45(10):1190-7.

170. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, et al. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nature medicine*. 1997 May;3(5):567-70.
171. Goude R, Amin AG, Chatterjee D, Parish T. The arabinosyltransferase *EmbC* is inhibited by ethambutol in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2009 Oct;53(10):4138-46.
172. Cui Z, Li Y, Cheng S, Yang H, Lu J, Hu Z, et al. Mutations in the *embC-embA* intergenic region contribute to *Mycobacterium tuberculosis* resistance to ethambutol. *Antimicrob Agents Chemother*. 2014 Nov;58(11):6837-43.
173. Ramaswamy SV, Amin AG, Goksel S, Stager CE, Dou SJ, El Sahly H, et al. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2000 Feb;44(2):326-36.
174. Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B, Alland D. Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2010 Jan;54(1):103-8.
175. Shi D, Li L, Zhao Y, Jia Q, Li H, Coulter C, et al. Characteristics of *embB* mutations in multidrug-resistant *Mycobacterium tuberculosis* isolates in Henan, China. *The Journal of antimicrobial chemotherapy*. 2011 Oct;66(10):2240-7.
176. Inawali HN, Hwang SC, Park YK, Kim H, Lee YS, Chung GT, et al. Characterization of mutations in multi- and extensive drug resistance among strains of *Mycobacterium tuberculosis* clinical isolates in Republic of Korea. *Diagnostic microbiology and infectious disease*. 2013 Jun;76(2):187-96.
177. Claudia Plinke HSC, Nana Zarkua, Hamraev A. Karimovich, Kai Braker, Roland Diel,, Sabine Ru"sch-Gerdes SFaSN. *embCAB* sequence variation among ethambutol-resistant *Mycobacterium tuberculosis* isolates without *embB306* mutation. *The Journal of antimicrobial chemotherapy*. 2010;65:1359-67.
178. Hazbon MH, Bobadilla del Valle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, et al. Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. *Antimicrob Agents Chemother*. 2005 Sep;49(9):3794-802.
179. Safi H, Sayers B, Hazbon MH, Alland D. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. *Antimicrob Agents Chemother*. 2008 Jun;52(6):2027-34.
180. Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O. Detection of *embB306* mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. *J Clin Microbiol*. 2002 Oct;40(10):3810-3.
181. Lee AS, Othman SN, Ho YM, Wong SY. Novel mutations within the *embB* gene in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2004 Nov;48(11):4447-9.
182. Plinke C, Walter K, Aly S, Ehlers S, Niemann S. *Mycobacterium tuberculosis embB* codon 306 mutations confer moderately increased resistance to ethambutol in vitro and in vivo. *Antimicrob Agents Chemother*. 2011 Jun;55(6):2891-6.
183. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2011 May;55(5):2032-41.
184. Laszlo A, Rahman M, Espinal M, Raviglione M, Laboratories WINoSR. Quality assurance programme for drug susceptibility testing of *Mycobacterium tuberculosis* in the

WHO/IUATLD Supranational Reference Laboratory Network: five rounds of proficiency testing, 1994-1998. *Int J Tuberc Lung Dis.* 2002 Sep;6(9):748-56.

185. Honore N, Cole ST. Streptomycin resistance in mycobacteria. *Antimicrob Agents Chemother.* 1994 Feb;38(2):238-42.

186. Finken M, Kirschner P, Meier A, Wrede A, Bottger EC. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular microbiology.* 1993 Sep;9(6):1239-46.

187. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature.* 2000 Sep 21;407(6802):340-8.

188. Sreevatsan S, Pan X, Stockbauer KE, Williams DL, Kreiswirth BN, Musser JM. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrob Agents Chemother.* 1996 Apr;40(4):1024-6.

189. Fukuda M, Koga H, Ohno H, Yang B, Hirakata Y, Maesaki S, et al. Relationship between genetic alteration of the *rpsL* gene and streptomycin susceptibility of *Mycobacterium tuberculosis* in Japan. *The Journal of antimicrobial chemotherapy.* 1999 Feb;43(2):281-4.

190. Nhu NT, Lan NT, Phuong NT, Chau N, Farrar J, Caws M. Association of streptomycin resistance mutations with level of drug resistance and *Mycobacterium tuberculosis* genotypes. *Int J Tuberc Lung Dis.* 2012 Apr;16(4):527-31.

191. Lipin MY, Stepanshina VN, Shemyakin IG, Shinnick TM. Association of specific mutations in *katG*, *rpoB*, *rpsL* and *rrs* genes with spoligotypes of multidrug-resistant *Mycobacterium tuberculosis* isolates in Russia. *Clin Microbiol Infect.* 2007 Jun;13(6):620-6.

192. Springer B, Kidan YG, Prammananan T, Ellrott K, Bottger EC, Sander P. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrob Agents Chemother.* 2001 Oct;45(10):2877-84.

193. Cuevas-Cordoba B, Cuellar-Sanchez A, Pasissi-Crivelli A, Santana-Alvarez CA, Hernandez-Illezcas J, Zenteno-Cuevas R. *rrs* and *rpsL* mutations in streptomycin-resistant isolates of *Mycobacterium tuberculosis* from Mexico. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi.* 2013 Feb;46(1):30-4.

194. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HI, Barry CE, 3rd. Mutations in *gidB* confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 2011 Jun;55(6):2515-22.

195. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, et al. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Molecular microbiology.* 2007 Feb;63(4):1096-106.

196. Organization WH. WHO treatment guidelines for drug-resistant tuberculosis: 2016 update. WHO Handbook. 2016;ISBN 978 92 4 154963 9(www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/resources/).

197. Heifets L, Lindholm-Levy P. Comparison of bactericidal activities of streptomycin, amikacin, kanamycin, and capreomycin against *Mycobacterium avium* and *M. tuberculosis*. *Antimicrob Agents Chemother.* 1989 Aug;33(8):1298-301.

198. Dooley KE, Mitnick CD, Ann DeGroot M, Obuku E, Belitsky V, Hamilton CD, et al. Old drugs, new purpose: retooling existing drugs for optimized treatment of resistant tuberculosis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2012 Aug;55(4):572-81.

199. Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL, et al. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 1998 May;42(5):1295-7.

200. Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2005 Aug;49(8):3192-7.
201. Sirgel FA, Tait M, Warren RM, Streicher EM, Bottger EC, van Helden PD, et al. Mutations in the rrs A1401G gene and phenotypic resistance to amikacin and capreomycin in *Mycobacterium tuberculosis*. *Microbial drug resistance*. 2012 Apr;18(2):193-7.
202. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PloS one*. 2012;7(3):e33275.
203. Malinga L, Brand J, Olorunju S, Stoltz A, van der Walt M. Molecular analysis of genetic mutations among cross-resistant second-line injectable drugs reveals a new resistant mutation in *Mycobacterium tuberculosis*. *Diagnostic microbiology and infectious disease*. 2016 Aug;85(4):433-7.
204. Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the rrs gene. *Antimicrob Agents Chemother*. 2009 Dec;53(12):5064-8.
205. Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. *Chemical reviews*. 2005 Feb;105(2):477-98.
206. Johansen SK, Maus CE, Plikaytis BB, Douthwaite S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2'-O-methylations in 16S and 23S rRNAs. *Molecular cell*. 2006 Jul 21;23(2):173-82.
207. Yuan X, Zhang T, Kawakami K, Zhu J, Li H, Lei J, et al. Molecular characterization of multidrug- and extensively drug-resistant *Mycobacterium tuberculosis* strains in Jiangxi, China. *J Clin Microbiol*. 2012 Jul;50(7):2404-13.
208. Sowajassatakul A, Prammananan T, Chairprasert A, Phunpruch S. Molecular characterization of amikacin, kanamycin and capreomycin resistance in M/XDR-TB strains isolated in Thailand. *BMC microbiology*. 2014;14:165.
209. Perdigao J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. *The Journal of antimicrobial chemotherapy*. 2010 Feb;65(2):224-7.
210. Reeves AZ, Campbell PJ, Willby MJ, Posey JE. Disparities in capreomycin resistance levels associated with the rrs A1401G mutation in clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2015 Jan;59(1):444-9.
211. Zaunbrecher MA, Sikes RD, Jr., Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 Nov 24;106(47):20004-9.
212. Gikalo MB, Nosova EY, Krylova LY, Moroz AM. The role of eis mutations in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates from the Moscow region. *The Journal of antimicrobial chemotherapy*. 2012 Sep;67(9):2107-9.
213. Engstrom A, Perskvist N, Werngren J, Hoffner SE, Jureen P. Comparison of clinical isolates and in vitro selected mutants reveals that tlyA is not a sensitive genetic marker for capreomycin resistance in *Mycobacterium tuberculosis*. *The Journal of antimicrobial chemotherapy*. 2011 Jun;66(6):1247-54.
214. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nature reviews Drug discovery*. 2013 May;12(5):388-404.
215. Yew WW, Chan CK, Leung CC, Chau CH, Tam CM, Wong PC, et al. Comparative roles of levofloxacin and ofloxacin in the treatment of multidrug-resistant tuberculosis: preliminary results of a retrospective study from Hong Kong. *Chest*. 2003 Oct;124(4):1476-81.

