Isoniazid resistance in *Mycobacterium tuberculosis*

Thesis

ISONIAZID RESISTANCE

IN

MYCOBACTERIUM TUBERCULOSIS

by

DAU QUANG THO

A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Sciences

Oxford University Clinical Research Unit

Hospital for Tropical Diseases

Ho Chi Minh City, Viet Nam

2012

DATE OF SUBMISSION : 18 APRIL 2012

DATE OF AWARD : 26 NOVEMBER 2012

APPENDICES EXCLUDED FROM DIGITISED THESIS

PLEASE REFER TO THE ORIGINAL TEXT TO SEE THIS MATERIAL

Abstract

Drug resistant tuberculosis (TB) is increasing worldwide and it is now estimated by the World Health Organisation (WHO) that 7% of TB cases globally are resistant to isoniazid (INH). INH is a key drug for the treatment of TB, alongside rifampicin (RIF). Understanding factors which influence the emergence and propagation of INH resistance and the influence of INH resistance on TB treatment success are vital in improving global TB control efforts.

Vietnam is ranked 12th of 22 high burden countries for TB and has a high prevalence of INH resistance (25%) with a relatively modest prevalence of TB resistant to both INH and RIF (multi-drug resistant, MDR TB) (2.7%)

The first study in this thesis developed rapid screening tests for both RIF and INH resistance in *Mycobacterium tuberculosis* isolates which showed high accuracy. The specificity and the sensitivity of the MAS-PCR test for INH resistance compared to the conventional phenotypic DST were 100% [95% CI 92.9-100%] and 90% [95% CI 82.4-95.1%], respectively.

The second study demonstrated that the minimum inhibitory concentration (MIC) for INH is influenced by both the mutation responsible for resistance to INH and the lineage backbone of the *M.tuberculosis* isolate. A two-way ANOVA of MIC including both strain lineage ($p=0.003$) and resistance mutation ($p<0.001$) showed highly significant independent effects of both factors on MIC level. MIC to INH of isolates with a *katG315* mutation (2μg/ml) was significantly higher compared to MIC to INH of isolates with an *inhA-15* mutation (0.25μg/ml) and wild-type isolates (0-0.1μg/ml) ($p<0.001$).The independent effect of the strain lineage on INH MIC was

predicted to correspond to a 2.68-fold (95% CI 1.52-4.73, p<0.001) increase for Beijing lineage strains and a 1.40-fold (95% CI 0.72-2.74, p=0.32) increase for Euro-American lineage strains in comparison with strains of the Indo-Oceanic lineage.

The third study investigated the impact of INH resistance on outcome in patients with HIV-associated TB meningitis (HIV/TBM) and showed that HIV/TBM patients infected with INH resistant *M.tuberculosis* which remains susceptible to RIF have significantly worse outcomes than patients infected with fully susceptible strains. The adjusted hazard ratio for MDR patients in comparison with fully susceptible or streptomycin (STR)-monoresistant isolates was 5.21 [95% CI 2.38-11.42], p<0.0001 compared with HR= 1.78 [95% CI 1.18-2.66], p=0.005 for patients with INH resistant isolates (+/-STR resistance).

The final study investigated the distribution of N-acetylator-2 (NAT2) phenotypes for INH metabolism in healthy volunteers from the Vietnamese Kinh population and correlated this with phenotypes using caffeine as a surrogate indicator for INH metabolism. This study showed that characterisation of 3 single nucleotide polymorphisms is sufficient to determine the acetylator status of individuals of Vietnamese Kinh ethnicity and that there is a predominance of fast acetylators (65%) in this population. A simple PCR-RFLP test was developed to enable rapid determination of acetylator status for further studies.

Collectively, these results contribute to our understanding of INH resistance in *M.tuberculosis* and provide molecular tools for further studies of this crucial drug.

Co-authorship

Dau Quang Tho, the author of this work, was responsible for conducting the experiments, data analysis and writing of all studies described in this thesis, with the exceptions and assistance detailed below.

The impact of INH resistance to the outcome in HIV/TBM patients (chapter 5) was investigated using isolates and data collected by the PI during a clinical trial and observational study as described in materials and methods (PI: Dr. Estee Torok, trial registration number: ISRCTN63659091).

Dr. Thomas Pouplin, Head of the pharmacology department, co-designed the caffeine analysis in the study of correlation between acetylator genotypes and phenotypes in Vietnamese Kinh population, chapter 6.

Ms. Do Dang Anh Thu completed all routine laboratory work of sample collection at the Hospital for Tropical Diseases related to smear microscopy and culture.

Dr. Marcel Wolbers assisted with statistical analysis in chapter 5.

Mr. Vo Sy Kiet did the laboratory work for genotyping test in chapter 4.

Ms. Hoang Thi Thu, Pharmacology group, measured caffeine metabolites for chapter 6.

Acknowledgements

Firstly, I would like to send a big thank to my beloved supervisor, Dr. Maxine Caws, who has put a lot of effort in encouraging, supporting and patiently reviewed my thesis.

I also would like to thank Prof. Jeremy Farrar, Director of the Oxford University Clinical Research Unit – Vietnam (OUCRU), Hospital for Tropical Diseases, who gave me the opportunity to conduct my PhD within the OUCRU.

Many thanks to Dr. Sarah Dunstan and Dr. Mary Chambers for your support and consideration to my study.

I very much appreciated and would like to thank Ms. Do Dang Anh Thu for helping me with the routine lab work. My thanks to Dr. Marcel Wolbers for revising my statistics analysis, Mr. Vo Sy Kiet for doing the spoligotyping tests, Ms. Hoang Thi Thu in the Pharmacology group for measuring caffeine metabolism. My PhD could not have been completed without a lot of technical support from all of you. Last but not least, many thanks to all of my colleagues working at the OUCRU.

Finally, I would like to send the biggest thank to my parents for their moral support. This thesis is for you both.

Abbreviations

µg	Microgram
µl	Microlitre
µM	Micromole
ACP	Acyl carrier protein
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ATP	Adenosine Tri-Phosphate
AUC	Area Under the concentration-time Curve
BCG	Baccilus Calmette Guerin
BLAST	Basic Local Alignment Search tool
bp	Base pairs
CART	Classification and regression tree
CDC	Centers for Disease Control and Prevention
CI	Confident interval
CSF	Cerebrospinal fluid
CT	Cranial computed tomography
CTAB	Cetyl-Trimethyl Ammonium Bromide
CXR	Chest X-Ray
ddNTP	Dideoxyribonucleotide triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOTS	Directly observed treatment strategy
DST	Drug susceptibility test
EAI	East-Asian-Indian
EBA	Early Bactericidal Activity
ELISA	Enzyme linked immuno-sorbent assay
EMB	Ethambutol
EQA	External quality assessment
FDC	Fixed-dose combination
FIND	The Foundation for Innovative New Diagnostics
FQ/FQN	Fluoroquinolone
GCS	Glasgow coma scale
GLC	Green Light Committee
GLI	Global Laboratory Initiate
HCM	Ho Chi Minh city
HIV	Human Immunodeficiency Virus
HTD	Hospital for Tropical Diseases
IGRA	Interferon-gamma release assays

INH	Isoniazid
IOM	International Organization for Migration
IPT	Isoniazid preventive therapy
IQR	Interquartile range
IUATLD	International Union Against Turberculosis and Lung Disease
LJ	Lowenstein Jensen
LPA	Line probe assays
LQAS	Lot quality assurance sampling
LRM	Logistic regression method
LSP	Large sequence polymorphism
MAS	Multiplex Allele Specific
MDR TB	Multi drug resistant TB
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum inhibitory concentration
MODS	Microscopic Observation Drug Susceptibility
MRC	Medical Research Council
MRI	Magnetic resonance imaging
M. tuberculosis	*Mycobacterium tuberculosis*
NAAT	Nucleic acid amplification test
NADH	Nicotinamide adenine dinucleotide
NALC	N-Acetyl L-Cysteine
NAT2	N-acetyltransferase 2
ng	Nanogram
NGOs	Non-government organizations
NPV/PPV	Negative / Positive predictive value
NTM	Non-tuberculous mycobacteria
NTP	National Tuberculosis Program
OADC	Oleic acid Albumin Dextrose Catalase
ORF	Open Reading Frame
OUCRU	Oxford University Clinical Research Unit in Vietnam
OXTREC	Oxford Tropical Research Ethics Committee
PAS	Para-aminosalicylic acid
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PNT	Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases
PPD	Purified protein derivative
PZA	Pyrazinamide
RCT	Randomised controlled trial
RD	Region of deletion

RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RRDR	Rifampicin-resistance determining region
SNP	Single nucleotide polymorphism
STAG	WHO Strategic and Technical Advisory Group
STR	Streptomycin
TB	Tuberculosis
TBM	Tuberculous Meningitis
TST	Tuberculin skin test
VNTR	Variable number tandem repeat
WHO	World Health Organisation
XDR TB	Extensively drug resistant tuberculosis
ZN	Zhiel Neelson

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Background

There is a lot of evidence showing that *Mycobacterium tuberculosis (M.tuberculosis)* has coexisted with humans since antiquity. The disease tuberculosis (TB) has been known under many different names, such as Phthisis (Greek), Consumptione (Latin) or White-plague (Europe, 17^{th}-mid19^{th} centuries) (Daniel, Bates et al. 1994). Without knowledge of the precise cause of the disease, people described the epidemic as a wasting disease or consumption. These diseases had all symptoms of TB with wasting, pale skin, fever, haemoptysis, sweats, and had been described by many ancient authors (Daniel and Daniel 1999), including Hippocrates. Mummies excavated from ancient tombs have shown skeletal traces of TB and molecular techniques have also confirmed the positive identification of *M.tuberculosis* infection (Crubezy, Ludes et al. 1998; Zink, Sola et al. 2003). It seems that TB has been a major cause of human mortality for thousands of years.

M.tuberculosis is an extremely successful pathogen and has evolved a number of mechanisms to evade the host immune response including the ability to survive within the acidic phagosome and tolerate oxidative radicals (Chan and Kaufmann 1994). *M.tuberculosis* transmission occurs when an infected individual coughs up infectious droplet nuclei which are inhaled by a susceptible individual. *M.tuberculosis*

may subsequently cause active symptomatic diseases or establish a latent infection in the host, which can reactivate later, especially when the host immune system has weakened due to age, malnutrition or disease (Smith and Moss 1994). With the discovery and development of chemotherapy for TB infection in the mid 1940s and 1950s, people was able to treat the disease effectively for the first time and the hope that TB would one day be eradicated were raised. However, only a few months after streptomycin (STR) was first administered to patients in 1947, the limitations of mono-therapy became apparent as individuals, initially responsive to therapy, relapsed (D'Arcy Hart 1999). Drug resistant strains were isolated from a number of patients in the first cohorts receiving the drug (McDermott 1948; Patterson and Youmans 1970). As further anti-tuberculous drugs, para-aminosalicylic acid (PAS, 1948), isoniazid (INH, 1952), rifampicin (RIF, 1963), ethambutol (EMB, 1962) and pyrazinamide (PZA, 1952), were discovered and introduced to TB treatment, these drugs were gradually incorporated into multidrug treatment regimens to prevent the emergence of drug resistance (Youmans, Raleigh et al. 1947; Dunner, Brown et al. 1949; Sarkar and Suresh 2011). A landmark series of trials by the British Medical Research Council (MRC) and the International Union Against Tuberculosis and Lung Disease (IUATLD) eventually led to the development of a 6-month Multi-drug regimen which is still in use today (WHO 1999; Volmink and Garner 2007). INH and RIF are the two most important drugs forming the backbone of standard first line therapy. The World Health Organisation (WHO) has supported a strategy of directly

observed treatment short-course (DOTS) to control TB through National TB Programmes (NTP) (WHO 1999).

The emergence of Human Immunodeficiency Virus (HIV) in the last decades has led to a resurgence in TB cases globally (Smith and Moss 1994). Infection with HIV leads to a gradual decline in an individual's immune function through the destruction of helper T cells or CD4 T cells; and eventual progression over approximately 10 years, to acquired immunodeficiency syndrome (AIDS) (Smith and Moss 1994). The continuing decline in cell-mediated immunity renders an individual more susceptible to TB and develops more severe disseminated forms of TB infection. The second major challenge to global TB control is the increasing prevalence of primary TB cases infected with drug resistant *M.tuberculosis* strains. In particular, multi-drug resistant TB (MDR TB), which is resistant to at least INH and RIF, requires a minimum of 18 months for treatment with second line drugs which are more expensive, higher toxicity and less potent.

Until recently, the number of TB infections worldwide incessantly increased as well as the emergence of multidrug resistant *M.tuberculosis*, that in 1993 WHO has announced TB as a Global Health Emergency (WHO 1993). Since then, coordinated actions between governments and international non-governmental organizations (NGOs), such as WHO, have been established to try to improve the effectiveness of treatment with DOTS, which increases the supply of TB drugs at affordable prices or increases global funding for the developing world for treatment and research on the disease. The Stop TB Strategy along with Millennium development Goals of WHO

with the target of reducing 50% of global burden of TB and death rate by 2015 relative to that in 1990, as well as set the goal of eliminating TB by 2050 (WHO 2006).

1.2 TB in the world

It has been estimated that one third of the world population is infected with *M.tuberculosis*. Only around 10% of infected individuals develop active symptomatic TB, the majority remain latently infected and form a reservoir of the disease. Approximately 5% will develop reactivated TB disease as the immune system becomes impaired due to age, disease or malnutrition (Raviglione, Snider et al. 1995; WHO 2009). TB kills approximately 2 million people each year (1.35 million deaths from TB in HIV negative individuals and 456,000 deaths in HIV co-infected patients in 2007). According to the WHO, the estimated global incidence of TB in 2007 was 9.27 million cases (139/100,000) and the estimated prevalence was 13.7 million cases (206/100,000) (WHO 2009). With all of the effort to control TB infection, the infection rate is slightly decreased with the estimated incidence of TB in 2010 was 8.8 million cases (128/100,000) and the estimated prevalence was 12 million cases (178/100,000) (WHO 2011).