216. Kam KM, Yip CW, Cheung TL, Tang HS, Leung OC, Chan MY. Stepwise decrease in moxifloxacin susceptibility amongst clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*: correlation with ofloxacin susceptibility. *Microbial drug resistance*. 2006 Spring;12(1):7-11.
217. Johnson JL, Hadad DJ, Boom WH, Daley CL, Peloquin CA, Eisenach KD, et al. Early and extended early bactericidal activity of levofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. *Int J Tuberc Lung Dis*. 2006 Jun;10(6):605-12.
218. Hu Y, Coates AR, Mitchison DA. Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2003 Feb;47(2):653-7.
219. Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, et al. A systematic review of *gyrase* mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed *gyrase* numbering system. *The Journal of antimicrobial chemotherapy*. 2012 Apr;67(4):819-31.
220. Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. *Antimicrob Agents Chemother*. 2004 Apr;48(4):1281-8.
221. Mdluli K, Ma Z. *Mycobacterium tuberculosis* DNA gyrase as a target for drug discovery. *Infectious disorders drug targets*. 2007 Jun;7(2):159-68.
222. Piton J, Petrella S, Delarue M, Andre-Leroux G, Jarlier V, Aubry A, et al. Structural insights into the quinolone resistance mechanism of *Mycobacterium tuberculosis* DNA gyrase. *PLoS one*. 2010;5(8):e12245.
223. Pantel A, Petrella S, Matrat S, Brossier F, Bastian S, Reitter D, et al. DNA gyrase inhibition assays are necessary to demonstrate fluoroquinolone resistance secondary to gyrB mutations in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2011 Oct;55(10):4524-9.
224. Malik S, Willby M, Sikes D, Tsodikov OV, Posey JE. New insights into fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional genetic analysis of *gyrA* and *gyrB* mutations. *PLoS one*. 2012;7(6):e39754.
225. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, et al. Extending the definition of the GyrB quinolone resistance-determining region in *Mycobacterium tuberculosis* DNA gyrase for assessing fluoroquinolone resistance in *M. tuberculosis*. *Antimicrob Agents Chemother*. 2012 Apr;56(4):1990-6.
226. Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, et al. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gyrA* and *gyrB*. *Antimicrob Agents Chemother*. 2009 Oct;53(10):4498-500.
227. Nosova EY, Bukatina AA, Isaeva YD, Makarova MV, Galkina KY, Moroz AM. Analysis of mutations in the *gyrA* and *gyrB* genes and their association with the resistance of *Mycobacterium tuberculosis* to levofloxacin, moxifloxacin and gatifloxacin. *J Med Microbiol*. 2013 Jan;62(Pt 1):108-13.
228. Li J, Gao X, Luo T, Wu J, Sun G, Liu Q, et al. Association of *gyrA/B* mutations and resistance levels to fluoroquinolones in clinical isolates of *Mycobacterium tuberculosis*. *Emerging microbes & infections*. 2014 Mar;3(3):e19.
229. Chen J, Chen Z, Li Y, Xia W, Chen X, Chen T, et al. Characterization of *gyrA* and *gyrB* mutations and fluoroquinolone resistance in *Mycobacterium tuberculosis* clinical isolates from Hubei Province, China. *The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases*. 2012 Mar-Apr;16(2):136-41.
230. Gagneux S. Fitness cost of drug resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect*. 2009 Jan;15 Suppl 1:66-8.
231. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. *The international journal of tuberculosis and lung*

disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2009 Dec;13(12):1456-66.

232. Borrell S, Teo Y, Giardina F, Streicher EM, Klopper M, Feldmann J, et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evolution, medicine, and public health*. 2013 Jan;2013(1):65-74.

233. Sirgel FA, Warren RM, Streicher EM, Victor TC, van Helden PD, Bottger EC. *gyrA* mutations and phenotypic susceptibility levels to ofloxacin and moxifloxacin in clinical isolates of *Mycobacterium tuberculosis*. *The Journal of antimicrobial chemotherapy*. 2012 May;67(5):1088-93.

234. Singh P, Jain A, Dixit P, Prakash S, Jaiswal I, Venkatesh V, et al. Prevalence of *gyrA* and B gene mutations in fluoroquinolone-resistant and -sensitive clinical isolates of *Mycobacterium tuberculosis* and their relationship with MIC of ofloxacin. *The Journal of antibiotics*. 2015 Jan;68(1):63-6.

235. Rueda J, Realpe T, Mejia GI, Zapata E, Rozo JC, Ferro BE, et al. Genotypic Analysis of Genes Associated with Independent Resistance and Cross-Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis* Clinical Isolates. *Antimicrob Agents Chemother*. 2015 Dec;59(12):7805-10.

236. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2011 Jan;55(1):355-60.

237. Rengarajan J, Sasseti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. *Molecular microbiology*. 2004 Jul;53(1):275-82.

238. Minato Y, Thiede JM, Kordus SL, McKlveen EJ, Turman BJ, Baughn AD. *Mycobacterium tuberculosis* folate metabolism and the mechanistic basis for para-aminosalicylic acid susceptibility and resistance. *Antimicrob Agents Chemother*. 2015 Sep;59(9):5097-106.

239. Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, et al. para-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. *The Journal of biological chemistry*. 2013 Aug 9;288(32):23447-56.

240. Zhao F, Wang XD, Erber LN, Luo M, Guo AZ, Yang SS, et al. Binding pocket alterations in dihydrofolate synthase confer resistance to para-aminosalicylic acid in clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2014;58(3):1479-87.

241. Zhang X, Liu L, Zhang Y, Dai G, Huang H, Jin Q. Genetic determinants involved in p-aminosalicylic acid resistance in clinical isolates from tuberculosis patients in northern China from 2006 to 2012. *Antimicrob Agents Chemother*. 2015 Feb;59(2):1320-4.

242. Desjardins CA, Cohen KA, Munsamy V, Abeel T, Maharaj K, Walker BJ, et al. Genomic and functional analyses of *Mycobacterium tuberculosis* strains implicate *ald* in D-cycloserine resistance. *Nature genetics*. 2016 May;48(5):544-51.

243. Zhang S, Chen J, Cui P, Shi W, Zhang W, Zhang Y. Identification of novel mutations associated with clofazimine resistance in *Mycobacterium tuberculosis*. *The Journal of antimicrobial chemotherapy*. 2015 Sep;70(9):2507-10.

244. Andries K, Villellas C, Coeck N, Thys K, Gevers T, Vranckx L, et al. Acquired resistance of *Mycobacterium tuberculosis* to bedaquiline. *PloS one*. 2014;9(7):e102135.

245. Almeida D, Ioerger T, Tyagi S, Li SY, Mdluli K, Andries K, et al. Mutations in *pepQ* Confer Low-Level Resistance to Bedaquiline and Clofazimine in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2016 Aug;60(8):4590-9.

246. Hillemann D, Rusch-Gerdes S, Richter E. In vitro-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob Agents Chemother*. 2008 Feb;52(2):800-1.

247. Beckert P, Hillemann D, Kohl TA, Kalinowski J, Richter E, Niemann S, et al. rplC T460C identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. *Antimicrob Agents Chemother*. 2012 May;56(5):2743-5.
248. Somoskovi A, Bruderer V, Homke R, Bloemberg GV, Bottger EC. A mutation associated with clofazimine and bedaquiline cross-resistance in MDR-TB following bedaquiline treatment. *The European respiratory journal*. 2015 Feb;45(2):554-7.
249. Gurusurthy M, Mukherjee T, Dowd CS, Singh R, Niyomrattanakit P, Tay JA, et al. Substrate specificity of the deazaflavin-dependent nitroreductase from *Mycobacterium tuberculosis* responsible for the bioreductive activation of bicyclic nitroimidazoles. *The FEBS journal*. 2012 Jan;279(1):113-25.
250. Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, et al. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*. 2006 Jan 10;103(2):431-6.
251. Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R, et al. PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. *Science*. 2008 Nov 28;322(5906):1392-5.
252. Haver HL, Chua A, Ghode P, Lakshminarayana SB, Singhal A, Mathema B, et al. Mutations in genes for the F420 biosynthetic pathway and a nitroreductase enzyme are the primary resistance determinants in spontaneous in vitro-selected PA-824-resistant mutants of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2015 Sep;59(9):5316-23.
253. Sacksteder KA, Protopopova M, Barry CE, 3rd, Andries K, Nacy CA. Discovery and development of SQ109: a new antitubercular drug with a novel mechanism of action. *Future microbiology*. 2012 Jul;7(7):823-37.
254. Jia L, Coward L, Gorman GS, Noker PE, Tomaszewski JE. Pharmacoproteomic effects of isoniazid, ethambutol, and N-geranyl-N'-(2-adamantyl)ethane-1,2-diamine (SQ109) on *Mycobacterium tuberculosis* H37Rv. *The Journal of pharmacology and experimental therapeutics*. 2005 Nov;315(2):905-11.
255. Tahlan K, Wilson R, Kastrinsky DB, Arora K, Nair V, Fischer E, et al. SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2012 Apr;56(4):1797-809.
256. Protopopova M, Hanrahan C, Nikonenko B, Samala R, Chen P, Gearhart J, et al. Identification of a new antitubercular drug candidate, SQ109, from a combinatorial library of 1,2-ethylenediamines. *The Journal of antimicrobial chemotherapy*. 2005 Nov;56(5):968-74.
257. Li W, Upadhyay A, Fontes FL, North EJ, Wang Y, Crans DC, et al. Novel insights into the mechanism of inhibition of MmpL3, a target of multiple pharmacophores in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2014 Nov;58(11):6413-23.
258. Lun S, Guo H, Onajole OK, Pieroni M, Gunosewoyo H, Chen G, et al. Indoleamides are active against drug-resistant *Mycobacterium tuberculosis*. *Nature communications*. 2013;4:2907.
259. Li K, Schurig-Briccio LA, Feng X, Upadhyay A, Pujari V, Lechartier B, et al. Multitarget drug discovery for tuberculosis and other infectious diseases. *Journal of medicinal chemistry*. 2014 Apr 10;57(7):3126-39.
260. STS/BMRC. Five-year follow-up of a clinical trial of three 6-month regimens of chemotherapy given intermittently in the continuation phase in the treatment of pulmonary tuberculosis. Singapore Tuberculosis Service/British Medical Research Council. *The American review of respiratory disease*. 1988 May;137(5):1147-50.
261. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. *Lancet*. 2010 May 22;375(9728):1830-43.

262. Almeida Da Silva PE, Palomino JC. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. The Journal of antimicrobial chemotherapy. 2011 Jul;66(7):1417-30.
263. Black PA, de Vos M, Louw GE, van der Merwe RG, Dippenaar A, Streicher EM, et al. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in *Mycobacterium tuberculosis* isolates. BMC genomics. 2015;16:857.
264. Spies FS, von Groll A, Ribeiro AW, Ramos DF, Ribeiro MO, Dalla Costa ER, et al. Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes. Tuberculosis. 2013 Mar;93(2):150-4.
265. Muller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. Trends in genetics : TIG. 2013 Mar;29(3):160-9.
266. Mariam SH, Werngren J, Aronsson J, Hoffner S, Andersson DI. Dynamics of antibiotic resistant *Mycobacterium tuberculosis* during long-term infection and antibiotic treatment. PloS one. 2011;6(6):e21147.
267. Warner DF, Koch A, Mizrahi V. Diversity and disease pathogenesis in *Mycobacterium tuberculosis*. Trends in microbiology. 2015 Jan;23(1):14-21.
268. Trauner A, Borrell S, Reither K, Gagneux S. Evolution of drug resistance in tuberculosis: recent progress and implications for diagnosis and therapy. Drugs. 2014 Jul;74(10):1063-72.
269. Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. Clin Microbiol Infect. 2007 Jan;13(1):5-18.
270. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nature genetics. 2013 Jul;45(7):784-90.
271. McGrath M, Gey van Pittius NC, Sirgel FA, Van Helden PD, Warren RM. Moxifloxacin retains antimycobacterial activity in the presence of *gyrA* mutations. Antimicrob Agents Chemother. 2014 May;58(5):2912-5.
272. Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. Evolutionary applications. 2015 Mar;8(3):273-83.
273. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nature reviews Microbiology. 2010 Apr;8(4):260-71.
274. Colijn C, Cohen T, Ganesh A, Murray M. Spontaneous emergence of multiple drug resistance in tuberculosis before and during therapy. PloS one. 2011;6(3):e18327.
275. Gao J, Ma Y, Du J, Zhu G, Tan S, Fu Y, et al. Later emergence of acquired drug resistance and its effect on treatment outcome in patients treated with Standard Short-Course Chemotherapy for tuberculosis. BMC pulmonary medicine. 2016 Feb 04;16:26.
276. Meacci F, Orru G, Iona E, Giannoni F, Piersimoni C, Pozzi G, et al. Drug resistance evolution of a *Mycobacterium tuberculosis* strain from a noncompliant patient. J Clin Microbiol. 2005 Jul;43(7):3114-20.
277. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. Genome biology. 2014;15(11):490.
278. Shamputa IC, Rigouts L, Eyongeta LA, El Aila NA, van Deun A, Salim AH, et al. Genotypic and phenotypic heterogeneity among *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients. J Clin Microbiol. 2004 Dec;42(12):5528-36.
279. Sun G, Luo T, Yang C, Dong X, Li J, Zhu Y, et al. Dynamic population changes in *Mycobacterium tuberculosis* during acquisition and fixation of drug resistance in patients. The Journal of infectious diseases. 2012 Dec 1;206(11):1724-33.

280. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, et al. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2012 Jun;56(6):3047-53.
281. Hillemann D, Kubica T, Rusch-Gerdes S, Niemann S. Disequilibrium in distribution of resistance mutations among *Mycobacterium tuberculosis* Beijing and non-Beijing strains isolated from patients in Germany. *Antimicrob Agents Chemother*. 2005 Mar;49(3):1229-31.
282. Hillemann D, Kubica T, Agzamova R, Venera B, Rusch-Gerdes S, Niemann S. Rifampicin and isoniazid resistance mutations in *Mycobacterium tuberculosis* strains isolated from patients in Kazakhstan. *Int J Tuberc Lung Dis*. 2005 Oct;9(10):1161-7.
283. Park YK, Shin S, Ryu S, Cho SN, Koh WJ, Kwon OJ, et al. Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of *Mycobacterium tuberculosis* in Korea. *Journal of microbiological methods*. 2005 Nov;63(2):165-72.
284. Qian L, Abe C, Lin TP, Yu MC, Cho SN, Wang S, et al. *rpoB* genotypes of *Mycobacterium tuberculosis* Beijing family isolates from East Asian countries. *J Clin Microbiol*. 2002 Mar;40(3):1091-4.
285. Bottger EC, Springer B. Tuberculosis: drug resistance, fitness, and strategies for global control. *European journal of pediatrics*. 2008 Feb;167(2):141-8.
286. Cohen T, Sommers B, Murray M. The effect of drug resistance on the fitness of *Mycobacterium tuberculosis*. *The Lancet infectious diseases*. 2003 Jan;3(1):13-21.
287. Du Q, Dai G, Long Q, Yu X, Dong L, Huang H, et al. *Mycobacterium tuberculosis* rrs A1401G mutation correlates with high-level resistance to kanamycin, amikacin, and capreomycin in clinical isolates from mainland China. *Diagnostic microbiology and infectious disease*. 2013 Oct;77(2):138-42.
288. Cohen KA, Abeel T, Manson McGuire A, Desjardins CA, Munsamy V, Shea TP, et al. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates from KwaZulu-Natal. *PLoS Med*. 2015 Sep;12(9):e1001880.
289. Kempker RR, Kipiani M, Mirtskhulava V, Tukvadze N, Magee MJ, Blumberg HM. Acquired Drug Resistance in *Mycobacterium tuberculosis* and Poor Outcomes among Patients with Multidrug-Resistant Tuberculosis. *Emerg Infect Dis*. 2015 Jun;21(6):992-1001.
290. Salvatore PP, Becerra MC, Abel zur Wiesch P, Hinkley T, Kaur D, Sloutsky A, et al. Fitness Costs of Drug Resistance Mutations in Multidrug-Resistant *Mycobacterium tuberculosis*: A Household-Based Case-Control Study. *The Journal of infectious diseases*. 2016 Jan 01;213(1):149-55.
291. Mokrousov I, Jiao WW, Sun GZ, Liu JW, Valcheva V, Li M, et al. Evolution of drug resistance in different sublineages of *Mycobacterium tuberculosis* Beijing genotype. *Antimicrob Agents Chemother*. 2006 Aug;50(8):2820-3.
292. Nguyen HQ, Nguyen NV, Contamin L, Tran TH, Vu TT, Nguyen HV, et al. Quadruple-first line drug resistance in *Mycobacterium tuberculosis* in Vietnam: What can we learn from genes? *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2017 Feb 21;50:55-61.
293. Mokrousov I, Vyazovaya A, Otten T, Zhuravlev V, Pavlova E, Tarashkevich L, et al. *Mycobacterium tuberculosis* population in northwestern Russia: an update from Russian-EU/Latvian border region. *PloS one*. 2012;7(7):e41318.
294. Mariam DH, Mengistu Y, Hoffner SE, Andersson DI. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2004 Apr;48(4):1289-94.
295. Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced *in vitro* in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 1999 Aug;43(8):1866-9.