Approximately 95% of TB patients and 98% of deaths due to TB are reported from low income countries; three quarters of TB patients are in the most economically productive ages (15-45 years old). For this reason, TB is often named a disease of poverty and the disease in turn intensifies poverty through morbidity in TB patients

unable to work and the financial burdens of TB treatment. Almost 80% of TB cases occurred in 22 high burden countries in 2004, of which Vietnam is one (WHO 2006). The distribution of TB prevalence varies widely among countries (Figure 1.1), apparently because of differences in wealth and health care systems. India had the largest total incidence (1.96 million estimated new cases each year) and also the biggest prevalence of TB (3.3 million estimated cases) in the world in 2007. China was ranked second with a prevalence and incidence of 1.3 million and 2.58 million cases, respectively. For this reason, Asia accounts for 55% of global TB cases. The African region accounts for 31%; the other three regions (the Americas, European and Eastern Mediterranean regions) account for small fractions of global cases (around 3-6%). However, when stratified by the number of prevalent TB cases per 100,000 population, African regions were among the countries with the highest rates, and the prevalence was also relatively high in Asia (WHO 2009).

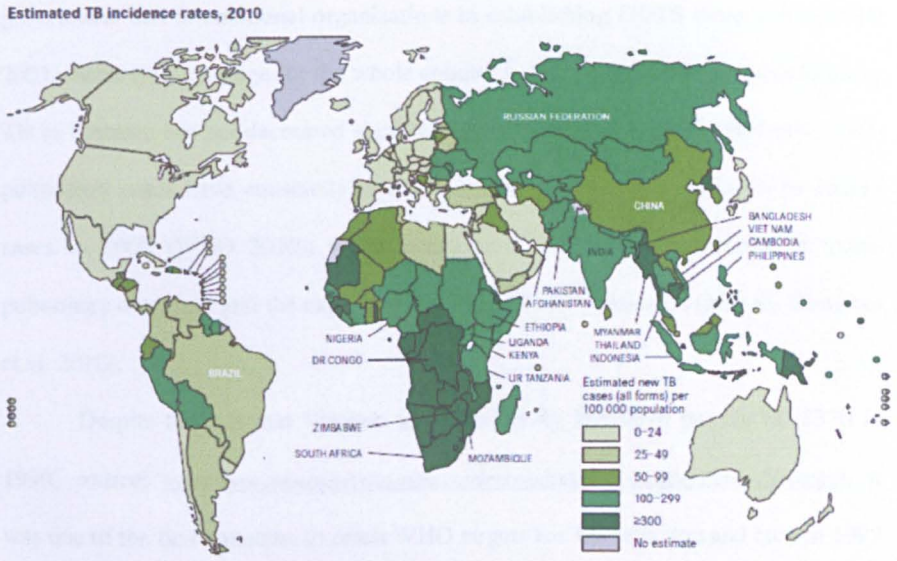


Figure 1.1: Estimated TB incidence rates by country in 2010, WHO 2011

According to WHO reports in 2009 and 2011, the overall treatment success worldwide was 85% in 2006 (based on treatment outcome), 86% in 2009, but the detection rate was only 57% in 2005, 65% in 2010 among new smear positive cases (WHO 2009; WHO 2011). There are a significant proportion of TB cases that are not detected - of particular concern is the 35% of sputum smear negative TB cases. These cases mostly remain untreated and become an infectious source for TB transmission.

1.3 TB in Vietnam

The estimated incidence of TB in Vietnam was 180,000 cases with 52,142 smear positive cases in 2010, for which Vietnam was ranked 12 of 22 high TB burden

countries in the world (WHO 2009; WHO 2011). Despite investment from the government and international organizations in establishing DOTS since 1985 (WHO 2001), achieving coverage for the whole country by 2000, the incidence of pulmonary TB in Vietnam has not decreased significantly (WHO 2009). The notified new extra-pulmonary cases have constantly increased since 1995 from 6,194 cases to 18,333 cases in 2009 (WHO 2010), which could be a result of improvement in extra-pulmonary diagnosis and the exponential increase in HIV infection (Marais, Thwaites et al. 2010).

Despite the fact that Vietnam had a relatively low GNP per capita ($370 in 1999, source: http://www.nationsencyclopedia.com/economies/Asia-and-the-Pacific/Vietnam), it was one of the first countries to reach WHO targets for TB detection and cure in 1999 (WHO 2001). The Vietnamese Ministry of Health has established a national fund for TB and also developed partnerships with international organizations to establish and obtain targets and to address the health system constraints of the country. The NTP had been established at the end of 1994 and rapidly scaled up DOTS coverage to 100% of the population in 2000. Principal targets for the NTP are to decrease TB related morbidity and mortality decrease TB infection and transmission, and eliminate the development and transmission of drug resistant strains. To achieve these objectives, case detection and treatment success must be improved (WHO 2001).

However, TB rates have not substantially declined in Vietnam, despite the notable achievements of the NTP and there are still many issues that need to be tackled. As globally, there is a desperate need for improved diagnostic tests to

increase case detection and operational research is required to determine how best to apply these tests within the existing NTP. Novel drugs are needed to improve existing treatment regimens and treat drug resistant forms. Neonatal vaccination with Baccilus Calmette Guerin (BCG) is applied in Vietnam but remains largely ineffective and an effective vaccine remains elusive (Andersen and Doherty 2005). Research is needed into the relative contribution of factors influencing the outcome of patients on standardised regimens. Drug resistance is known to be a major contributing factor to poor outcome, alongside adherence (Quy, Lan et al. 2003). The relative contributions of host genetic and pathogen virulence factors of different TB genotypes are only beginning to be studied (Caws, Thwaites et al. 2008; Thuong, Dunstan et al. 2008). Resistance to the first line drugs is prevalent, although MDR TB rates remain relatively low (2.7%), it is slowly increasing (Quy, Buu et al. 2006). Contributing factors are likely to be the treatment of TB in the private sector, undetected relapse and a lack of available funds to manage drug resistant TB. Many patients go to the private sector for TB treatment which is a well-documented risk for poorly standardised treatment (Lonnroth, Thuong et al. 2003) and the private sector is likely to increase in importance as Vietnam transitions to a middle-income nation. Treatment for multi-drug resistant TB in the NTP has only become available in 2010 at one centre in Southern Vietnam (Pham Ngoc Thach Hospital) and many patients carrying multi drug resistance are still being treated with WHO standard re-treatment regimen with first-line drugs (Quy, Lan et al. 2003), which is known to be ineffective (Burugina Nagaraja, Satyanarayana et al. 2011).

1.4 TB and HIV

TB incidence has increased in many parts of the world in conjunction with the HIV epidemic. Globally, WHO reported that there were 1.1 million new HIV-positive TB cases in 2010 (out of a total of 8.8 million incident cases of TB) (WHO 2011). The African Region accounts for 79% of estimated HIV-positive TB cases; most of the remaining cases (11%) are in the South-East Asia Region (WHO 2009). People infected with HIV are more likely to develop active TB when they are infected with the *M.tuberculosis* bacteria. Compared with an individual who is not infected with HIV, an individual infected with HIV has 10 times increased risk of developing active TB (WHO 2004). In fact, the HIV pandemic had increased the number of TB patients by at least 10% (WHO 2004).

With sixty percent of the global population within the most sexually active age group (15-49 years), Asia is home to one of the fastest-growing HIV epidemics in the world (Ruxrungtham, Brown et al. 2004). Despite many efforts from WHO, governments and other nongovernmental organizations, which has led to one-third of those needing antiretroviral therapy (ART) being put on treatment, the majority of patients in these developing countries are still unable to access ART. As a result, most HIV-infected patients in Asia present with an opportunistic infection, usually TB (WHO and UNAIDS 2009). This not only increases the burden of TB in the region but also increases morbidity and mortality in HIV patients with TB co-infection (Gandhi, Moll et al. 2006; Quy, Cobelens et al. 2006).

The diagnosis of TB is complicated in a patient with HIV co-infection. Further, HIV co-infected patients often develop more severe disseminated forms of the disease and are less likely to have a successful outcome even when diagnosed correctly. HIV-infected patients are more likely to have either disseminated, genitourinary, intra-abdominal, lymph node or pulmonary TB (Shafer, Kim et al. 1991). Diagnosis of TB in HIV infected patients is difficult and often delayed because these individuals are more likely to be sputum smear negative or have atypical chest X-ray presentation (Samb, Sow et al. 1999; Swaminathan, Paramasivan et al. 2004). The differential diagnosis is also broader. HIV/TB patients who are on ART also suffer from increased toxicity and drug:drug interaction because of the large number of drugs; thus present further treatment adherence and failure problems in this population (Amuha, Kutyabami et al. 2009).

1.5 TB and HIV in Vietnam

Estimates extrapolated from sentinel surveillance data suggested that about 122,000 people in Vietnam were infected with HIV in 2000, with a prevalence rate of 0.29% in the 15-49 year-old age-group (WHO and UNAIDS 2009). As in other settings with an emerging epidemic HIV infection, the survey was focused on the high-risk groups, including injecting drug users and commercial sex workers. The number of HIV infected people in Vietnam was rising exponentially in 1995 and has been increasing slightly during the last fifteen years, partly due to improvement in access to HIV treatment (WHO and UNAIDS 2009). Effective ART dramatically

reduces the viral load of an HIV-infected individual, reducing the probability of transmission occurring (Wagner, Kahn et al. 2010; Williams, Lima et al. 2011). However, as HIV-infected individuals on ART remain healthy for longer, and are likely to remain sexually active, the long term impact on HIV transmission is not known (Wagner, Kahn et al. 2010). To date, the epidemic remains focused in core high-risk groups: commercial sex workers, intravenous drug users and men who have sex with men. Rising HIV prevalence rates in Vietnam have complicated TB control efforts and are likely to be a major contributing factor to the slow rate of decline in TB incidence. According to the WHO in 2010, the estimated incidence of TB in Vietnam was 180,000 cases (199/100,000) and the estimated prevalence was 290,000 cases (334/100,000). Of those, 7,600 cases were in HIV co-infected patients (4.2% of total TB incidence cases) (WHO 2011). The increasing rate of HIV in this population not only fuels TB prevalence but also decreases the effectiveness of the TB control program. HIV infected individuals with TB are more likely to be smear negative and present a normal chest X-ray (CXR), therefore TB often remains undetected in HIV-infected individuals, resulting in high mortality (Quy, Cobelens et al. 2006). A multi-country study has been conducted to develop a diagnostic algorithm to improve TB diagnosis in HIV patients in Cambodia, Thailand and Vietnam (Cain, McCarthy et al. 2010). Globally, the mortality of TB/HIV co-infection is extremely high with 33% mortality among HIV-positive incident TB cases (WHO 2009).

1.6 Global TB phylogeny

M.tuberculosis is a fascinating pathogen. Despite a long history of co-existence with humans, *M.tuberculosis* genomes appear to be quite preserved or have low diversity in comparison with other pathogen genomes (Garnier, Eiglmeier et al. 2003). Various typing schemes have been devised for *M.tuberculosis*, including IS*6110* restriction fragment length polymorphism (RFLP), spoligotyping, variable number tandem repeat (VNTR) typing and large sequence polymorphism (LSP) typing. Early typing systems for *M.tuberculosis,* RFLP and spoligotyping, were devised largely by examining diversity within isolates from Europe or North America and the discriminatory power of such systems was therefore low for isolates from Asia. The global diversity of *M.tuberculosis* was therefore underestimated.

The differences between different *M.tuberculosis* genotypes are very small, which are >99.95% identical at the nucleotide level in genome comparison (Garnier, Eiglmeier et al. 2003; Zheng, Lu et al. 2008) meaning extensive translocations, large deletions or insertions rarely occur. Unlike other bacilli, *M.tuberculosis* does not appear to acquire DNA by horizontal transfer and genome variation occurs by homologous recombination or deletion events, often mediated by mobile repetitive elements such as IS*6110* (Flores, Van et al. 2007; Zheng, Lu et al. 2008).

Spoligotyping is a typing method based on the detection of deletions in the direct repeat region which consists of 36 base pair repeated elements interspersed with variable 'spacer' regions. The presence/absence of the variable spacers is detected to generate a 'spoligotype' for the strain. This method is discriminatory for

M.tuberculsois isolates among the Euro-American lineage, but does not discriminate the Beijing lineage, which is predominate in Asia (de Jong, Antonio et al. 2009). LSP detects the absence of large DNA sequences (region of deletion - RD) and defines 6 major *M.tuberculosis* lineages, which are named after the geographic region in which they predominate (Flores, Van et al. 2007). Both spoligotyping and LSP have been used widely for both phylogeny and molecular epidemiological studies of *M.tuberculosis* (Gagneux, DeRiemer et al. 2006). Recent studies have examined the diversity within the Beijing genotype family. Using LSP typing, the Beijing genotype is a sub-group of the East-Asian lineage. The RD105 deletion defines the East-Asian lineage. This deletion when presents in additional with RD207 deletion (which containing spacers 1-34), defines the Beijing genotype on spoligotyping.

Beijing/W genotype is a *M.tuberculosis* lineage which has a unique spoligotype pattern with spacers 1-34 missing on standard 43-spacer spoligotyping (Kremer, Glynn et al. 2004). The Beijing genotype has generated significant interest due to the high prevalence of this strain in some regions of Asia and its association with an outbreak of drug resistant TB in the United States (Niemann, Diel et al. 2010) and many parts of Eastern Europe (Cox, Kubica et al. 2005). Beijing genotype has also been reported to be associated with MDR TB in Vietnam (Caws, Thwaites et al. 2006; Buu, Huyen et al. 2009). Animal models infection with this genotype have suggested that the Beijing lineage is hypervirulent in comparison with other strains of *M.tuberculosis* (Tsenova, Ellison et al. 2005); however, the evidence for this hypervirulent is inconsistent and controversial (Parwati, van Crevel et al. 2010).

1.7 TB phylogeny in Vietnam

Three of the six major global lineages of *M.tuberculosis* are found in Vietnam; the Beijing, Indo-Oceanic and Euro-American lineages. Beijing genotype is a predominant genotype in Asia and is increasing in prevalence in other regions of the world, such as South Africa (Johnson, Warren et al. 2006). The prevalence of Beijing genotype in Vietnam is around 40-50% (Caws, Thwaites et al. 2006; Buu, Huyen et al. 2009). Several studies in Vietnam showed that Beijing genotype was associated with MDR TB (Caws, Thwaites et al. 2006; Buu, Huyen et al. 2009).