296. Bottger EC, Springer B, Pletschette M, Sander P. Fitness of antibiotic-resistant microorganisms and compensatory mutations. *Nature medicine*. 1998 Dec;4(12):1343-4.
297. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant *Mycobacterium tuberculosis*. *Nature genetics*. 2013 Oct;45(10):1183-9.
298. Osorio NS, Rodrigues F, Gagneux S, Pedrosa J, Pinto-Carbo M, Castro AG, et al. Evidence for diversifying selection in a set of *Mycobacterium tuberculosis* genes in response to antibiotic- and nonantibiotic-related pressure. *Molecular biology and evolution*. 2013 Jun;30(6):1326-36.
299. Huitric E, Werngren J, Jureen P, Hoffner S. Resistance levels and *rpoB* gene mutations among in vitro-selected rifampin-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob Agents Chemother*. 2006 Aug;50(8):2860-2.
300. Morlock GP, Plikaytis BB, Crawford JT. Characterization of spontaneous, In vitro-selected, rifampin-resistant mutants of *Mycobacterium tuberculosis* strain H37Rv. *Antimicrob Agents Chemother*. 2000 Dec;44(12):3298-301.
301. den Hertog AL, Sengstake S, Anthony RM. Pyrazinamide resistance in *Mycobacterium tuberculosis* fails to bite? *Pathogens and disease*. 2015 Aug;73(6):ftv037.
302. Muller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, et al. Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. *PloS one*. 2013;8(8):e70919.
303. Brandis G, Wrande M, Liljas L, Hughes D. Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. *Molecular microbiology*. 2012 Jul;85(1):142-51.
304. Levin BR, Perrot V, Walker N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics*. 2000 Mar;154(3):985-97.
305. Reynolds MG. Compensatory evolution in rifampin-resistant *Escherichia coli*. *Genetics*. 2000 Dec;156(4):1471-81.
306. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nature genetics*. 2012 Jan;44(1):106-10.
307. de Vos M, Muller B, Borrell S, Black PA, van Helden PD, Warren RM, et al. Putative compensatory mutations in the *rpoC* gene of rifampin-resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. *Antimicrob Agents Chemother*. 2013 Feb;57(2):827-32.
308. Song T, Park Y, Shamputa IC, Seo S, Lee SY, Jeon HS, et al. Fitness costs of rifampicin-resistance in *Mycobacterium tuberculosis* are amplified under conditions of nutrient starvation and compensated by mutation in the beta' subunit of RNA polymerase. *Molecular microbiology*. 2014 Jan 14.
309. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE, 3rd, et al. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science*. 1996 Jun 14;272(5268):1641-3.
310. Brandis G, Hughes D. Genetic characterization of compensatory evolution in strains carrying *rpoB* Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. *The Journal of antimicrobial chemotherapy*. 2013 Nov;68(11):2493-7.
311. Li QJ, Jiao WW, Yin QQ, Xu F, Li JQ, Sun L, et al. Compensatory Mutations of Rifampin Resistance Are Associated with Transmission of Multidrug-Resistant *Mycobacterium tuberculosis* Beijing Genotype Strains in China. *Antimicrob Agents Chemother*. 2016 May;60(5):2807-12.
312. Phillips PC. Epistasis--the essential role of gene interactions in the structure and evolution of genetic systems. *Nature reviews Genetics*. 2008 Nov;9(11):855-67.
313. Lehner B. Molecular mechanisms of epistasis within and between genes. *Trends in genetics : TIG*. 2011 Aug;27(8):323-31.

314. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive epistasis drives the acquisition of multidrug resistance. *PLoS genetics*. 2009 Jul;5(7):e1000578.
315. Borrell S, Gagneux S. Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect*. 2011 Jun;17(6):815-20.
316. Nhung NV, Hoa NB, Sy DN, Hennig CM, Dean AS. The fourth national anti-tuberculosis drug resistance survey in Viet Nam. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease*. 2015 Jun;19(6):670-5.
317. Vietnam national tuberculosis control programme N. Guidline for management of multi-drug resistance tuberculosis. 2009.
318. Hoa NB, Nhung NV, Khanh PH, Hai NV, Quyen BT. Adverse events in the treatment of MDR-TB patients within and outside the NTP in Pham Ngoc Thach hospital, Ho Chi Minh City, Vietnam. *BMC research notes*. 2015;8:809.
319. Hoa Binh Nguyen NVN, Huong Thi Giang Tran, Hai Viet Nguyen and Quyen Thi Tu Bui. Prevalence of resistance to second-line tuberculosis drug among multidrugresistant tuberculosis patients in Viet Nam, 2011. www.prowhoint/wpsar. 2016;WPSAR Vol 7, No 2, 2016(doi: 10.5365/wpsar.2016.7.2.002).
320. Hoang TT, Nguyen NV, Dinh SN, Nguyen HB, Cobelens F, Thwaites G, et al. Challenges in detection and treatment of multidrug resistant tuberculosis patients in Vietnam. *BMC public health*. 2015;15:980.
321. Phuong NT, Nhung NV, Hoa NB, Thuy HT, Takarinda KC, Tayler-Smith K, et al. Management and treatment outcomes of patients enrolled in MDR-TB treatment in Viet Nam. *Public health action*. 2016 Mar 21;6(1):25-31.
322. Caws M, Duy, P.M., Tho, D.Q., Lan, N.T., Hoa, D.V. and Farrar, J. Mutations prevalent among rifampin and isoniazid resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam. *J Clin Microbiol*. 2006;44: 2333-7.
323. Hung NV, Ando H, Thuy TT, Kuwahara T, Hang NT, Sakurada S, et al. Clonal expansion of *Mycobacterium tuberculosis* isolates and coexisting drug resistance in patients newly diagnosed with pulmonary tuberculosis in Hanoi, Vietnam. *BMC research notes*. 2013;6:444.
324. Huyen MN, Tiemersma EW, Lan NT, Cobelens FG, Dung NH, Sy DN, et al. Validation of the GenoType MTBDRplus assay for diagnosis of multidrug resistant tuberculosis in South Vietnam. *BMC infectious diseases*. 2010;10:149.
325. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, et al. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. *Emerg Infect Dis*. 2000 May-Jun;6(3):302-5.
326. Van Anh Thi Nguyen MC, Duy Hung Nguyen, Thanh Hoa Thi Tran, Kim Lien Thi Pham, Phuong Thao Thi Dinh, Jules Philippe, Thai Son Nguyen, Minh Ly Ho, Sang Van Tran, Anne-Laure Bañuls, Duc Anh Dang. High Prevalence of Beijing and EAI4-VNM Genotypes among *M. tuberculosis* Isolates in Northern Vietnam: Sampling Effect, Rural and Urban Disparities. *PloS one*. 2012;7((9)): e45553. doi:10.1371/journal.pone.0045553.
327. Buu TN, van Soolingen D, Huyen MN, Lan NT, Quy HT, Tiemersma EW, et al. Increased transmission of *Mycobacterium tuberculosis* Beijing genotype strains associated with resistance to streptomycin: a population-based study. *PloS one*. 2012;7(8):e42323.
328. Huyen MN, Kremer K, Lan NT, Buu TN, Cobelens FG, Tiemersma EW, et al. Clustering of Beijing genotype *Mycobacterium tuberculosis* isolates from the Mekong delta in Vietnam on the basis of variable number of tandem repeat versus restriction fragment length polymorphism typing. *BMC infectious diseases*. 2013;13:63.
329. Maeda S, Hang NT, Lien LT, Thuong PH, Hung NV, Hoang NP, et al. *Mycobacterium tuberculosis* strains spreading in Hanoi, Vietnam: Beijing sublineages, genotypes, drug susceptibility patterns, and host factors. *Tuberculosis*. 2014 Dec;94(6):649-56.

330. Buu TN, Huyen MN, Lan NN, Quy HT, Hen NV, Zignol M, et al. *Mycobacterium tuberculosis* genotype and case notification rates, rural Vietnam, 2003-2006. *Emerg Infect Dis*. 2009 Oct;15(10):1570-7.
331. Hang NT, Maeda S, Lien LT, Thuong PH, Hung NV, Thuy TB, et al. Primary drug-resistant tuberculosis in Hanoi, Viet Nam: present status and risk factors. *PLoS one*. 2013;8(8):e71867.
332. Nguyen VA, Nguyen HQ, Vu TT, Nguyen NA, Duong CM, Tran TH, et al. Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2015 Dec;21(12):1084-92.
333. WHO. Guidelines of surveillance of drug resistance in tuberculosis. Second Edition. WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance Geneva 2003. 2003;WHO/CDS/CSR/RMD/2003.3.
334. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of clinical microbiology*. 1997 Apr;35(4):907-14.
335. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. *The New England journal of medicine*. 2003 Sep 18;349(12):1149-56.
336. Allix-Beguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol*. 2008 Aug;46(8):2692-9.
337. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2006 Dec;44(12):4498-510.
338. Frothingham R, Meeker-O'Connell WA. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology*. 1998 May;144 (Pt 5):1189-96.
339. Allix-Beguec C, Fauville-Dufaux M, Supply P. Three-year population-based evaluation of standardized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2008 Apr;46(4):1398-406.
340. Allix-Beguec C, Wahl C, Hanekom M, Nikolayevskyy V, Drobniewski F, Maeda S, et al. Proposal of a consensus set of hypervariable mycobacterial interspersed repetitive-unit-variable-number tandem-repeat loci for subtyping of *Mycobacterium tuberculosis* Beijing isolates. *J Clin Microbiol*. 2014 Jan;52(1):164-72.
341. Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTRplus: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. *Nucleic acids research*. 2010 Jul;38(Web Server issue):W326-31.
342. Shriver MD, Jin L, Boerwinkle E, Deka R, Ferrell RE, Chakraborty R. A novel measure of genetic distance for highly polymorphic tandem repeat loci. *Molecular biology and evolution*. 1995 Sep;12(5):914-20.
343. Chevenet F, Brun C, Banuls AL, Jacq B, Christen R. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC bioinformatics*. 2006 Oct 10;7:439.
344. Chevenet F, Jung M, Peeters M, de Oliveira T, Gascuel O. Searching for virus phylotypes. *Bioinformatics*. 2013 Mar 1;29(5):561-70.
345. Ali A, Hasan R, Jabeen K, Jabeen N, Qadeer E, Hasan Z. Characterization of mutations conferring extensive drug resistance to *Mycobacterium tuberculosis* isolates in Pakistan. *Antimicrob Agents Chemother*. 2011 Dec;55(12):5654-9.

346. Rodwell TC, Valafar F, Douglas J, Qian L, Garfein RS, Chawla A, et al. Predicting extensively drug-resistant *Mycobacterium tuberculosis* phenotypes with genetic mutations. *J Clin Microbiol*. 2014 Mar;52(3):781-9.
347. Zhao LL, Chen Y, Liu HC, Xia Q, Wu XC, Sun Q, et al. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolates from China. *Antimicrob Agents Chemother*. 2014;58(4):1997-2005.
348. Theron G, Peter J, Richardson M, Barnard M, Donegan S, Warren R, et al. The diagnostic accuracy of the GenoType((R)) MTBDRsl assay for the detection of resistance to second-line anti-tuberculosis drugs. *The Cochrane database of systematic reviews*. 2014;10:CD010705.
349. Handel A, Regoes RR, Antia R. The role of compensatory mutations in the emergence of drug resistance. *PLoS computational biology*. 2006 Oct;2(10):e137.
350. Jagielski T, Bakula Z, Roeske K, Kaminski M, Napiorkowska A, Augustynowicz-Kopec E, et al. Detection of mutations associated with isoniazid resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. *The Journal of antimicrobial chemotherapy*. 2014 Sep;69(9):2369-75.
351. Zhang Z, Pang Y, Wang Y, Liu C, Zhao Y. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with linezolid resistance in multidrug-resistant and extensively drug-resistant tuberculosis in China. *Int J Antimicrob Agents*. 2014 Mar;43(3):231-5.
352. Ebrahimi-Rad M, Bifani P, Martin C, Kremer K, Samper S, Rauzier J, et al. Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerg Infect Dis*. 2003 Jul;9(7):838-45.
353. Li D, Hu Y, Werngren J, Mansjo M, Zheng X, Drobniewski F, et al. Multicenter Study of the Emergence and Genetic Characteristics of Pyrazinamide-Resistant Tuberculosis in China. *Antimicrob Agents Chemother*. 2016 Sep;60(9):5159-66.
354. Whitfield MG, Streicher EM, Dolby T, Simpson JA, Sampson SL, Van Helden PD, et al. Prevalence of pyrazinamide resistance across the spectrum of drug resistant phenotypes of *Mycobacterium tuberculosis*. *Tuberculosis*. 2016 Jul;99:128-30.
355. Zignol M, Dean AS, Alikhanova N, Andres S, Cabibbe AM, Cirillo DM, et al. Population-based resistance of *Mycobacterium tuberculosis* isolates to pyrazinamide and fluoroquinolones: results from a multicountry surveillance project. *The Lancet infectious diseases*. 2016 Oct;16(10):1185-92.
356. Willby M, Sikes RD, Malik S, Metchock B, Posey JE. Correlation between GyrA substitutions and ofloxacin, levofloxacin, and moxifloxacin cross-resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2015 Sep;59(9):5427-34.
357. Aono A, Chikamatsu K, Yamada H, Kato T, Mitarai S. Association between *pncA* gene mutations, pyrazinamidase activity, and pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2014 Aug;58(8):4928-30.
358. Fonseca JD, Knight GM, McHugh TD. The complex evolution of antibiotic resistance in *Mycobacterium tuberculosis*. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*. 2015 Mar;32:94-100.

Appendix

Table A 1. Critical concentrations for first-line and second-line drug susceptibility testing (Source from WHO 2012)

Drug group ^a	Drug	DST method available	DST critical concentrations (µg/ml)			
			Löwenstein-Jensen ^b	Middlebrook 7H10 ^b	Middlebrook 7H11 ^b	MGIT960
Group 1 First-line oral anti-TB agents	Isoniazid	Solid, liquid	0.2	0.2	0.2	0.1
	Rifampicin ^c	Solid, liquid	40.0	1.0	1.0	1.0
	Ethambutol ^d	Solid, liquid	2.0	5.0	7.5	5.0
	Pyrazinamide	Liquid	-	-	-	100.0
Group 2 Injectable anti-TB agents	Streptomycin ^e	Solid, liquid	4.0	2.0	2.0	1.0
	Kanamycin	Solid, liquid	30.0	5.0	6.0	2.5
	Amikacin	Solid, liquid	30.0	4.0	-	1.0
	Capreomycin	Solid, liquid	40.0	4.0	-	2.5
Group 3 Fluoroquinolones	Ofloxacin ^f	Solid, liquid	4.0	2.0	2.0	2.0
	Levofloxacin	Solid, liquid	-	1.0	-	1.5
	Moxifloxacin ^g	Solid, liquid	-	0.5/2.0	-	0.5/2.0
	Gatifloxacin ^h	Solid	-	1.0	-	-
Group 4 ⁱ Oral bacteriostatic second-line anti-TB agents	Ethionamide	Solid, liquid	40.0	5.0	10.0	5.0
	Prothionamide	Solid, liquid	40.0	-	-	2.5
	Cycloserine	Solid	30.0	-	-	-
	<i>P</i> -aminosalicylic acid	Solid, liquid	1.0	2.0	8.0	4.0
Group 5 ^j Antituberculosis agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients)	Clofazimine	Liquid	-	-	-	-
	Amoxicillin/clavulanate	None	-	-	-	-
	Clarithromycin	None	-	-	-	-
	Linezolid	Liquid	-	-	-	1.0

^a WHO *Guidelines for the programmatic management of drug-resistant tuberculosis*.

^b Indirect proportion method recommended. Other solid media methods (resistance ratio) have not been adequately validated for second-line drugs. Concentrations for the absolute concentration method were not evaluated.

^c Rifampicin borderline resistance more frequently missed by MGIT. Prevalence and geographical distribution of borderline resistance not clear, final LJ interpretations should be made after 6 weeks

^d Ethambutol 5µg/ml in MGIT is not equivalent to other methods. Ethambutol testing in 7H11 not equivalent to 7H10. There is insufficient evidence to recommend a change in concentration for any method

^e Streptomycin has a bimodal distribution of MIC values. Insufficient evidence to recommend a change.

^f Ofloxacin concentration in LJ media increased to 4.0µg/ml. Insufficient data to extrapolate change in 7H10 or 7H11 methods.

^g Moxifloxacin. Two concentrations proposed. In programmes using both ofloxacin/levofloxacin and moxifloxacin, possible testing is for moxifloxacin only at both concentrations OR test ofloxacin/levofloxacin and moxifloxacin at higher concentration. In programmes using ofloxacin/levofloxacin only test only these drugs. In programmes using only moxifloxacin test at higher concentration of moxifloxacin only.

^h Gatifloxacin only to be used in exceptional circumstances.

ⁱ Routine DST for group 4 and 5 drugs is not recommended. Linezolid suitable for testing in MGIT only.