The Indo-Oceanic lineage is the predominant genotype in Indonesia, the Philippines and other regions of South East-Asia. Isolates of this lineage are designated with East-Asian-Indian (EAI) spoligotype classifications (Comas, Homolka et al. 2009). The Indo-Oceanic lineage is also prevalent in Vietnam, accounting for approximately 40% of *M.tuberculosis* strains. A single strain of this lineage, which has a single IS*6110* copy on RFLP typing and a unique spoligotype (EAI-VNM) has been referred to in the literature as the 'Vietnam genotype' or the 'Ha Noi genotype' and appears to be a geographically specific genotype in this region (Buu, Huyen et al. 2009). However, there is limited data on *M.tuberculosis* genotypes in the surrounding countries or regions (Cambodia, Laos, Southern Thailand and Southern China), and so the geographical extent of this genotype is not clear.

The Euro-American lineage strains of *M.tuberculosis* are also present in Vietnam and account for approximately 20% of strains. A limited diversity of Euro-

American lineage isolates is found, possibly due to a relatively recent introduction to the region in the last two centuries. Euro-American lineage strains show spoligotype patterns: LAM, Haarlem, T1 and T3 (Caws, Thwaites et al. 2006; Buu, Huyen et al. 2009). It is not clear which strains are emerging in Vietnam due to a lack of historical material for analysis and further study would help to elucidate the evolution of the *M.tuberculosis* population in Vietnam.

1.8 TB pathogenesis

The genus mycobacteria contain two major human pathogens: *Mycobacteria leprae*, which causes leprosy in humans and *M.tuberculosis*, which causes TB. Other mycobacteria of the *M.tuberculosis* complex *(M.africanum, M.bovis, M.canettti)* can also cause TB disease. The non-tuberculous mycobacteria (NTM) are classified in two sub-groups as slow or rapid growers. Some species are also pathogenic in humans, particularly in immune compromised individuals. Of these, the most important ones are *M.avium-intracellulare* and *M.kansassii* which cause pulmonary disease and *M.ulcaerans*, which causes buruli ulcer. Others, such as *M.marinum*, can cause opportunistic infection in humans (Tonjum, Welty et al. 1998; Corbett, Churchyard et al. 1999).

M.tuberculosis is a rod-shaped bacillus, 2-4μm in length and 0.2-0.5μm in width, non-motile, non-spore forming, obligate aerobe which stains weakly with Gram dyes. Mycobacteria are classified as acid-fast bacilli because the lipid-rich cell wall renders them relatively resistant to acid and this property is exploited in acid fast-staining techniques such as Ziehl-Neelsen (ZN) smear. *M.tuberculosis* is a slow

growing bacillus with a generation time of 16-24 hours (for comparison, *Escherichia coli* has a generation time of 20 minutes) (Wayne 1994). Despite its slow growth, *M.tuberculosis* is one of the deadliest pathogens.

M.tuberculosis is transmitted when a patient with active TB coughs, sneezes or spits, expelling infectious aerosol droplets containing *M.tuberculosis* bacilli (Smith and Moss 1994). Destruction or survival of the bacillus depends on the interplay between the bacilli and the host. A strong immune response can destroy or inhibit a weak bacillus before replication occurs. If the bacilli are not successfully eliminated, a latent infection may be established in which the bacilli remain in the body but the infection does not progress and the individual stays asymptomatic (latent TB) or individuals may progress to active pulmonary TB (primary TB).

In individuals with latent TB, the infection may 'reactivate' after years or decades, most commonly when the immune system is weakened by other co-morbidities or infections (such as diabetes or HIV infection), or the use of immunosuppressive drugs, such as anti-TNFα (Dannenberg, Jr. et al. 1994).

Some individuals develop active TB directly following initial infection, especially those with immune compromise. In others, active TB develops after many years of latent TB infection. At this stage, the bacteria begin to actively multiply within the body. The growth of *M.tuberculosis* creates lesions in the lungs principally via immune destruction of surrounding tissue. As immune cells are recruited to the site of infection, a granuloma is formed. If containment is not established at this stage, individuals develop active TB and gradually become infectious and able to transmit

M.tuberculosis as bacilli are expectorated in the sputum until the infection is effectively treated (Dannenberg, Jr. et al. 1994; Schluger 2005).

Without appropriate treatment, the majority of TB patients will die. One study showed that 90% of TB patients, who did not receive appropriate chemotherapy, often due to delayed diagnosis, died (Chiang, Lee et al. 2009). This report demonstrates the importance of early diagnosis and instigation of appropriate chemotherapy in preventing death from TB.

1.9 Clinical symptoms of TB

Symptoms of pulmonary TB include a productive prolonged cough for more than three weeks, chest pain, haemoptysis, shortness of breath, fever, chills, tiredness, night sweats, appetite loss, weight loss and pallor (Hopewell 1994).

Extra-pulmonary TB may co-exist with pulmonary TB, thus may present as 'classic' pulmonary TB. Weight loss, fever and night sweats are common symptoms among all forms of TB. Other symptoms and signs of extra-pulmonary TB depend on the affected organs; for example, lymphadenopathy occasionally with pus drainage when lymph nodes are infected or arthralgia when joints are involved (Hopewell 1994). Patients with TB meningitis may present with headache, stiffness of the neck and decreased consciousness (Hopewell 1994). All these symptoms are non-specific and are only suggestive for extra-pulmonary TB (WHO 2009). Demonstration of *M.tuberculosis* in clinical samples is the gold-standard for TB diagnosis, but is often difficult, even in settings with culture facilities, due to the difficulty of obtaining

adequate samples and the frequently paucibacillary nature of clinical samples in extra-pulmonary disease.

1.10 Tuberculous meningitis (TBM)

TBM is the most severe form of TB infection, causing death or disability in more than half of those infected (Hosoglu, Geyik et al. 2002). TBM is caused by dissemination of *M.tuberculosis* to the brain resulting in inflammation of the meninges. The severity of the disease is thought to be largely a consequence of the immune response, rather than the bacilli themselves, since the bacterial load within the cerebrospinal fluid (CSF) is thought to be very low, although bacilli may be sequestered and therefore not present in the sampled fluid (Radhakrishnan and Mathai 1993). Death from TBM is strongly associated with delays in diagnosis and treatment (Hosoglu, Geyik et al. 2002). The stage of TBM is normally defined by the Glasgow coma scale (GCS). GCS is measured by performing clinical assessment of eye opening (1–4 points), best verbal response (1–5 points) and best motor function (1–6 points), where lower values indicate more severe neurological dysfunction. TBM severity is also assessed by the MRC grades 1-3 for TBM. The MRC grading system defines patients in grade I if they have a GCS of 15 (fully conscious) and no focal neurology; patients in grade II have a GCS of between 11 and 14 and/or focal neurological deficit; and patients in grade III have severe coma with a GCS of less than 11 (Donald and Schoeman 2004).

Treatment regimens for TBM have never been optimized through adequately powered randomized controlled trials (RCT), and are extrapolated from trials of

pulmonary TB treatment. As a consequence, national and international guidelines for TBM treatment vary widely (Thwaites, Fisher et al. 2009; Kadhiravan and Deepanjali 2010), and generally recommend extended treatment duration, due to the severe consequences of relapse. It is unlikely that these regimens represent optimal treatment, due to the poor penetration of many first line anti-TB drugs across the blood:brain barrier (Donald 2010). Of the existing drugs, only INH and PZA penetrate well, while EMB, STR, and crucially RIF, penetrate very poorly, particularly if the meninges is not inflamed (Ellard, Humphries et al. 1993; Thwaites, Lan et al. 2005). Despite use of the standard anti-TB chemotherapy, around 30% of patients still die and many of the survivors have a significant neurological deficit (Hosoglu, Geyik et al. 2002; Thwaites, Lan et al. 2005). Neurological sequelae are caused largely by infarction and subsequent damage to brain tissue. Adjunctive steroids have long been advocated for TBM treatment, but their efficacy has been disputed, especially in patients with MRC grade 1 disease, which is those with a GCS of 15. A RCT of adjunctive dexamethasone in 545 patients with TBM in Vietnam has shown a 30% reduction in mortality but despite this, uptake of the recommendation into guidelines has been slow (Thwaites, Nguyen et al. 2004; Prasad and Singh 2008).

HIV infection increases individual risk of developing extra-pulmonary disease, especially TBM (Berenguer, Moreno et al. 1992). Consequently, TBM has become a common and serious clinical problem in populations with a high prevalence of both TB and HIV infection, such as Africa and South East Asia. Co-infection with HIV results in increased mortality in TBM (Thwaites, Duc Bang et al. 2005; Torok,

Chau et al. 2008). For HIV-infected patients in the dexamethasone study the relative risk of death was, 2.91, 95% CI [2.14 - 3.96], $p<0.001$) in comparison to HIV-uninfected individuals. However, none of the patients in this trial received ART (Thwaites, Duc Bang et al. 2005). A subsequent trial of immediate versus delayed (8 weeks) ART in antiretroviral naïve TBM patients showed no overall impact on mortality in HIV/TBM patients. However, the median CD4 count in these patients was extremely low, which may have contributed to the findings of the trial. This study is discussed further in chapter 4. The WHO recommends treating extra-pulmonary TB with the same regimens as pulmonary TB (2HRZS/4HR), except for TBM. As TBM is more severe form of extra-pulmonary TB, some experts recommend a prolonged treatment from 9-12 months. WHO also suggests that EMB should be replaced by STR in TBM patients (WHO 2009). British Infection Society guidelines recommend 2HRZE/10HR regimen and adjunctive corticosteroids (either dexamethasone or prednisolone) should be given to all patients with TBM, regardless of disease severity (Thwaites, Fisher et al. 2009).

1.11 TBM in Vietnam and challenges

TBM is difficult to diagnose due to a broad differential diagnosis and non-specific symptoms at the early period of the disease (Donald and Schoeman 2004). At the point where the signs of TBM are clear such as neurologic deficits, loss of consciousness or convulsions, the disease is already at the severe state, when diagnosis starts to take place (Donald and Schoeman 2004). The CSF is paucibacillary

in TBM and therefore the diagnosis is rarely confirmed microbiologically, through smear or culture, in most laboratories (Bhigjee, Padayachee et al. 2007). Delays in treatment are the strongest predictor of mortality and poor outcome, and therefore early diagnosis and instigation of appropriate treatment is paramount (Donald and Schoeman 2004).

The Hospital for Tropical Diseases (HTD) and Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases (PNT) in Ho Chi Minh City (HCM) treat approximately 700 cases of adult TBM each year, due to the referral patterns within the Vietnamese health system. The largest RCT of TBM was completed at these two hospitals, enrolling 545 adults with TBM. This randomized, double-blinded, placebo-controlled trial showed that adjunctive dexamethasone treatment was associated with a reduced risk of death (relative risk of death, 0.69; 95%CI [0.52 - 0.92]; p=0.01) (Thwaites, Nguyen et al. 2004). As a result of this study, adjunctive dexamethasone has been adopted as part of routine care for the treatment of TBM in Vietnam and other countries (Thwaites, Fisher et al. 2009) and incorporated into WHO guidelines. The mortality rate in HIV infected patients in this study was 65%, compared with 32% in HIV-negative patients.

Consistent with the literature, results from previous studies at Oxford University Clinical Research Unit in Vietnam (OUCRU) show that early diagnosis and early treatment have a significant positive impact on the outcome (Thwaites, Nguyen et al. 2004). The classical diagnostic methods to confirm a diagnosis for TBM are smear microscopy and culture from CSF samples. Smear is a fast and

specific way to detect mycobacteria in clinical samples; however, the sensitivity of the test is low, approximately 50% for pulmonary TB and less than 10% for TBM in many settings. Culture methods can improve sensitivity but have a long turn-around time. Conventional solid culture on Lowenstein-Jensen (LJ) media takes 20-28 days to become positive (Coban, Birinci et al. 2004). Commercial automated culture, such as Bactec system (Beckton Dickinson, USA), has a median time to positive of approximately 13 days (Ha, Lan et al. 2009). However, these turn-around times are too long to be clinically useful for the diagnosis of TBM, and at best are used to confirm a diagnosis and provide isolates for drug susceptibility testing. An accurate, quicker method to identify TBM is urgently needed. Attempts to rapidly diagnose TBM through nucleic acid amplification tests (NAAT) or serology have met limited success (Pai, Flores et al. 2003; Deshpande, Kashyap et al. 2007) because of the low sensitivity or specificity, respectively, of the tests. Other tests such as adenosine deaminase assay or bromide partition test have been proposed but are not specific for the differential diagnosis of bacterial meningitis (Girgis, Farid et al. 1990; Tuon, Higashino et al. 2010).

Clinical features are the principal method for TBM diagnosis in the absence of microbiological confirmation. Common clinical signs are headache, fever and meningismus with a prodromal stage exceeding 7 days. Typically, the CSF protein is raised, blood:CSF glucose ratio is low with a lymphocyctic predominance (Bhigjee, Padayachee et al. 2007). However, atypical CSF findings are common and a high neutrophil count has often been reported in HIV-associated TBM (Bhigjee,

Padayachee et al. 2007). Neuroimaging, contrast computerized tomography (CT) and magnetic resonance imaging (MRI) can be informative with classic indicators of TBM including enlarged ventricles, hydrocephalus and basal enhancement, although features are non-specific (Murthy 2010). CXR for concurrent pulmonary TB may also inform the diagnosis of TBM, although in areas highly endemic for TB, should be interpreted with caution.

1.12 TB diagnosis

Early detection and effective treatment of TB reduces morbidity and mortality and interrupts chains of transmission. NTPs detect TB cases using sputum smear microscopy, which has a poor sensitivity for active TB, particularly in patients with HIV co-infection. Sputum-smear negative cases may be diagnosed based on clinical presentation, CXR findings and disease history. Diagnosis by clinical factors and CXR can be subjective and depends on the skill and experience of the treating clinician. There is an internationally recognized need for improved, simple diagnostic tests for TB which can be applied at the point of care in resource limited, high burden settings.