196	Srd51	2009	PNT	HRSE	1101111111111011111001111100001011111111	16	EAI-like	322221434144867262333263	130
197	S117	2008	PNT	HRSE	1101111111101111111111111100001011111111	15	EAI	623241425341256242333253	174
198	S118	2008	PNT	HRSE	1111111111111111111111100100001011111111	52	EAI	923241425247256242333363	190
199	S300	2008	PNT	HRSE	1111111111111111111111100100001011111111	52	EAI	923241425247256242333352	189
200	S246	2005	PNT	HRSE	1111111110111111100000000000000000000111	27	EAI	623241425247256242333353	171
201	S224	2008	PNT	HRSE	1111111111111111111111100100000011111111	49	EAI	622241425247256242333253	151
202	S247	2005	PNT	HRSE	1111111111111111100000000000000000000111	41	EAI	623241425246256242333353	166
203	S255	2006	PNT	HRSE	1111110011111111111111100000000000000111	23	EAI-like	1232412352471064042333253	5
204	Srd24	2008	PNT	HRSE	111111111101111111111111101001011111111	35	EAI	633241415247246252333253	183
205	S221	2008	PNT	HRSE	11111111111111111111111000000111111111	59	EAI	623241425247274242333253	173
206	N23S	2007	NLH	Sensitive	1111011111111111111110111111100001111111	64	T	252232344242525122335252	34
207	N40S	2008	NLH	Sensitive	11111111110111111111111011000011111111	68	T	212433512442137112234252	18
208	Nrd2	2008	NLH	Sensitive	101111111111111111111110100001110111	63	T	253232233143337112334252	39
209	Nrd28	2008	NLH	Sensitive	111111111111111111111110000111111111	76	T	212433513343133122314252	20
210	Nrd37	2008	NLH	Sensitive	111111111111111111111110000111111111	76	T	452222233564518122234252	134
211	N32	2008	NLH	Sensitive	11111111111111111111101100001110000	73	T	254232332243533122334252	49
212	N36	2008	NLH	Sensitive	111111111110111111111111100001110000	71	T	254232332243533122334252	49
213	S151	2008	PNT	Sensitive	1111111111111111111111100001110000	75	T	254232343242525122334252	50
214	S156	2005	PNT	Sensitive	111111111101111111111111100001110111	69	T	25142333333526132334252	32
215	S167	2005	PNT	Sensitive	1111111111110000000000111111100001111111	70	T	233122432322236122334262	24
216	S170	2005	PNT	Sensitive	111111111111111111111110000111111111	76	T	252242232342326122335252	36
217	Nrd23	2008	NLH	H	111111111111111111111110000111111111	76	T	212433513243137122334252	19

No of spoligotype (in this study): numbering of spoligotypes found in this study (coded from 1 – 101)

No of MIRU-VNTR genotype (in this study): numbering of MIRU-VNTR genotypes found in this study (coded from 1 – 192)

Table A 3. Mutation patterns of the 260 clinical *Mycobacterium tuberculosis* isolates in Vietnam

Strain	Family	FLD susceptibility phenotype	SLD susceptibility phenotype	Mutation patterns
Nrd77	Beijing	H	NA	katG315Thr
N2	Beijing	H	NA	katG315Thr
N3	Beijing	H	NA	katG315Thr
Nrd83	Beijing	HRSE	KAC	katG315Thr & rpoB531Leu & rpsL88Arg & pncA deletion & rrs1484T & rrs1484T & rpoC483Gly
Nrd95	Beijing	HRSE	Sensitive	katG315Thr & rpoB572Phe & rpsL43Arg & embB497Pro
Nrd11	Beijing	HRSE	KAC	katG315Thr & inhA15T & rpoB531Leu & embB360Met & pncA82Gln & rrs1401G & rpoC416Ser
Srd87	Beijing	H	NA	katG315Thr
S98	Beijing	HRS	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & rpoC448Glu
N36S	Beijing	Sensitive	NA	WT
N50	Beijing	S	NA	rrs514C
S134	Beijing	H	Sensitive	katG315Thr
S56	Beijing	HRSE	OM	katG394Ala & inhA15T & inhA94Ala & rpoB531Leu & rrs514C & embB497Arg & pncA116Pro & gyrA94Tyr
S70	Beijing	HRSE	C	katG315Thr & rpoB511Pro & rrs514C & embB306Leu & pncA102Val
S120	Beijing	HRSE	Sensitive	inhA15T & inhA94Ala & rpoB531Leu & rrs514C & embB497Arg
S126	Beijing	HRSE	KACOM	inhA15T & inhA94Ala & rpoB531Leu & rrs514C & embB406Ser & pncA108Stop & rrs1401G & gyrA90Val
Nrd27	Beijing	HRSE	Sensitive	katG315Thr & rpoB516Val & rpsL88Arg & embB306Val
S80	Beijing	HRS	Sensitive	katG315Thr & rpoB516Val & rpsL88Arg & pncA10Pro & pncA10Pro & rpoC416Ser
S8	Beijing	HRSE	OM	katG315Thr & rpoB531Leu & rpsL88Arg & embB306Val & pncA96Glu & gyrA94Gly

S132	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL88Arg & embB306Ile & embB360Met & pncA49Ala & rpoC525Asn
S30	Beijing	HRSE	NA	katG315Thr & rpoB146Phe & rpsL88Arg & embB306Val & pncA11G & gyrA90Val & rpoC483Gly
C108	Beijing	HRS	NA	katG315Thr & rpoB533Pro & rpsL88Arg & pncA11G
Nrd7	EAI	H	NA	katG315Thr
N5	EAI	H	NA	katG315Thr
N44d	EAI	HRSE	NA	katG315Thr & rpoB531Leu & rrs514C & embB306Ile & pncA104Arg & gyrA90Val
S188	EAI	H	Sensitive	katG315Thr
C214	EAI	H	NA	katG315Thr
S112	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & rpoC485Asn
Srd57	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Ile & pncA1Arg
Srd83	Beijing	HRSE	NA	katG315Thr & rpoB533Pro & rpsL43Arg & embB306Val & pncA62Leu & gyrA94Gly
Srd85	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL88Arg & embB330Ser & pncA130 insertion(GG) & rpoA183Ala
N38S	EAI	Sensitive	NA	WT
N39S	EAI	Sensitive	NA	WT
N40S	T	Sensitive	NA	WT
Nrd23	T	H	NA	katG315Thr
N17	EAI	S	NA	inhA21Val & rpsL43Arg & rrs517T
N46	T	S	NA	WT
N13	EAI	S	NA	rpsL88Met
Nrd9	T	HS	NA	inhA15T
N2d	T	HRS	NA	katG315Thr & rpoB526Asp & rrs514C & pncA7Phe
Nrd86	Beijing	HRSE	Sensitive	katG315Thr & rpoB526Tyr & rpsL43Arg & embB306Ile
N48d	Beijing	HRSE	Sensitive	katG315Thr & rpoB516Val & rpoB526Asp & rpsL88Arg & pncA164Stop
N50d	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rrs517T & embB306Val & pncA156Pro
Nrd47	T	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB354Ala & embB497Lys & pncA134Val

S161	EAI	S	Sensitive	WT
S246	EAI	HRSE	KACO	katG315Thr & rpoB526Asp & rrs514C & embB306Val & embB561Thr & pncA103Ser & rrs1401G & gyrA88Ala
C138	Beijing	HRS	NA	katG315Thr & katG710Phe & rpoB516Val & rpsL88Arg
C146	Beijing	HRSE	NA	katG315Thr & katG710Phe & rpoB516Val & rpsL88Arg & embB306Val & pncA96Glu
C147	Beijing	HRSE	NA	katG315Thr & katG710Phe & rpoB516Val & rpsL88Arg & embB306Val & pncA96Glu
Nrd74	EAI	Sensitive	NA	WT
N32	T	Sensitive	NA	WT
N36	T	Sensitive	NA	WT
Nrd80	Beijing	HS	NA	katG315Thr & rpsL43Arg & pncA128Gly
Nrd5	Beijing	HS	NA	katG315Asn & rpoB526Asn & rpsL43Arg & pncA28Thr & pncA140 deletion(G)
N17d	Beijing	HRS	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & pncA2Trp
N21d	U	HRS	NA	katG315Asn & rpoB516Val
N23d	U	HRS	Sensitive	katG315Asn & rpoB516Val
Nrd90	Beijing	HRSE	Sensitive	katG315Thr & rpoB572Phe & rpsL43Arg & embB497Arg & pncA19 deletion©
Nrd21	Beijing	HRSE	Sensitive	katG315Thr & rpoB526Asp & rpsL43Arg & embB306Val & pncA57Tyr
Nrd76	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & pncA141Pro & rpoA181Ala
N19	Beijing	S	NA	rpsL43Arg
S173	Beijing	Sensitive	NA	WT
S196	Beijing	Sensitive	Sensitive	WT
S211	EAI	Sensitive	Sensitive	WT
S107	Beijing	H	Sensitive	inhA15T
Srd89	Beijing	H	NA	katG315Thr & pncA149Ser
S165	EAI	H	Sensitive	katG315Thr
S130	Beijing	HRE	Sensitive	inhA15T & inhA194Thr & rpoB531Leu & embB306Val
Srd86	Beijing	HRS	NA	katG315Thr & rpoB516Val & rpsL43Arg & rrs151G & pncA130Gly

Srd81	EAI	HR	NA	inhA15T & inhA21Val & rpoB531Leu & embB577Ala & pncA155Gly & rrs1401G & gyrA94Gly
Srd82	Beijing	RSE	NA	katG285Ser & katG389Gly & rpoB516Val & rpoB757Ala & rpsL43Arg & embB306Ile
S202	Beijing	HRSE	KAC	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & pncA68Arg
S205	Beijing	HRSE	KAC	katG315Thr & rpoB572Phe & rpsL43Arg & embB306Val & pncA103Stop & rrs1001T & rrs1401G & rpoA202Thr
S85	Beijing	HRSE	KACOM	katG315Thr & rpoB516Val & rpsL43Arg & embB328Tyr & embB338Ser & pncA51Arg & rrs1401G & gyrA94Gly
Srd90	Beijing	HRSE	NA	katG315Thr & rpoB526Ser & rpoB527Gln & rpsL43Arg & embB306Ile
S27	Beijing	HRSE	Sensitive	katG315Thr & rpoB526Tyr & rpsL43Arg & embB306Ile & pncA deletion & rpoC448Glu
S224	EAI	HRSE	KACOM	inhA15T & inhA21Val & rpoB531Leu & embB577Ala & pncA155Gly & rrs1401G & gyrA94Gly
S247	EAI	HRSE	KACOM	katG315Thr & rpoB526Arg & rpsL43Arg & embB306Ile & rrs1401G & rpoC566Val
S221	EAI	HRSE	NA	katG315Thr & rpoB526Thr & rpsL88Arg & pncA130Gly
C84	Beijing	S	NA	rpsL43Arg
C209	EAI	HS	NA	katG315Thr & rrs517T & embB561Arg & pncA119Cys
C293	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL43Arg & pncA164 insertion(C)
Nrd39	Beijing	Sensitive	NA	WT
N22S	Beijing	Sensitive	NA	pncA103Asp
N45S	Beijing	Sensitive	NA	pncA138Ser
Nrd26	Beijing	HS	Sensitive	katG315Thr & rpsL43Arg
Nrd67	Beijing	HS	NA	katG315Thr & rpsL88Arg
Nrd96	Beijing	HS	NA	katG315Thr & rpsL43Arg & pncA142Ala
Nrd69	Beijing	HS	NA	katG315Thr & rpsL43Arg
Nrd6	Beijing	HRS	Sensitive	katG315Thr & rpoB522Leu & rpoB531Leu & rpsL43Arg & pncA11G
Nrd93	Beijing	HRSE	Sensitive	katG315Thr & rpoB533Pro & rpsL88Arg & pncA deletion
Nrd18	Beijing	HRSE	KACOM	katG315Thr & rpoB526Asp & rpsL43Arg & embB306Ile & gyrA94Ala
Nrd40	Beijing	HRSE	Sensitive	katG315Thr & rpoB533Pro & rpsL43Arg & embB306Ile & pncA11G
N22d	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB497Arg & pncA138Arg

S163	Beijing	Sensitive	Sensitive	WT
Srd58	Beijing	HRE	NA	katG315Thr & katG430Pro & rpoB531Leu & embB306Val & pncA120Pro
C109	Beijing	S	NA	rpsL88Arg
N2S	Beijing	Sensitive	NA	WT
N4S	EAI	Sensitive	NA	WT
N16S	EAI	Sensitive	NA	WT
N42S	EAI	Sensitive	NA	WT
N23S	T	Sensitive	NA	WT
Nrd52	EAI	Sensitive	NA	WT
Nrd65	EAI	Sensitive	NA	WT
Nrd68	EAI	Sensitive	NA	WT
Nrd79	EAI	Sensitive	NA	WT
Nrd98	EAI	Sensitive	NA	WT
Nrd2	T	Sensitive	NA	WT
Nrd28	T	Sensitive	NA	WT
Nrd34	EAI	Sensitive	NA	WT
Nrd37	T	Sensitive	NA	WT
Nrd25	EAI	Sensitive	NA	WT
Nrd56	U	Sensitive	NA	WT
Nrd59	U	Sensitive	NA	WT
Nrd66	U	Sensitive	NA	WT
N21S	U	Sensitive	NA	rrs295C
Nrd73	U	Sensitive	NA	WT
Nrd97	U	Sensitive	NA	WT
N14	U	Sensitive	NA	WT

Nrd55	LAM	Sensitive	NA	pncA49Glu
Nrd85	Beijing	H	NA	katG315Thr
N7	Beijing	H	NA	katG315Thr
N4	EAI	H	NA	katG315Thr
N1	U	H	NA	inhA15T
N15	EAI	S	NA	rrs517T
N8	T	S	NA	WT
N21	T	S	NA	rpsL43Arg
N38	T	S	NA	WT
N48	Beijing	S	NA	rpsL88Arg
N25	EAI	S	NA	rrs517T
N33	EAI	S	NA	rrs239T
Nrd19	Beijing	HS	Sensitive	katG315Thr & rpsL43Arg
Nrd57	Beijing	HS	NA	katG315Thr & rrs514C
Nrd87	Beijing	HS	NA	katG315Thr & rpsL88Arg
Nrd42	U	HS	NA	katG315Thr
N1d	EAI	HRS	NA	katG315Thr & inhA15T & rpoB526Arg
N15d	U	HRS	Sensitive	katG315Thr & rpoB531Leu & rpsL88Arg
Nrd62	Beijing	HRSE	Sensitive	katG315Thr & rpoB526Asp & rpsL43Arg & embB497Arg
Nrd36	Beijing	HRSE	NA	katG315Thr & rpoB526Asn & rpoB533Ser & rpsL43Arg & embB406Ala & pncA142Ala
Nrd38	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL88Arg & embB306Val & pncA76Pro & rpoC483Gly
Nrd35	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL88Arg
Nrd10	Beijing	HRSE	Sensitive	katG315Asn & rpoB526Asp & rrs514C & embB306Val
Nrd14	Beijing	HRSE	Sensitive	katG315Thr & rpoB515Ile & rpoB516Val & rpsL43Arg & embB306Ile & pncA137Arg
Nrd16	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB497Arg & pncA155Gly

Nrd51	Beijing	HRSE	K	katG315Thr & rpoB531Leu & rpsL43Asn & rrs514C & embB306Ile & embB497Lys & pncA47Ala & rpoC483Gly
Nrd53	Beijing	HRSE	KAC	katG315Thr & rpoB526Asp & rpoB562Ala & embB370Arg & embB497Lys & & rrs1401G
Nrd63	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg
Nrd92	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & pncA68Arg
N9d	Beijing	HRSE	OM	katG315Thr & rpoB572Phe & rpsL43Arg & embB497Arg & pncA19 deletion(C) & gyrB461Asn
N26d	EAI	HRSE	Sensitive	inhA15T & rpoB526Asp & rpsL88Arg & embB306Ile & pncA62Ser
N30d	EAI	HRSE	Sensitive	inhA15T & rpoB526Asp & embB306Ile & pncA62Ser
N36d	EAI	HRSE	NA	katG315Thr & inhA15T & rpoB526Asp & rrs908C & embB306Ile
N42d	EAI	HRSE	NA	katG315Thr & rpoB531Leu & rrs514C & embB306Ile & pncA58Val & gyrA90Val
N19d	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & pncA119Stop & rpoA187Ala
N20d	T	HRSE	OM	inhA15T & inhA94Ala & rpoB531Leu & rpsL43Arg & embB306Val & pncA161 insertion(G) & gyrA94Gly
N43d	T	HRSE	KACOM	inhA15T & inhA94Ala & rpoB531Leu & rpsL43Arg & embB306Val & pncA161 insertion(G) & gyrA94Gly
Nrd33	EAI	HRSE	Sensitive	katG315Asn & rpoB516Val & rrs878A & embB306Val
Nrd78	U	HRSE	Sensitive	katG480Ala & rpoB531Trp & rpoB616Ala & rrs517T & embB306Ile & pncA51Tyr
Nrd82	EAI	HRSE	Sensitive	katG315Asn & rpoB516Val & rrs878A & embB330Leu & pncA12G
N42	Beijing	S	NA	WT
Nrd8	Beijing	S	NA	rpsL43Arg
Nrd70	Beijing	S	NA	katG497Ser & rpsL43Arg
S158	Beijing	Sensitive	Sensitive	WT
S168	Beijing	Sensitive	Sensitive	WT
S178	Beijing	Sensitive	Sensitive	WT
S182	Beijing	Sensitive	Sensitive	WT
S231	Beijing	Sensitive	Sensitive	WT
S169	EAI-like	Sensitive	Sensitive	WT
S172	EAI-like	Sensitive	Sensitive	WT