A wide range of techniques have been applied in the development of diagnostic tests for TB. However, no test has yet achieved sensitivity and specificity approaching 100%. The Foundation for Innovative New Diagnostics (FIND) (website:http://www.finddiagnostics.org/programs/tb/tb_diagnostics.html) has developed a systematic approach to evaluation of novel diagnostic tests for TB, aiming to generate an evidence base for adoption, implementation and scale up of

these tests in resource-limited settings. FIND collaborative studies to date have resulted in a series of recommendations by WHO Strategic and Technical Advisory Group (STAG) for TB in the last decade (Pai, Ramsay et al. 2008). TB diagnostic tests are classified into direct and indirect tests according to the method of detection.

TBM is difficult to diagnose and remains a serious health problem in the developing world and regions of high TB prevalence. The number of the bacteria found in CSF of TBM patients are lower than in sputum of pulmonary TB patients (Radhakrishnan and Mathai 1993), thus more difficult to identify for diagnosis. Difficulty in early diagnosis of TBM is one of the major barriers to improving outcomes. Many tests have been proposed to detect TBM because the delay in diagnosis is strongly associated with mortality however none have proven effective (Hosoglu, Geyik et al. 2002).

1.12.1 Direct tests

Direct tests for TB have lower sensitivity than indirect tests but the specificity is high (approaching 100%) because they detect the *M.tuberculosis* bacteria or acid fast bacilli directly.

Sputum smear microscopy is the most widely applied technique, usually ZN-staining method (Heifets and Good 1994). The principle of the method is based on the acid-fast nature of the lipid-rich cell wall of the bacilli. This property allows an acid wash step to remove carbol fuschin dye stain from other bacilli while leaving the mycobacteria stained red. A blue methylene blue counter-stain is usually used to

highlight the red organisms against a blue background. ZN-staining is a low-cost method, approximately $1 USD per test, and can be applied in most settings as it requires little in the way of capital equipment. The specificity is extremely high however the sensitivity of this technique varies depending on the disease stage, the skill of the technicians, the processing method for sputum (most importantly whether direct or homogenous smear is applied) and the time available for examining each slide (Pai, Ramsay et al. 2008). Recently much effort has focused on optimizing the sensitivity of smear microscopy through application of alternative sputum processing methods such as bleach decontamination (Merid, Yassin et al. 2009; Cattamanchi, Davis et al. 2010), application of fluorescence microscopy and implementation of quality control systems such as lot quality assurance sampling (LQAS) or external quality assessment (EQA) of smear microscopy in NTPs (Selvakumar, Murthy et al. 2005). Sensitivity of smear microscopy is generally reported to be around 50% of all clinical TB cases, however, in patients who are HIV positive, smear microscopy has lower sensitivity and the global HIV pandemic has therefore increased the need for a more sensitive TB diagnostic test which can be applied in resource-poor settings.

Bacterial culture on LJ or using commercial liquid culture systems such as MGIT is the gold standard method for bacterial confirmation because of its high sensitivity and specificity (Cruciani, Scarparo et al. 2004). These methods are more expensive and ideally require an appropriate biological containment laboratory to perform, to prevent laboratory acquired nosocomial infection. In Europe and the US, *M.tuberculosis* is cultured at biohazard level 3 containment (Drobniewski, Hoffner et

al. 2006). However, in most resource-poor settings, *M.tuberculosis* culture is performed in laboratories of biosafety level 2 or lower. Worldwide, there is a lack of laboratories able to perform TB culture for diagnosis and the Global Laboratory Initiate (GLI, website: http://www.stoptb.org/wg/gli/) has recently been developed to address this issue, Microscopic Observation Drug Susceptibility (MODS) is a new cultural method which had been developed in Peru (Moore, Evans et al. 2006) (website: http://www.modsperu.org/) and evaluated in many places, including PNT hospital in southern Vietnam (Ha, Lan et al. 2009). It shows a similar specificity to the conventional culture methods but the result returning time is shorter (around 7 days).

Many nucleic acid amplification tests (NAAT) have been developed to diagnose TB as well as to rapidly detect drug resistance (Pai, Flores et al. 2003). These PCR-based tests detect *M.tuberculosis* by amplifying target pathogen-specific DNA sequences in the clinical sample. NAAT have several advantages in performance such as reducing risk of infection, reduction in turn-around times and increased sensitivity. However, NAAT can suffer from contamination problem; reducing specificity and PCR based tests for mycobacteria have suffered from sub-optimal sensitivities compared to tests for other bacteria, probably due to the difficulties of obtaining high DNA yields from mycobacteria with simple sample-processing protocols. The sensitivity and the specificity of commercial PCR-based tests for TB are normally low for routine diagnostic tests (Greco, Girardi et al. 2006).

The commercial line probe assays (LPA) are recommended by WHO for rapid screening of TB, MDR TB and XDR TB suspects (Albert, Bwanga et al. 2010). However, these tests are only applicable on smear-positive sputum and therefore cannot replace conventional testing. The commercial MTBDR*plus* test (Hain Lifesciences, Germany) is a LPA endorsed by WHO as a rapid test for TB and the mutations which confer INH and RIF resistance. MTBDR*plus* amplifies target genes associated with drug resistance (*katG, inhA* and *rpoB*) and uses reverse hybridization to specific probes to identify the genes amplicons and the relevant mutations. This test has an advantage of detecting both RIF and INH resistant isolates directly from smear positive samples. The sensitivity and specificity in detecting RIF resistance were 98.1% and 97.8%, respectively; and for INH 90.2% sensitivity and 100% specificity (Hillemann, Rusch-Gerdes et al. 2007).

In 2010, a novel commercial diagnostic test, the Xpert MTB/RIF for use in the GeneXpert system (Cepheid company, US) was reported to show promising results in identifying *M.tuberculosis* in clinical samples (Helb, Jones et al. 2010). The advantage of Xpert MTB/RIF is to minimize the sample preparation process by automating the concentration, purification, extraction, amplification and detection of *M.tuberculosis* DNA directly from clinical samples within a closed cartridge based system. Pre-processing of the sample is limited to addition of a sample liquefaction reagent with intermittent manual shaking at room temperature for 15 minutes. The real-time PCR performed within the cartridge to amplify the *rpoB* gene, which sequence is unique for *M.tuberculosis*. The test uses probes to detect mutations

conferring RIF resistance and reports the results on screen. An evaluation study in South Africa showed that the sensitivity and the specificity of MTB/RIF test were 90.2-99.8% and 98.1-99.2%, respectively, compared with *M.tuberculosis* culture in clinical sputum samples (Boehme, Nabeta et al. 2010).

1.12.2 Indirect tests

Indirect tests normally have higher sensitivity but lower specificity. They are generally used in TB screening or to detect TB forms that are difficult to confirm with direct tests, such as latent TB or extra-pulmonary TB.

Tuberculin skin test (TST)

The Tuberculin skin test is a traditional test which was developed in the 1920s (Tisdall and Brown 1926). The test is based on the immune response to mycobacterial proteins after infection. Purified protein derivative (PPD), a precipitate of non-specific polypeptides obtained from *M.tuberculosis* cultures, is injected intradermally in both the Mantoux and Heaf tuberculin skin tests. The individual returns to the clinic 2-3 days after injection to check for a delayed-type hypersensitivity reaction visible as raised rash at the site of injection. The circumference of the rash is used to indicate whether that person has been infected with *M.tuberculosis*. Positive TST results do not generally discriminate well between old infection, current active infection, latent TB or BCG vaccination, although different cut-off measures are generally used in areas with and without universal BCG vaccination and to discriminate between latent or active TB(Ewer, Deeks et al. 2003). Specificity of the test is higher in places with low TB prevalence and low in high burden areas. The test is also confounded by

cross-reaction with atypical mycobacteria and specificity is lower in areas where exposure to mycobacteria is high, such as tropical settings (Farhat, Greenaway et al. 2006). The TST is cheap and simple to perform. The test is still widely used in most places, and is used in TB prevalence surveys.

Serodiagnostics

Enzyme linked immuno-sorbent assay (ELISA) can be used to detect specific anti-mycobacterial antibodies or antigens in blood, urine or other clinical samples. A recent WHO evaluation of 19 commercial serodiagnostic tests for TB concluded that none reached a sensitivity or specificity which justified their use for TB diagnosis (WHO 2008) and has led to an unprecedented negative statement by WHO on a diagnostic test recommending against their use (WHO 2011). Use of the tests is especially widespread in the private sector in India, despite there being no diagnostic value to the tests. Efforts are ongoing to find improved biomarkers for use in serodiagnostic tests since such a test could potentially be used as a low-cost, simple and rapid near-patient test. However, despite large studies, no strong leading candidates have emerged for development to date.

Interferon-gamma release assays (IGRA)

In vitro interferon-gamma release assays (including QuantiFERON–GOLD (Cellestis Limited, Australia) and T-SPOT (Oxford Immunotec Limited, UK)) measure the IFN-gamma released from whole blood or isolated white blood cells after incubating them with *M.tuberculosis* specific antigens. These tests use *M.tuberculosis* specific antigens such as ESAT-6 and CFP-10 and are therefore more specific than

TST with the ability to discriminate between TB infection and prior BCG vaccination. However, IGRA have limited value in resource-limited settings and WHO has recommended against their application in endemic settings (WHO 2011).

1.12.3 Other TB diagnosis tools

CXR is a widely-applied tool for TB diagnosis and provides vital information for TB diagnosis, alongside microbiology and clinical history. However, CXR changes can be non-specific and interpretation is highly dependent on the skill and experience of the radiologist or clinician reading the CXR. The CXR is useful to assess the extent of disease and response to treatment.

Cranial computed tomography (CT) and magnetic resonance imaging (MRI) scan are also applied for TB diagnosis. CT imaging of the lungs can provide a more detailed picture of lesions. MRI and CT scanning can assist the diagnosis of extrapulmonary TB and radiological features can often assist the differential diagnosis of TBM. The cost of MRI imaging limits its application in resource-limited settings. Algorithms can assist in the diagnosis of TB in settings with limited laboratory facilities. Several have been developed for application to paucibacillary forms of TB which are hard to confirm by smear microscopy, such as pediatric TB, HIV-associated TB and TBM. A simple clinical algorithm for TBM evaluates factors including patient age, blood white cell count, history of illness, CSF white cell count, CSF neutrophil percentage and showed high sensitivity (99%) but limited specificity (68%) in prospective evaluation (Torok, Nghia et al. 2007).

1.13 The WHO DOTS treatment strategy for TB

DOTS is the central WHO strategy recommended to combat TB (WHO 2009). Under DOTS, the average global treatment success rate was 83% among the new smear-positive TB in 2003 (WHO 2006) and increased to 85% in 2006 (WHO 2009). By contrast, if untreated, the mortality rate of active pulmonary TB cases is more than 60%, ranging from 83% in HIV-positive smear-positive to 20% in HIV-negative smear-negative cases (WHO 2009); showing the crucial role of DOTS in TB control. The DOTS strategy includes 5 main strands or 'pillars':

1) Political commitment from the government with increased and sustained financing.
2) Case detection by smear microscopy and quality-assured bacteriology.
3) Standardised treatment with direct observation by a healthcare worker or community health worker for at least the first two months (the intensive treatment phase).
4) A sustainable, uninterrupted and effective drug supply and management system.
5) A standardised monitoring and evaluation system with recording and reporting to allow assessment of treatment outcomes.

In the DOTS strategy, WHO has defined targets for detection of new sputum smear positive TB cases and treatment cure rates of at least 70% and 85%, respectively. The target was defined base on the ZN smear, the most widely used diagnostic test for TB, which needs at least 10,000 bacilli per milliliter in sputum as a

threshold of detection (Hobby, Holman et al. 1973). Further, estimated case detection is based on often inaccurate estimates of total smear positive cases. HIV/TB co-infection and MDR-TB have also been identified as challenges for TB control. Interventions to reduce HIV/TB co-infection and development of MDR-TB treatment programmes have been implemented by collaboration and management among NTPs and international organizations but scale-up is challenging. An effective TB control programme should have specific strategies to address vulnerable populations such as prisoners, refugees and homeless people (WHO 2006).

In addition to increasing coverage by the DOTS strategy research on development of new diagnostics, drugs and vaccines has been intensified. Many studies are developing new diagnostics or drugs in the attempt to control TB more effectively and move towards eventual eradication (Hillemann, Rusch-Gerdes et al. 2007; Helb, Jones et al. 2010; Sarkar and Suresh 2011). The BCG vaccine has very poor effectiveness in adults, ranging from 0%-80% with adults in high TB burden countries receiving the lowest protection level from BCG, possibly due to prior exposure to atypical mycobacteria (Andersen and Doherty 2005), and a novel vaccine with enhanced effectiveness in developed settings is urgently required. A WHO report in 2009 showed that the global pulmonary TB case detection rate was estimated to be 63% in 2007 and the treatment success rate apparently reached the target 85% in 2006. Even though the WHO targets for detection and treatment were not reached by 2005, the overall prevalence of TB worldwide started decreasing slightly from 13.9 million cases in 2006 to 13.7 million cases in 2007 (WHO 2009). The TB incidence

rate trends vary in different WHO geographic regions. For example in Central Europe, and Latin America rates are decreasing but increasing in Eastern Europe, Eastern Mediterranean and Africa (WHO 2009), suggesting that further sustained, intensified efforts are required to have substantial impacts on TB globally.

1.14 Chemotherapy for TB treatment

DOTS is the standard short course therapy for TB treatment with the combination of four to five drugs: STR (or S), INH (or H), RIF (or R), EMB (or E), PZA (or Z). The standard regimen is a 6-month regimen in which patients are treated with INH, RIF, EBM and PZA daily for two months, followed by four months of INH and RIF given daily or intermittently (2HREZ/4HR). An alternative eight-month regimen (2SHRZ/6HE) was the standard regimen in use in Vietnam (Quy, Cobelens et al. 2006) until 2010 when a phased transition to the 6 month regimen was introduced, following a revision of WHO recommendations to recommend phase-out of the 8-month regimen (WHO 2009). The six-month regimen was demonstrated to have lower failure and relapse rates in a clinical trial conducted by the IUATLD and referred to as 'study A' (Jindani, Nunn et al. 2004).

The eight-month regimen consists of an intensive phase of two months using four drugs SHRZ and a continuation phase of six months using two drugs H and E given daily (2SHRZ/6HE) (Quy, Cobelens et al. 2006). A minimum of three drugs to which the bacilli is susceptible is essential to treat TB due to spontaneous drug resistance mutations. Four drugs are used in NTP treatment programmes to maintain

efficacy if undetected resistance to one of the agents is present due to the lack of capacity for programmatic drug susceptibility test (DST).

The 6-month regimen is also recommended to treat extra-pulmonary TB, except that of the central nervous system, bone or joint for which a longer duration of therapy is recommended (WHO 2009). Cases with meningial involvement are recommended to receive 9–12 months of chemotherapy but the evidence base for this recommendation is weak and principally due to the severe consequences of a relapse in the case of TBM (Thwaites, Fisher et al. 2009).

According to the National TB treatment guidelines of Vietnam (NTP-Vietnam 2008), new TB patients receive 2SHRZ/6HE, which is being phased out in favour of 2HRZE/4HR. Retreatment patients receive the category II regimen: 2SHREZ/1HREZ/5$R_3H_3E_3$. TBM cases receive the same drugs as pulmonary TB with a longer duration and EMB is replaced by STR. HIV infected TB patients are treated with 6 months regimen of pulmonary TB, and receive EMB in place of STR with daily dosing recommended during continuous phase (WHO 2009). TB treatment regimens in children are also different with 2HRZ/4HR for smear negative, 2SHRZ/4HR for smear positive and 2SHREZ/1HREZ/5HRE for retreatment patients.

1.14.1 First line drugs

STR was the first effective anti-tuberculous drug to be administered in the mid 1940s. STR is an aminocyclitol glycoside antibiotic which inhibits the translation process of protein synthesis by targeting the *M.tuberculosis* ribosome. The drug targets a small subunit of the ribosome which is formed by the RpsL protein and 16S

sRNA (*rrs* gene). However, the bactericidal activity of STR is not very potent. Within a few months of its use, resistance was reported to this drug and the failure of monotherapy was recognized (McDermott 1948).

INH (isonicotinic acid hydrazide) was first synthesized in 1912 by Meyer and Malley during a doctoral thesis. It was subsequently forgotten for nearly 40 years. The anti-TB properties of INH were discovered in 1951 by pharmaceutical companies (Mitchell 1955). After the first clinical trial in 1951, the enormous potential of INH with high potency against *M.tuberculosis* and *M.bovis* was reported to the public in 1952 (Mitchell 1955; Mitchison 2005). Despite the very simple structure, INH is a very powerful anti-TB drug with a very complex mechanism of action which is only active against mycobacteria. As a prodrug INH needs to be activated by the bacterial catalase-peroxidase enzyme. The activated reactive components attack multiple sites and pathways in the cell. Well-known targets of activated INH are enzymes involved in cell wall mycolic acid synthesis (InhA, KasA).

With the discovery of para-aminosalicylic acid (PAS) in 1946 and the rediscovery of the anti-tuberculous property of INH in 1951 (Youmans, Raleigh et al. 1947; Dunner, Brown et al. 1949), combination therapy was developed in a series of landmark clinical trials and became the standard for TB treatment from the mid 1950s.

EMB was discovered in 1961 and showed anti-TB effects in mice (Thomas, Baughn et al. 1961). The ocular toxicity of EMB which was evident in the first clinical trial, limits the dosage and thus the potency of the drug (Carr and Henkind

1962). EMB inhibits *M.tuberculosis* cell wall synthesis via a different targeted pathway from INH, thus cross-resistance between INH and EMB is limited. EMB inhibits biosynthesis of arabinogalactan, the major polysaccharide in *M.tuberculosis* cell wall, which has a vital role in growth and replication (Takayama and Kilburn 1989). For this reason, EMB is only active against the replicating bacilli.

Despite the ocular toxicity, combination therapy containing EMB showed better tolerance and efficacy than combinations containing STR (Bobrowitz 1971). STR has the further drawback of requiring administration by injection and therefore EMB now widely replaces STR in first-line regimens, especially for HIV/TB patients, in whom injection carries higher risks (WHO 2009).

RIF is a semi-synthenthic drug which was produced in 1959 and was introduced to TB treatment in 1967(Baronti and Lukinovich 1968). It showed very strong bacterial clearance effects in TB patients who received TB combination chemotherapy containing RIF. The antituberculous activities of RIF are stronger than INH and serum levels are more stable (Canetti, Le Lirzin et al. 1968). RIF has high affinity to the beta-subunit of bacterial RNA polymerase, thus inhibits activity of this crucial enzyme and prevents RNA transcription. When the RNA polymerase enzyme is inhibited, no mRNA is produced and consequently no protein is synthesized and the bacterium is killed.

With the introduction of EMB in 1962 and RIF in 1967, TB treatment was shortened to 9 months with a new combination of RIF, INH and STR (Nitti 1972).

PZA was first synthesized in 1936 in Germany and was soon being tested as an anti-TB candidate in early 1950s due to the analogue structure to nicotinamide component. The testing results from animal models and in clinical trial (Cordice, Hill et al. 1953) showed good anti-TB activity of PZA. Similar to nicotinamide and INH, PZA is a prodrug which only has antituberculous activities after activation. The definitive target of PZA after activation is still unclear. Antituberculous activity of PZA was observed *in-vivo* but not *in-vitro*, due to the need for the acidic *in-vivo* environment. Activated PZA may target the cell membrane and dysfunction ion exchange membrane channels. This change eventually changes the pH in the cell, thus disrupts enzymes activities in *M.tuberculosis* and kills the cell (Salfinger, Crowle et al. 1990).

When initially used in a combination with INH, PZA gave good sputum conversion, however severe hepatotoxicity was observed and thus PZA was abandoned as a first line drug for TB treatment and was used only as a second line TB drug (Somner and Brace 1962). In the 1980s, with the rediscovery of PZA sterilizing activity against dormant *M.tuberculosis* and the trial of a new multi drug regimen, TB chemotherapy was further reduced to 6 months (Steele and Des Prez 1988), establishing the standard short course chemotherapy in use today.

The targets of first line anti-TB drugs is described in Figure 1.2

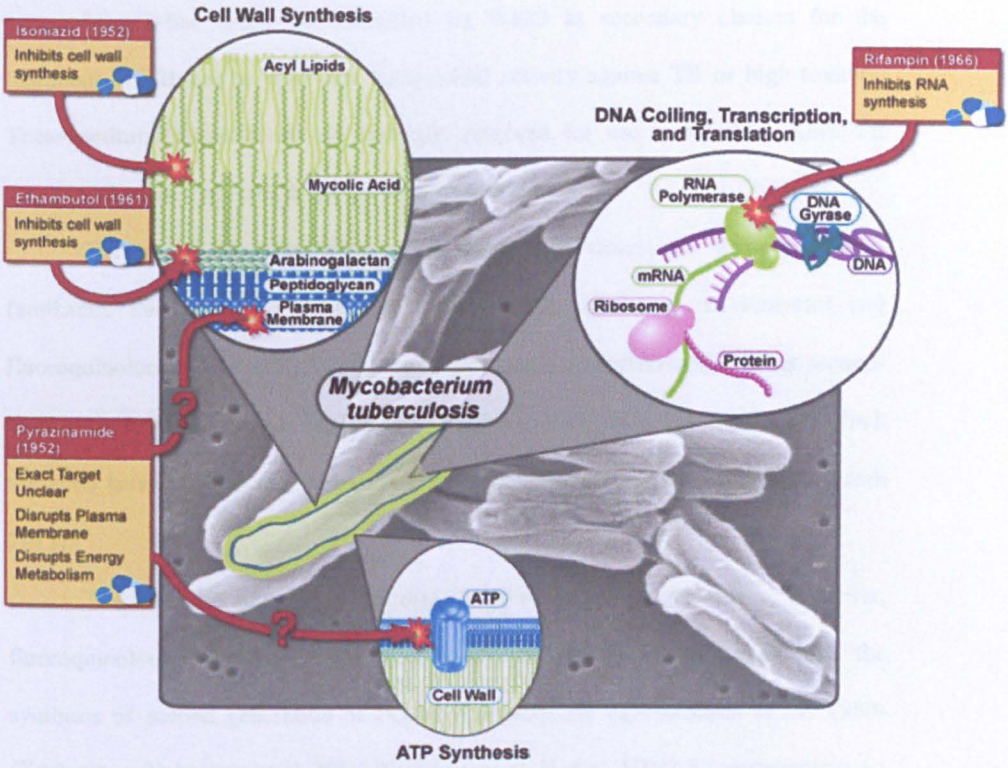


Figure 1.2: First line anti-TB drugs and activities in *M.tuberculosis* cell.

Source: http://www.niaid.nih.gov/topics/tuberculosis/Understanding/WhatIsTB/ScientificIllustrations/Pages/firstLineIllustration.aspx

1.14.2 Second-line drugs

Second-line drugs are classified by WHO as secondary choices for the treatment of TB due to their low bactericidal activity against TB or high toxicity. These antituberculous drugs are normally reserved for use in MDR or XDR TB patients when the first-line drugs are no longer effective.

The most important classes of second-line drugs are aminoglycosides (amikacin, kanamycin), polypeptides (capreomycin, viomycin, enviomycin) and fluoroquinolones (ofloxacin, levofloxacin, gatifloxacin, moxifloxacin). Other second-line drugs are thioamides (ethionamide, prothionamide), cycloserine and PAS which are even less effective and more toxic than the previous group(Sarkar and Suresh 2011).

The first fluoroquinolone (nalidixic acid) was discovered in 1960s. However, fluoroquinolones (FQN) were not used as an anti-tuberculous agent until after the synthesis of second generation of FQNs, ofloxacin and ciprofloxacin in the 1980s (Tsukamura, Nakamura et al. 1985; Truffot-Pernot, Ji et al. 1991). Ciprofloxacin is no longer recommended for the treatment of TB due to its weak bactericidal activity and potential for generating resistance to the more effective FQNs. The later generation FQNs levofloxacin, gatifloxacin and moxifloxacin show very high antituberculous activities with mild adverse effects, similar to first line drugs. Despite this high potency in TB treatment, FQNs are currently used as second line drugs. Treatment trials are underway to establish the potential role of these compounds in first-line

treatment (Nuermberger, Yoshimatsu et al. 2004), with the hope that a 4-month FQN-containing standard regimen can replace the current 6-month regimen.

FQNs have been used as broad spectrum antibiotics for both gram-positive and gram-negative bacterial infection for a long time. They are often administered as a broad spectrum antibiotic in patients with cough to aid differential diagnosis. This wide use of FQN in general bacterial infection leads to the accumulation of many FQN resistant bacteria, include FQN resistant *M.tuberculosis*. A study in Johns Hopkins showed a rapid development of FQN resistance in *M.tuberculosis* in an HIV patient after 13 days of mono-FQN treatment for an urinary tract infection (Ginsburg, Woolwine et al. 2003).

1.15 Isoniazid preventive therapy (IPT)

HIV infection increases the risk of TB development approximately 10 fold (WHO 2004). Treatment of latent TB in HIV-infected individuals can reduce TB incidence and also help to prevent onward transmission.

Prophylactic treatment options for HIV infected patients or latent TB include INH daily (6H, 9H) or twice weekly ($6H_2$, $9H_2$) for 6-9 months. Because of the long duration of IPT, adherence can be a problem. To shorten the treatment period, some novel combinations have been tested, such as a 2-month regimen of daily RIF and PZA. However, these alternatives increased the fatal risk of severe hepatotoxicity in some cases (Kaplan, Benson et al. 2009). When INH is given intermittently (twice a week) it should be administered only as DOTS.

Treatment with INH for 6 months reduces the overall risk of TB by 33% in individuals with HIV infection (WHO 2009). The effect is much greater when using with other combinations such as co-trimoxazole preventive therapy for opportunistic infection (Thuy, Shah et al. 2007) or ART. IPT is generally safe and well-tolerated but it is important to rule-out active TB before commencement of IPT, due to the risk of INH resistance if IPT is given to a patient with active TB disease. It can be difficult to rule-out active TB in HIV-infected individuals due to the low sensitivity of smear microscopy in these patients. A recent multi-centre study in Vietnam, Thailand and Cambodia developed an algorithm which can rule-out TB with 93% sensitivity and 36% specificity in HIV-infected patients and is therefore suitable as a screening tool prior to implementation of IPT while waiting for culture confirmation, in these settings (Cain, McCarthy et al. 2010). The generalizability of this algorithm to other settings remains to be evaluated.

1.16 Drug resistance

Drug resistant TB is a global issue and the prevalence of drug resistance is increasing all over the world (WHO 2008). Soon after the introduction of new drugs, resistant isolates were identified in most early clinical trials using mono or dual therapy (McDermott 1948; Dunner, Brown et al. 1949). In *M.tuberculosis*, drug resistance is generated by spontaneously occurring mutations at a rate of $1/10^6$-$1/10^8$ bacilli, depending on the drug. It was rapidly established that triple therapy with high adherence is required to prevent the emergence of resistance. The DOTS programme

was developed by WHO specifically to manage TB treatment through NTPs (WHO 2006). In fact, drug resistance usually arises as a result of non-adherence to standardized TB treatment, incomplete or inappropriate treatment (Amuha, Kutyabami et al. 2009), or substance abuse (Matthys, Rigouts et al. 2009). This phenomenon happens more frequently in the private sectors in many settings where treatment is often non-standardised and is not controlled by the NTP (Quy, Lonnroth et al. 2003; Choi, Lim et al. 2007). The use of fixed-dose combination (FDC) tablets has recently been advocated to reduce the potential for partial drug adherence to multi-drug regimens. In a multi-centre trial, known as 'study C', FDC tablets were shown to be effective (Lienhardt, Cook et al. 2011; Monedero and Caminero 2011) and are now advocated for use by NTPs by IUATLD, WHO and others.