S177	EAI	Sensitive	Sensitive	WT
S151	T	Sensitive	Sensitive	WT
S156	T	Sensitive	Sensitive	WT
S167	T	Sensitive	Sensitive	WT
S170	T	Sensitive	Sensitive	WT
S150	U	Sensitive	Sensitive	WT
S193	EAI-like	Sensitive	Sensitive	WT
S195	EAI	Sensitive	Sensitive	WT
S209	Beijing	Sensitive	Sensitive	WT
S152	LAM	Sensitive	Sensitive	WT
S174	Beijing	H	OMG	inhA15T & gyrA94Tyr
Srd91	Beijing	H	NA	katG315Thr
S157	EAI-like	H	Sensitive	inhA15T
S233	EAI	H	Sensitive	katG315Thr
S276	EAI	H	Sensitive	katG325Leu & ahpC-52T
Srd92	EAI-like	H	NA	pncA13T
Srd48	T	H	NA	katG315Thr
S212	U	H	Sensitive	inhA15T
S128	U	R	Sensitive	rpoB526Leu
S208	H	R	Sensitive	rpoB531Leu
S242	Beijing	S	Sensitive	rpsL88Arg
S214	Beijing	S	Sensitive	rrs514C
S192	Beijing	S	Sensitive	rpsL43Arg
S228	Beijing	S	KAC	rrs1401G
S164	EAI	S	Sensitive	WT

S171	EAI	S	Sensitive	WT
S191	EAI	S	Sensitive	WT
S277	EAI	S	Sensitive	WT
S141	EAI	S	Sensitive	WT
S61	U	S	Sensitive	WT
S113	Beijing	S	Sensitive	rpsL43Arg
S230	U	S	Sensitive	WT
Srd15	Beijing	E	NA	embB306Val
S119	EAI	HR	NA	katG112Ile & inhA15T & rpoB511Pro
Srd94	EAI	HR	NA	inhA95Val & rpoB526Tyr & rrs796G & pncA12Arg
S266	LAM	HR	OM	gyrA94Tyr & rpoC542Ala
S142	Beijing	HS	Sensitive	katG315Thr & rpsL88Arg
Srd28	EAI	HS	NA	katG315Thr & rpsL43Arg
Srd97	EAI-like	HS	NA	katG315Thr
Srd54	T	HS	NA	rpoB516Tyr & rrs513T & embB505Val & pncA11G
S76	EAI	HRE	Sensitive	katG315Thr & rpoB511Pro & rpoB531Leu & embB306Ile & pncA47Ile & rpoC491Val
S109	EAI	HRE	Sensitive	katG439Pro & rpoB531Leu & pncA6Leu
Srd37	EAI	HRE	NA	inhA15T & rpoB531Leu & embB406Ser & pncA76Pro
Srd47	EAI	HRE	NA	katG315Thr & rpoB513Pro & embB497Arg & pncA deletion
S57	Beijing	HRS	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & pncA6Thr
S84	Beijing	HRS	Sensitive	inhA15T & rpoB531Leu & rpsL43Arg & pncA96Thr
S124	Beijing	HRS	Sensitive	katG315Thr & rpoB526Tyr & rrs514C
S127	Beijing	HR	Sensitive	katG315Thr & rpoB531Leu & pncA103Ser
Srd29	EAI	HRS	NA	inhA8C & rpoB526Asn & rrs905G & pncA61Pro
Srd50	EAI	HRS	NA	katG315Thr & rpoB526Asp

S250	EAI	HRS	KAC	katG315Thr & rpoB531Gln & embB603Arg & pncA deletion12nu(-18)(-7) & rrs1401G
Srd52	EAI	HRS	NA	katG315Thr & rpoB516Tyr & rpoB518His & rrs517T & embB439Val & pncA63Gly
Srd99	Beijing	HSE	NA	katG315Thr & rpsL88Arg & embB306Val
S256	Beijing	HRSE	KACOM	katG315Thr & rpoB531Leu & rpsL43Arg & embB406Asp & rrs1401G
S90	Beijing	HRSE	OM	katG315Thr & rpoB531Leu & rpsL43Arg & rpoB306Ile & embB454Thr & pncA11G & gyrA94Gly
S114	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & pncA141Pro & rpoC483Ala
S116	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & rpoC452Ser
S201	Beijing	HRSE	Sensitive	katG315Thr & rpoB531Leu & rpsL88Met & embB330Leu & pncA146Pro
S104	Beijing	HRSE	KACOM	katG315Thr & rpoB516Val & rpsL88Arg & embB450Leu & pncA104Arg & rrs1401G & gyrA94Gly
S204	Beijing	HRSE	KACOM	katG315Thr & inhA8C & rpoB530Met & rpoB531Phe & rpsL43Arg & embB306Ile & gyrA94Ala
S55	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Ile & pncA35Pro & rrs1401G & gyrA94Tyr
Srd55	Beijing	HRSE	NA	katG315Thr & inhA15T & rpoB531Leu & rpsL88Arg & embB497Arg & pncA154Gly
Srd56	Beijing	HRSE	NA	katG315Thr & rpoB511Pro & rpoB526Asp & rpsL88Arg & embB306Val
Srd70	Beijing	HRSE	NA	katG315Thr & rpoB516Val & rpsL43Arg & embB306Val & pncA181Stop
Srd80	Beijing	HRSE	NA	katG315Thr & rpoB526Asp & rpoB533Pro & rpsL43Arg & embB306Val & pncA142Arg & rrs1401G & gyrA94Gly
Srd51	EAI-like	HRSE	NA	katG33Asp & katG315Thr & rpoB531Leu & rrs517TembB306Ile & pncA49Glu
S117	EAI	HRSE	Sensitive	katG257Ile & inhA15T & rpoB531Gln & rrs514C & embB354Ala
S118	EAI	HRSE	Sensitive	katG315Thr & rpoB531Leu & rrs517T & pncA63Ala
S300	EAI	HRSE	Sensitive	katG315Thr & rpoB526Asp & rpsL88Thr & embB306Val & pncA59Phe
Srd59	T	HRSE	NA	rpoB512 Insertion(Arg) & rpsL43Arg & embB406Ser & pncA54Gln
S83	T	HRSE	Sensitive	katG315Thr & rpoB526Tyr & embB306Val & rpoC448Glu
S199	T	HRSE	NA	katG315Thr & rpoB490Arg & rpoB531Trp & rpsL88Met & embB306Ile & pncA11G
S16	T	HRSE	OM	katG315Thr & rpoB531Leu & embB306Val & pncA57Pro & gyrA94Gly
S220	T	HRSE	KOM	katG315Thr & rpoB518 deletion(Asn) & pncA54Leu & gyrB504Val
Srd23	U	HRSE	NA	katG232Ala & rpoB531Leu & embB354Ala & embB497Pro & pncA10Arg

S38	Beijing	HRSE	Sensitive	katG315Thr & rpoB516Val & rrs514C & embB306Ile & pncA130 insertion(GG)
S51	Beijing	HRSE	Sensitive	katG315Thr & rpoB526Tyr & rpsL88Arg & embB306Val & pncA78 insertion(G)
S255	EAI-like	HRSE	NA	katG127Pro & inhA15T & rpoB526Tyr & rrs514C & embB306Ile & embB406Ala & gyrA90Val
Srd24	EAI	HRSE	NA	katG315Thr & rpoB526Asp & rpsL88Arg & embB406Asp & embB651Asn & pncA138Ser
Srd43	U	HRSE	NA	katG315Thr & rpoB513Leu & rpoB753Phe & rpsL43Arg & embB406Asp & pncA97Asp & pncA183 deletion(T)
S244	U	HRSE	KA	rpoB531Leu & pncA63 insertion(10nu) & pncA107Lys & ahpC-52T
C159	EAI	Sensitive	NA	WT
C79	EAI	H	NA	katG315Thr
C130	EAI	H	NA	katG169Ser & inhA15T
C01	U	HS	NA	katG315Thr
C116	Beijing	S	NA	rpsL88Arg
C254	EAI	HR	NA	katG31Asp & inhA15T & rpoB531Leu
C199	Beijing	HS	NA	katG315Thr & rpsL43Arg
C213	Beijing	HS	NA	katG315Thr & rpsL88Arg
C117	Beijing	HRS	NA	katG315Thr & rpoB526Arg & rrs908C & pncA deletion
C172	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL43Arg & embB306Val & pncA58Ser
C225	Beijing	HRSE	NA	katG315Thr & rpoB531Leu & rpsL88Met & embB306Val & pncA58Ser

NA: not available

WT: wild type (no drug resistance-associated mutation)