MDR TB is caused by *M.tuberculosis* which is resistant to at least the two key first-line drugs, INH and RIF. MDR TB has been a global concern for many years because treatment is more complex, toxic, expensive and outcomes worse than in drug-sensitive TB. Treatment for MDR TB requires administration of more toxic and less effective drugs that are given for at least three times longer than standardised regimens (Pablos-Mendez, Gowda et al. 2002). The success rate in MDR treatment is relatively low compared to treatment for drug-sensitive TB; MDR treatment success is highly variable, with reports as low as 37% (Ohkado, Aguiman et al. 2006; Johnston, Shahidi et al. 2009; Matthys, Rigouts et al. 2009). A recent meta-analysis reported a pooled treatment success of 62% of 31 treatment programs from 21 countries (Johnston, Shahidi et al. 2009); even though some studies impressively

showed similar success rate of MDR with susceptible TB (72-86%) (Ward, Marciniuk et al. 2005; Karagoz, Yazicioglu Mocin et al. 2009). The cost for MDR treatment is, however, extremely high in comparison with a treatment regimen for susceptible TB ($8,700-$160,000 comparing to $350; 25-450 times) (Resch, Salomon et al. 2006). There has never been a RCT for MDR TB and these studies are crucial to allow treatment scale-up and reverse transmission trends. A recent study from Bangladesh reported a novel MDR treatment regimen developed in stepwise modification of the regimen in serial small cohorts (Van Deun, Maug et al. 2010). The final regimen, often referred to as 'the Bangladesh regimen' is now being trialed against the current WHO recommended regimen of 18 months in a multi-country trial funded by the MRC and Treat-TB. This trial, "Standardised treatment regimen" of anti-TB drugs for patients with MDR TB (STREAM trial - Registration code: ISRCTN78372190) is expected to commence recruitment in Vietnam in 2012.

MDR TB prevalence is increasing in many countries; and recently the emergence of extensively drug resistant TB (XDR TB) threatens the attempt to control TB worldwide (Gandhi, Moll et al. 2006; Velayati, Masjedi et al. 2009). XDR TB is MDR TB which has further resistance to a FQN and one of the injectable second-line drugs (amikacin, capreomycin, or kanamycin) (WHO 2007). Although XDR TB is curable in HIV negative patients (Mitnick, Shin et al. 2008), the success rate is very low (48%) (Keshavjee, Gelmanova et al. 2008) that it is often described as virtually untreatable (2006; Chauhan 2007).

Data from WHO showed that there were around 30,000 notified MDR cases in 2007, whereas the number of estimated MDR cases was approximately 500,000 globally. The data suggested that at least 100,000 MDR TB patients (including 10,000 patients with XDR TB) worldwide need to be on specific MDR treatment to combat the development of drug resistance and reverse upward trends in MDR prevalence (WHO 2009). This means TB detection rates should be increased (more than current 63% (WHO 2009) alongside capacity building for MDR detection. With the attempt to curb the increasing rate of MDR TB the principal 2010 WHO targets for MDR TB have been set: (i) to offer diagnostic DST to all previously treated and chronic TB cases as well as to 90% of new TB cases with a high risk of having MDR TB (for example, contacts of MDR TB cases and those for whom treatment is failing after three months); and (ii) to enroll all those in whom MDR TB is diagnosed in Green Light Committee (GLC) (WHO 2009) approved or equivalent treatment programs. GLC is a subgroup of the MDR TB Working Group of the Stop TB Partnership to promote access to MDR TB treatment. It helps reduce the significant costs of second line drugs for government programs by bulk-purchase agreements. Despite the progress that has been made in some countries, the number of MDR TB patients notified in 2007 and the number of MDR TB patients to be enrolled on treatment in 2008 and 2009 fall far behind the expectations of the Global Plan in that only a third of notified MDR TB were enrolled on the treatment (WHO 2009).

1.17 Anti-tuberculous drug resistance in Vietnam

The Vietnamese WHO national drug resistance survey in 1996 showed that the prevalence of MDR TB in Vietnam was 2.3% in new cases (WHO 2006). According to the International Organization for Migration (IOM) in Vietnam, reviewing data from 1989 to 2000, the prevalence of MDR TB was around 4.5% (Ward, Marciniuk et al. 2005). This high prevalence of MDR in the latter study might be caused by bias in the selected population, whereas the national drug resistance survey sampled NTP patients throughout all regions of Vietnam, the IOM patients are more likely to be urban, where drug resistance rates are higher, and wealthy enough to afford private treatment.

The most recent WHO survey showed an estimated prevalence of MDR TB in Vietnam of 2.7% in 2007 (WHO 2009). Despite some inconsistencies in these data, the indication is that MDR TB prevalence in Vietnam is increasing, along with other forms of drug resistance. The fourth Vietnamese National Drug resistance survey was conducted in 2011, but has not finish yet.

INH, which is widely used globally, plays a key role in TB prevention and treatment. Resistance to INH is normally the first step in the development of MDR TB since mono-resistance to RIF is rare. In addition, Vietnam has a high burden of primary drug resistance with a prevalence of resistance to any TB drug of 32% in new patients in 1996 (WHO 2004). This prevalence increased to 36.6% by 2000. In a sample collection from pulmonary TB cases in HCM city from 1995 to 2000, the prevalence of INH resistance was 25% among primary TB patients (one of the highest

rates of first line drug resistance in the world) (Quy, Buu et al. 2006). In previously treated patients, the prevalence of resistance to any drug and INH was 65.8% and 54.5%, respectively (Quy, Buu et al. 2006). It is not known whether the high rate of primary INH resistance in this country would increase treatment failure, but data from other settings suggest it is a contributing factor (Faustini, Hall et al. 2005; Quy, Cobelens et al. 2006; Lew, Pai et al. 2008; Menon, Lodha et al. 2011). A better understanding of the resistance mechanisms to INH and their impact on clinical treatment outcomes is essential to combat the development of drug-resistant strains.

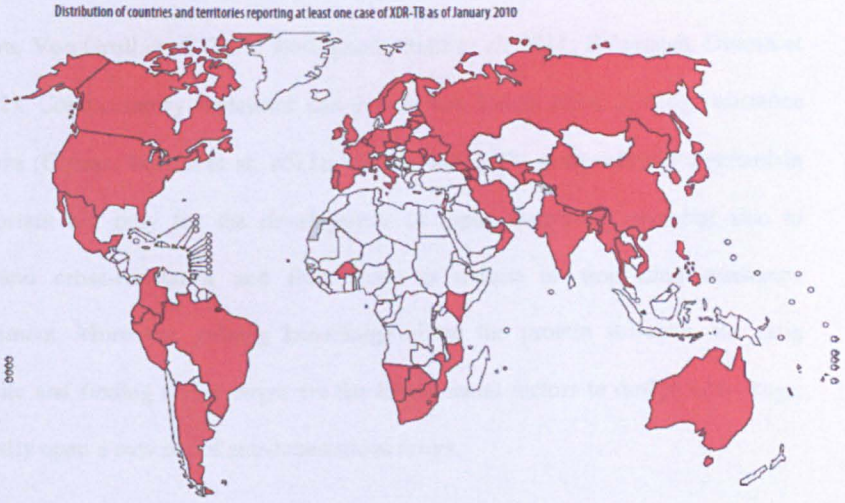


Figure 1.3: WHO mapping countries with XDR cases in 2010

Recently, Vietnam has reported the detection of XDR to WHO and is now one of 58 countries with more than one cases of XDR TB were identified (Figure 1.3) (WHO 2010). The exact number of XDR TB cases in Vietnam is still unknown; due

to lack of surveillance; yet the burden of untreated MDR TB in this population implies that XDR number might be significant and more research is urgently required to determine the extent of XDR TB in Vietnam.

1.18 Drug resistance mechanism (RIF and INH)

Drug resistance in *M.tuberculosis* is caused by single-step acquisition of single nucleotide polymorphisms in genomic DNA. *M.tuberculosis* does not carry plasmids, and so does not acquire resistance by lateral transfer. The role of efflux pump mechanisms is not clear but is likely to contribute to resistance to some drug classes (da Silva, Von Groll et al. 2011; Rodrigues, Ainsa et al. 2011; Balganesh, Dinesh et al. 2012). Compensatory mutations can reduce the fitness costs of drug resistance mutations (Comas, Borrell et al. 2011). Understanding the drug resistant mechanism is important not only for the development of rapid diagnostic tests but also to understand cross-resistance and find ways to reduce or stop drug resistance development. Moreover, gaining knowledge about the protein structure, the drug target site and finding a new target are the fundamental factors to design new drugs, potentially open a new era of anti-tuberculous drugs.

1.18.1 RIF resistance mechanism

RIF is one of the most important agents used to treat TB. Resistance to this drug arises due to mutations in the beta-subunit of DNA-dependent RNA polymerase encoded by the *rpoB* gene. The emergence of mutations associated with RIF

resistance follows a single-step mutation that occurs spontaneously at the frequency of $1/10^9$ which can lead to high level of RIF resistance (Lee, Cho et al. 1998). Single-base substitutions, insertions and deletions have been documented worldwide in a region of 81 bp on *rpoB* gene in which 96% of all these mutations can be found and has been termed the rifampicin-resistance determining region (RRDR) (Gillespie 2002).

Worldwide, the three most prevalent single-mutations were observed in three codons: 531, 526 and 516 (Nikolayevsky, Brown et al. 2004; Sajduda, Brzostek et al. 2004; Hillemann, Rusch-Gerdes et al. 2007). A previous study on RIF resistant isolates in Vietnam also showed a similar distribution of mutations in *rpoB* with the three hot spot codons 531 (43%), 526 (31%) and 516 (15%) (Caws, Duy et al. 2006).

Understanding the frequency and distribution of these mutations helps researchers develop accurate and rapid tests for detecting RIF resistant *M.tuberculosis* isolates which is very helpful in clinical diagnosis. Until now, there are many reports about new mutations in the *rpoB* gene; however, most of the RIF resistance mutations are located in the 81-bp RRDR region which implies that the development of rapid tests should focus mainly on the RRDR. However, since the frequency and distributions of the mutations have been shown to vary geographically, rapid tests should be evaluated in the location where they will be applied.

1.18.2 INH resistance mechanism

Although INH has been used since 1952 and the first clinical INH-resistant isolates were reported in the early 1970s (Lancet_report 1973), its mechanism of action and the development of resistance to this drug are still not fully understood.

There are at least four genes that are known to contribute to the resistance to this drug (Hazbon, Brimacombe et al. 2006), of which mutations at *katG* and *inhA* have been well investigated confirmed to be associated with INH resistance (Zhang and Telenti 2000). Mutations of *katG*, normally at loci 315, or deletion of the whole gene (which is rare), confer resistance to INH at high MIC, whereas mutation in the *inhA* promoter confers an intermediate level of resistance (Lavender, Globan et al. 2005).

A previous study from Vietnam reported a very high prevalence of mutations at *katG*315 (79%) (Caws, Duy et al. 2006). This dominant genotype in INH resistance population may suggest that *katG*315 mutation has a low fitness cost as compared with other INH resistance mutations.

INH is a prodrug which requires conversion into active forms by the bacterial catalase-peroxidase enzyme encoded by *katG* gene. After being activated, the drug agents target multiple sites and pathways in the cell. One of the target proteins of this drug is NADH-dependent enoyl ACP reductase, an enzyme encoded by *inhA* (Zhang and Telenti 2000). Thus, mutations at these genes consequently confer resistance to INH.

1.18.2.1 katG

Catalase-peroxidase, the enzyme encoded by the *katG* gene, has a central role in the activation of the pro-drug INH; therefore mutations in the *katG* gene region encoding the active site can confer resistance to INH. These mutations can be: deletions (of the whole gene or several codons), insertions or single-base substitutions. A study using site-directed mutagenesis (Rouse, DeVito et al. 1996) showed that the Arginine to Leucine substitution reported in some isolates at residue 463 is a phylogenetic mutation and has little effect on either enzymatic activity or resistance to INH, whereas substitution of Threonine for Serine at position 315 does confer resistance and is the most prevalent genotype among clinical isolates of INH resistant *M.tuberculosis.*

Other rare *katG* mutations have been reported at residues 270, 275, 315, 321, 381, 138, and 148 may play a direct role in eliminating enzymatic activity, while others such as R104L or H108Q may act primarily by inducing protein conformation changes which are unstable in the mycobacterial host (Zhang and Telenti 2000). The net effect is still the same: a loss of enzymatic catalase-peroxidase activity which subsequently causes resistance to INH because the prodrug remains inactivated or is activated at a reduced efficiency.

Deletion of the whole *katG* gene results in a high level of INH resistance (MIC>50μg/ml) (Musser 1995). However, further studies showed that deletion of this gene is normally observed via *in vitro* mutation generation experiments (Bergval, Schuitema et al. 2009) but is very rarely detected among clinical isolates (Guo, Seet et

al. 2006; Hazbon, Brimacombe et al. 2006). The reason for this is likely to be a reduced fitness of *katG* deleted bacilli since the catalase-peroxidase activity is essential to resist oxidative stress in the macrophage during host infection.

Many studies have shown that INH resistant isolates which have either mutation at *katG*315 or total deletion of *katG* acquire high level resistance to INH which are 5-10μg/ml or >50μg/ml, respectively (van Soolingen, de Haas et al. 2000).

1.18.2.2 inhA

Figure 1.4: *inhA* gene orientation in the operon and the promoter containing INH resistance mutation.

Molecular genetic studies have identified a two-gene operon with a contiguous Open Reading Frame (ORF) designated *fagG1* and *inhA*, which encodes for mycolic acid biosynthesis and is involved in resistance to both INH and ETH (Figure 1.4). The protein product of *fagG1* (or alternatively named *mabA)* has greatest identity with 3-oxoacyl-acyl carrier protein (ACP) reductase encoded by *fabG* of *E.coli*, *M.smegmatis, M.avium*, an enzyme involved in fatty acid biosynthesis pathway (first reduction step). The mycobacterial *inhA*-encoded enoyl ACP reductase, is an enzyme involved in the second reductive step in fatty acid biosynthesis. Protein InhA from

M.tuberculosis was purified, crystallized, and shown to be an NADH-dependent enoyl-ACP reductase with specificity for long-chain enoylthioester substrates. The sum of evidence suggests that FabG1 and InhA participate in mycolic acid biosynthesis. Mutations in the promoter of this two-gene operon or mutations inside *inhA* confer resistance to INH and ETH at low level (Larsen, Vilcheze et al. 2002).

INH is a prodrug which requires activation to generate the active compounds. One of the active agents, isonicotinic acyl NADH, has been identified and shown to have an extremely high affinity to the InhA protein (Lei, Wei et al. 2000). This provides the strong evidence that InhA is the target of INH, and explains the observed association between mutation in the *inhA* promoter and INH resistance phenotype.

Mutations in the promoter region (-8 and –15 upstream from start site of *fabG1* translation) in INH resistant strains which carry wild type *katG* suggests that these mutations result in increased InhA protein expression; thereby compensating for the InhA damaged protein cause by activated INH. This was proved by cloning and transformation experiment on *M. smegmatis* and *M.tuberculosis* (Banerjee, Dubnau et al. 1994). The results indicated that the mutations had no measurable effect on INH resistance in *M.smegmatis* but had a modest effect on *M.tuberculosis*.

Many mutations in the *inhA* structural gene have been identified in low-level INH resistant isolates without *katG* mutations. Crystal structure analysis and enzymatic characterization has shown that all amino acid substitutions would be located in the NADH binding site and INH resistance in these strains is related to the reduced NADH binding affinity for enoyl reductase (Dias, Vasconcelos et al. 2007).

Even though *fagG1* and *inhA* are located in the same operon and have similar function involved in long-chain fatty acid biosynthesis, all INH resistant mutations occur outside encoding sequence of *fagG1* and the gene remains intact. Experimental results from over expression of FagG1 from *M.tuberculosis* suggest it does not confer the ability to titrate either INH or ETH (Banerjee, Sugantino et al. 1998). This rules out the possibility of FagG1 being a direct target *in vivo* for active INH derivative, like isonicotinic acyl-NADH/NADPH. This result is consistent with the fact that, until now, no mutations have been mapped on *fagG1* gene in INH-resistant organisms (Banerjee, Sugantino et al. 1998).

1.18.2.3 Mutations in *oxyR-ahpC* intergenic region

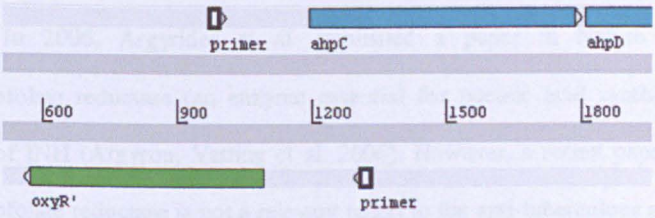


Figure 1.5: *oxyR-ahpC* intergenic region showing the region characterized between two primers

Further investigation of INH resistant *M.tuberculosis* isolates has shown that the *oxyR-ahpC* intergenic region (or the *ahpC* promoter sequence, Figure 1.5) contains many mutations. The *ahpC* gene encodes alkyl hydroperoxide reductase, a protein involved in the oxidative stress response. It is proposed that increased AhpC activity could compensate for the loss of catalase-peroxidae activity in the

detoxification of organic peroxides. Some mutations in this region were found in INH resistant isolates only (Kelley, Rouse et al. 1997). Western blot analysis showed that several of these mutations had enhanced transcriptional activity. However, some common mutations found in *ahpC* exist in both INH sensitive and resistant isolates and thus are polymorphisms (Baker, Brown et al. 2005). Other mutations are relatively rare among a broad spectrum of INH resistant strains and most of them occur in catalase-negative INH resistant isolates. These facts suggest that these mutations appear to be compensatory and are presumably selected after reduction in catalase-peroxidase activity attributable to *katG* changes arising with INH therapy. AhpC itself does not appear to directly mediate INH resistance.

1.18.2.4 A new target protein

In 2006, Argyrides et al. published a paper in Nature showing that dihydrofolate reductase (an enzyme essential for nucleic acid synthesis) is a new target of INH (Argyrou, Vetting et al. 2006). However, a recent paper showed that dihydrofolate reductase is not a relevant target to the anti-tuberculous activity of INH (Wang, Jain et al.). The discrepant result indicates that INH activity and its resistance mechanism is not simple. The fact is that sometimes the *in vitro* results do not reflect the actual effects of a drug *in vivo,* such as is the case with PZA (Bergval, Schuitema et al. 2009).

1.18.2.5 Other INH resistance mechanisms

A study in Vietnam in a set of 100 INH resistant isolates (Caws, Duy et al. 2006) showed that no single mutation was found in the resistant associated genes,

katG and *inhA*, among 10 INH resistant isolates (10%). Similar results have been shown worldwide with 15-25% of INH resistant clinical isolates having both wild-type *katG* and *inhA* genotypes. The implication is that mutations in *katG* or *inhA* do not account for all INH resistant strains, and suggests that some other unknown mechanisms may lead to INH resistance.

Unlike other forms of drug resistance, for example RIF resistance which has a hot-spot region conferring resistance in more than 70-98% of RIF resistant isolates (Heep, Brandstatter et al. 2001; Garcia de Viedma, del Sol Diaz Infantes et al. 2002; Cavusoglu, Turhan et al. 2006), resistance to INH can occur via alterations in several different genes including enzymatic structural genes as well as alteration in promoter regions. The relationships of *katG* and *inhA* mutations with INH resistance were well characterized. However, the evidence of mutations in other genes such as *ahpC, kasA, oxyR, ndh, glf* and *nat* is still unclear. Further investigations in the search for new mechanism of drug resistance in TB are necessary.

1.19 Drug resistance diagnosis

Knowledge of drug susceptibility patterns improves treatment outcomes by allowing appropriate therapy. However, drug susceptibility testing is not usually available for patients in resource limited settings such as Vietnam. DST is often limited by resource constraints to failure and retreatment patients, and in many high-burden regions is not available at all.

Within Vietnam, DST is only officially available in two reference laboratories in Ha Noi and HCM city and is only performed following the request of the physicians, usually after treatment failure or relapse. When the conventional DST is applied, it takes at least 2 weeks (when using MGIT) and several months (when using LJ media) to return the result. This results in delays to diagnosis which leads to the delay in giving appropriate chemotherapy. Direct DST on MGIT or by the MODS method can be used to shorten the turnaround period because the method can do DST by inoculated directly from sputum (Moore, Mendoza et al. 2004). However, the MODS technique is human-resource intensive because plates must be examined manually and repeatedly to detect growth of *M.tuberculosis* cords under inverse-microscope and MGIT DST is prohibitively expensive. Thus, a cheap and rapid molecular DST for TB is urgently needed.

So far, some fast methods for DST such as Mycobacteria-phage and NAAT based techniques have been developed. In the phage-based assay, live *M.tuberculosis* is detected in clinical samples by infection with phage. The sample is plated onto a lawn of *M.smegmatis* where plaques of lysed bacilli indicate the presence of phage which has replicated inside any *M.tuberculosis* in the clinical sample. The addition of drug to incubation media can be used to detect resistance. In contrast, NAAT methods amplify the target gene(s) and detect mutations which confer resistance to the drug of interest. LPA are also widely used for drug resistance testing. With the ability to differentiate multiple mutations using multiple specific probes, LPA report consistent

results with high sensitivity and specificity. The commercial MTBDRplus test is a successor of LPA that is recommended by WHO and widely used to screen MDR TB.

1.20 Pharmacokinetics of INH

INH is eliminated via acetylation by N-acetyltransferase (NAT2) enzyme system in kidneys. The speed of INH metabolism shows inter-individual variation due to genetic variation in the N-acetyltransferase genes (*NAT2*). Individuals classified as fast metabolizers have lower C_{max} values of INH in the sera.

Although pharmacokinetic-pharmacodynamic (PK-PD) index has been used for dosing, little information was available to define the PK-PD index of anti-TB drugs until the past 5 years. Microbial PK-PD concepts relate to the shape of the concentration-time profile and the minimum inhibitory concentration (MIC) which was used to optimize the efficacy of the drug toward the pathogen (Ambrose, Bhavnani et al. 2007).

A recent population pharmacokinetic analysis revealed that 88% of the variation in INH serum clearance is driven by *NAT2* gene; ethnicity, sex, and body weight accounting for very little to the variability (Kinzig-Schippers, Tomalik-Scharte et al. 2005). INH effect is best explained by peak concentration C_{max}:MIC ratio, and INH doses should be combined together and administered as high intermittent doses. Thus the area under the concentration-time curve (AUC) of INH is mostly driven by the dose of INH intake and the patient genotype. A study by Donald *et al.* showed that with INH administration over 10mg/kg, the INH serum concentration reaches C_{max}

despite the genotypes, but with the lower INH oral dose (3mg/kg) the contribution of genotype became predominant (Donald, Parkin et al. 2007). This suggests that in some ethnic groups where *NAT2*4* genotypes are dominant (associated with a high serum clearance), higher doses of INH may be needed, whereas people carrying *NAT2* SS genotype and being severity attacked by the side effect should be modified to lower doses that remain effective but minimize toxicity.

The pharmacokinetics of INH in children was also not well known until recently (McIlleron, Willemse et al. 2009). Interestingly, many studies showed that the process to eliminate INH in children is much faster than that of adults, therefore INH serum concentration in children is much lower when administered the same doses with adults, according to body weight (Schaaf, Parkin et al. 2005).The treatment guidelines for pediatric TB have been recently revised to reflect this (WHO 2010).

1.21 Pharmacogenetics of INH in Vietnamese

INH is eliminated by NAT2 enzyme system in humans. It is known that NAT2 genotype affects the serum concentration of INH (Donald, Parkin et al. 2007), therefore may also impact on the efficacy of INH therapy in some TB patients. The Vietnamese are likely to be predominantly fast acetylators as Chinese, Japanese and Thai ethnic groups (Zhao, Seow et al. 2000; Kita, Tanigawara et al. 2001), in contrast to USA, where the majority are slow acetylators. The acetylator status may play an important role in TB treatment based on its role in INH metabolism.

Caffeine and INH share the same pathway of metabolism in humans in which NAT2 is the key enzyme; therefore caffeine can be used to investigate acetylator phenotype in healthy volunteers.

Aims of the Thesis

The specific aims of this thesis are:

1. Develop rapid diagnostic tests for MDR TB and INH resistance based on identifying gene mutations (*rpoB*, *katG*, *inhA* and *ahpC*).

2. Confirm the association of *katG*315 mutation with higher MIC to INH compared to *inhA*-15 mutations in Vietnamese strains and determine the impact of the genotype backbone on MIC to INH.

3. Determine if INH resistance without RIF resistance is associated with poor outcome in HIV-associated TBM patients.

4. Determine the correlation between genotype and phenotype for NAT2 acetylation in Vietnamese Kinh population.

Chapter 2

Materials and Methods

2.1 General methods

2.1.1 Study location

Laboratory work for studies in chapter 3, 4, 5 and 6 of this thesis was conducted at OUCRU. Clinical isolates were collected from patients at two hospitals in HCM city, Vietnam: HTD and PNT.

HTD is a 500-bed tertiary reference hospital for infectious diseases, serving a population of 38 million in southern Vietnam.

PNT is a 800-bed tertiary reference hospital in HCM city which also houses the WHO accredited national reference TB laboratory for southern Viet Nam. Clinical isolates from pulmonary TB patients were collected at PNT hospital for chapters 3 and 4.

TBM patients were recruited at both hospitals for enrolment into the clinical trial which provided the isolates for the work described in chapter 5.

OUCRU is funded by the Wellcome Trust, UK. The Unit was established in 1991 with in HTD as a collaboration center between HTD and Oxford University to study tropical diseases, initially to study malaria but subsequently diversifying to include groups working on dengue, typhoid, influenza, TB, enterics, and zoonosses

supported by pharmacology, statistics, bioinformatics, host genetics and modeling departments.

2.1.2 Sample preparation.

Sputum sample decontamination

Sputum samples were decontaminated by NALC-NaOH decontamination using sputa prep kit from Nam Khoa company, Vietnam. The kit contains 4% NaOH and 2.9% sodium citrate and N-Acetyl L-Cysteine (NALC) powder.

NALC powder (0.5g) was dissolved in 100 ml of 4% sodium hydroxide-2.9% sodium citrate solution. The solution was vortexed until completely dissolved and used as decontamination solution within 24 hours after adding NALC.

Sputum sample (less than 10ml) was collected in a 50 ml clean capped container. An equal volume of decontamination solution was added to the container to obtain the final concentration of 2% NaOH. Both NaOH and NALC act to liquefy and decontaminate the sputum. The container was left to stand for 2-3 minutes for liquefaction before transferring the specimen from the container to a plastic 20ml centrifuge tube. The tube was tightly capped, vortexed and left to stand for 15-20 minutes for optimal decontamination. A balance must be achieved to obtain maximal killing of organisms other than *M.tuberculois* in the sputum without rendering the *M.tuberculosis* non-viable. The specimen was later diluted with sterile phosphate buffer solution (pH 6.8) or sterile distilled water to lessen the viscosity and decrease alkaline concentration to a final volume of 45ml prior to centrifugation for 15 minutes at a speed of 3,000rpm.

The supernatant was subsequently discharged carefully and the pellet was resuspended with sterile phosphate buffer solution (pH 6.8) or sterile distilled water to achieve a final volume of 0.5-1ml. This deposit was divided for smear preparation, inoculation of culture and an aliquot was stored in Eppendorf tube at -20°C.

CSF sample preparation

Two to ten mililitres of CSF was collected by lumbar puncture into a 20 ml clear sterile tube from all patients with suspected TBM. The sample was centrifuged at 3000rpm for 15 minutes, followed by culture and smear as soon as possible after processing. CSF samples are sterile and therefore decontamination is not required.

After centrifugation most of the supernatant was removed using a sterile pipette, and stored in an Eppendorf at -20°C. The deposit was vigorously resuspended in the remaining 300-400µl of supernatant. Two drops (approximately 100µl each drop) were used for smear, two drops were frozen at -20°C for later use and the remaining deposit was divided equally for culture on LJ and MGIT media.

2.1.3 ZN or AFB smear

Two drops of sample deposit was applied onto a clean glass slide. The slide was laid on a heater block at 80°C for approximately five minutes until dry in order to fix the material onto the slide. Carbol-Fuchsin was applied to cover the sampling area. The slide was then moved in a circle over a flame until the Carbol-Fuchsin steamed without boiling and laid on a bench for further 5 minutes for optimal penetration of the dye. Carbol-Fuchsin on the slide was washed with running tap water, 1% acid 70% alcohol solution and again with running tap water before applying Methylene

Blue solution (1.4mg/ml). After 30 seconds, the slide was rinsed with water, dehydrated and covered with a cover slip and observed under the microscope to read the result. A positive AFB sample shows a bright red rod shape of mycobacteria bacilli in the blue background of other material which is stained blue with Methylene Blue. A negative slide shows only blue background.

2.1.4 *M.tuberculosis* culture

Sputum and CSF samples were processed as detailed above (section 2.1.2) prior to inoculation for culture.

LJ culture

The LJ tube (Becton Dickinson, USA) was labeled with the study code and date of the sample. Around 100µl of sample deposit was used to inoculate on LJ media. The LJ media tube was capped and gently turned so that the inoculated suspension was spread across the sloped surface of the solid LJ media. The tube was secured to stand in a rack and kept in an incubator at 37°C. The tubes were first visually checked for positive growth two weeks after the innoculation. Negative tubes were continually incubated and checked weekly for positive samples. A sample was reported negative only if no growth was observed after 8 weeks of incubation.

MGIT culture using BACTEC 960 system

MGIT culture (Becton Dickinson, USA) was performed using the commercial kits according to manufacturer's instructions. Each MGIT tube was labeled with study code and date of the tested sample. Eight hundred microliters of OADC (Oleic-acid-albumin-dextrose-catalase) supplement (Becton Dickinson, USA) was added into the

MGIT tube. With sputum samples an additional 100ul of PANTA antibiotic supplement (Becton Dickinson, USA) was added into each MGIT tube to inhibit the growth of micro organisms other than mycobacteria. One hundred microlitres PANTA contains polymixin B (100 units), amphotericin (10 units), nalidixic acid (40 units), trimethoprim (10 units), azlocillin (20 units).

Around 100ul of decontaminated sample deposit was inoculated into the MGIT tube. The tube was tightly capped and inverted twice to mix the solution well with the innoculum. The bar code on MGIT tube was scanned before the tube was put into the indicated slot in BACTEC960 system. BACTEC960 is an automatic system that incubates MGIT tubes at 37°C, monitors the growth of micro-organisms in MGIT tubes every hour and automatically reports positive samples during 42 days of incubation. Growth is detected via an increased fluorescence, emitted in response to a decrease in oxygen concentration within the tube, caused by oxygen consumption of growing micro-organisms. A positive signal on MGIT does not confirm the presence of mycobacteria and positive samples must be confirmed by AFB microscopy, and *M.tuberculosis* identification. Negative samples are only reported after 42 days (6 weeks) when no growth is recorded. The initial incubation date, positive date and negative date are recorded and reported by the system.

2.1.5 Phenotypic DST

Conventional phenotypic DST on solid LJ media by 1% proportion method was performed at PNT according to standard WHO methods (WHO 2009). *M.tuberculosis* isolates are considered resistant if growth occurs on the media

containing the relevant drug at ≥1% of the growth observed on the drug-free media. Critical drug concentrations are 0.2 μg/ml INH, 40 μg/ml RIF, 2 μg/ml ETB and 4 μg/ml STR in accordance with the WHO standard protocol (Canetti, Fox et al. 1969). The microbiology laboratory is a WHO international reference laboratory undergoing and consistently passing supranational quality assurance with the WHO supervisory reference laboratory in Adelaide, Australia. In principle, one sample is inoculated equally on both drug-containing medium and drug-free medium. The number of colonies growing on drug-free medium is compared with the number on drug-containing medium and the proportion of resistant organisms is calculated. A resistant isolate is defined if the number of colonies on the drug-containing medium is 1% or more of the number developing on the drug-free medium.

Indirect DST in liquid media was performed using the MGIT SIRE kit on BACTEC960 system (Becton Dickinson, USA) at the HTD according to the manufacturer's guidelines. Standard critical concentrations in liquid media are lower than those for solid media testing: INH (0.1μg/ml), RIF (1μg/ml), ETB (5 μg/ml) and STR (1 μg/ml). In principle, one sample is inoculated into both drug-containing medium and drug-free medium with different amount of inoculum.

Innoculation suspensions were prepared using the manufacturers' guidelines for preparation from MGIT culture. Positive cultures were used on undiluted for preparation of organism suspension on days 1-2, cultures on day 3-5 positivity were diluted 1:5 in sterile saline solution. 0.1ml organism suspension was pipetted into 10ml sterile saline to prepare 1:100 growth control suspension, which was then mixed

by vortex. 0.5ml of growth control suspension was then inoculated into the drug free media and 0.5ml organism suspension to the drug containing wells.

A resistant isolate is defined based on the proportional growth relative to the inoculum size. The control-growth tube (drug-free) is only incubated with 1% of the bacilli in comparison with that used in the drug-containing medium. Once the control-growth tube becomes positive, detection of bacterial growth in the drug-containing medium identifies a resistant strain.

These DST results were used as gold standard for comparison with molecular tests in our studies.

2.1.6 Extraction of *M.tuberculosis* chromosomal DNA

DNA extraction of *M.tuberculosis* was performed using CTAB/chloroform method after culturing on LJ media (Honore-Bouakline, Vincensini et al. 2003). The purified DNA was dissolved in Tris-EDTA (TE, 10 mM Tris and 1 mM EDTA) buffer, quantified, diluted to the final 15ng/μl concentration and stored at -20°C

DNA extraction was performed when sufficient *M.tuberculosis* grew on LJ media. First, 0.6ml TE buffer was dispensed into labelled screw-cap tubes. The colonies of *M.tuberculosis* were scraped with a plastic disposable loop and dispersed in the tube with TE. The tubes were placed in a hot block and heated at 80°C for 20 minutes.

The tubes were allowed to cool before adding 50µl fresh lysozyme solution (10mg/ml) (Roche, Viet Nam). The mixture was vortexed before incubation at 37°C overnight.

On the following day, 10µl proteinase K (10mg/ml) (Roche, Viet Nam) and 35µl SDS 20% (w/v) (Sigma, Viet Nam) was added to the extraction tube, vortexed to mix and incubated at 55°C for 30 minutes. Then 100µl 5M NaCl (Merck, Viet Nam)and 100µl 5% CTAB solution (Sigma, Viet Nam) was added to the mixture, vortexed until opaque and incubated at 65°C for 15 minutes.

The tube was left to stand to cool to room temperature before adding 700µl chloroform:isoamyl alcohol (24:1) (Sigma, Viet Nam), vortexed and centrifuged at 12,000rpm for 5 minutes. At this stage, the mixture inside the tube has separated into three phases: aqueous phase (top), interphase (middle) and the organic phase (bottom). The top aqueous layer, containing DNA, was carefully transferred to a fresh Eppendorf (600µl). DNA precipitation was followed by adding 360µl (0.6 volume) of isopropanol to the Eppendorf. The solution was mixed well by inverting several times, left at –20°C for at least 30 minutes, then centrifuged at 12,000rpm for 15 minutes. At this stage, the precipitated DNA was gathered at the bottom of the Eppendorf and is visible by eye.

The supernatant was discarded and the pellet washed again with 1.0 ml of cold 70% ethanol to dissolve the salts remaining in the pellet without dissolving the DNA. Then the tube was centrifuged for 7 minutes at 12,000rpm. The supernatant was discarded again and the tube was left to stand with the lid open at room temperature

until the last traces of liquid evaporated. An appropriate volume of TE was added to each tube and left at room temperature for 2 hours or placed at 4°C overnight until the pellet was completely dissolved. All extracted DNA was stored at –20°C for later analysis.

2.1.7 Polymerase Chain Reaction (PCR)

PCR reaction mix contained 10-100ng of extracted genomic DNA (section 2.1.6), 10mM Tris pH 8.3, 50mM KCl, 1–3mM $MgCl_2$, 200 µM dNTPs, 200nM of each primer and 0.75 unit of Taq DNA polymerase. The concentration of individual reagents and annealing temperature was optimized for each PCR test.

To investigate RIF resistant isolates and amplify RIF resistance associated gene, *rpoB*, the primers and condition in Table 2.1 were used.

Table 2.1: Primers for *rpoB* PCR and sequencing for RIF resistance

Primer	Sequence (5'-3')	Ta (oC)	Conc (nM)	Target in *rpoB* gene	Product length
RPOBF	GGG AGC GGA TGA CCA CCC A	65	150	Flank RRDR	350bp
RPOBR[1]	GCG GTA CGG CGT TTC GAT GAA C	65	150		
TB146-F	CTT CTC CGG GTC GAT GTC GTT G	64	300	N-terminal	365bp
TB146-R [2]	CGC GCT TGT CGA CGT CAA ACT C	64	300	region	
RpoB-C3F	GAG TAC GTG CCC TCG TCT GA	56	300	*rpoB* cluster	319bp
RpoB-C3R	ACT TGC GCA TCC GGT AGG TA	56	300	3 region	

[1]: (Kapur, Li et al. 1994), [2]: (Heep, Brandstatter et al. 2001)

To investigate INH resistant isolates and amplify the genes associated with INH resistance, the primers and condition in Table 2.2 were used.

Table 2.2: Primers for PCR and sequencing of INH-resistance associated regions in *M.tuberculosis*

Primer names	Sequence (5'-3')	Ta °C	Conc. (nM)	Target	Product length
katGwgF1	GTC CTC TAT ACC GGA CTA CGC	61	120 nM each	*katG* whole gene	2899bp
katGwgR1	TCG CAC ATC CAG CAC ATT TC				
katGP5	GGT CGA CAT TCG CGA GAC GTT	68	150 nM each	*katG* codon 315, sequencing primers	519bp
katGP6 [1]	CGG TGG ATC AGC TTG TAC CAG				
TB92	CCT CGC TGC CCA GAA AGG GA	56	150 nM each	*inhA* promoter region	248bp
TB93 [2]	ATC CCC CGG TTT CCT CCG GT				
AhpC1	GCC TGG GTG TTC GTC ACT GGT	56	150 nM each	*oxyR-ahpC* intergenic region	359bp
AhpC2 [3]	CGC AAC GTC GAC TGG CTC ATA				

[1]:(Marttila, Soini et al. 1996), [2]: (Telenti, Honore et al. 1997), [3]:(Silva, Senna et al. 2003)

To investigate NAT2 genotype, *NAT2* gene was PCR with two primers NAT2F 5'-TGGGCTTAGAGGCTATTT and NAT2R 5'-GAGTTGGGTGA-TACATACAC which flank the area of 768bp, with the best annealing temperature of this primer pair is 65°C.

All of the reactions were amplified under the same thermocycling program: 95°C for 1 min, following by 30 cycles of 95°C for 10 sec, the right annealing temperature (or Ta°C, Table 2.1 and Table 2.2) for 30 sec, 72°C for 30 sec and a final step of 72°C for 5 mins.

Negative control (distilled water instead of DNA template) was included in every 'batch' of PCR to control and detect cross-contamination during processing.

PCR products were analysed by electrophoresis on 1% agarose gel at 150V from 30-60 mins and visualise in UV light.

2.1.8 Sequencing

Prior of sequencing process, the target DNA fragments were PCR in order to obtain enough material for sequencing.

RIF resistance gene

RIF resistant isolates were sequenced in the RRDR and N-terminal of the *rpoB* gene with primers showed in Table 2.1. Half volume of CEQ Quick Start kit (Beckman Coulter) was used to sequence from both strands of a 350bp fragment containing the 81bp sequence of RRDR.

INH resistance genes

INH resistant isolates were sequenced in *katG* gene. The resistant isolates with no mutation in the *katG* gene were also sequenced at the junction in *oxyR-ahpC* intergenic region and a junction in the *inhA* promoter. Using half volume of CEQ Quick Start kit (Beckman Coulter), DNA was sequenced from both strands of a 519bp fragment of *katG*, 359bp fragment of *ahpC* and 248bp fragment of *inhA*.

Acetylator NAT2 gene

The DNA of 37 healthy volunteers enrolled into the caffeine study was sequenced 768bp region in the second exon of *NAT2* gene which contains most polymorphisms associated with changes in acetylator phenotype. Using half volume of BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystem), sequence was obtained from both strands of this *NAT2* gene.

2.1.8.1 CEQ Quick Start Kit (Beckman Coulter, Singapore)

The CEQ8000 is a capillary electrophoresis sequencing machine which analyses sequencing reactions generated by CEQ™ Quick Start Kit. PCR products were used as templates in the sequencing reactions after an initial purification process.

- *Template preparation*

DNA templates used for sequencing in this thesis were generated from PCR products. The QIAquick PCR purification Kit (Qiagen, UK) was used to purify PCR products generated by a standard PCR. The concentration of purified PCR product was estimated by electrophoresis along with mass standard ladder (Biorad, USA) or by UV absorbance measured by NanoDrop (Thermo Fisher Scientific, USA) (Figure 2.1). Approximately 50 – 100ng of purified PCR products were used for one sequencing reaction.

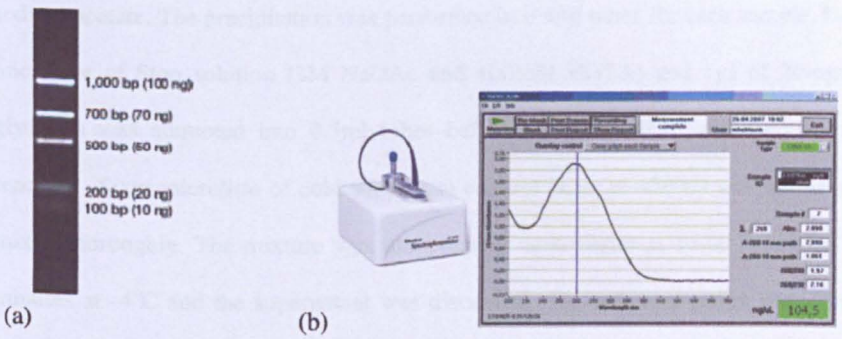


Figure 2.1: a) Mass standard ladder. b) NanoDrop equipment and software to measure DNA concentration.

- *Preparation of the DNA sequencing reaction*

Each fragment of DNA of interest was sequenced from both strands to confirm the accuracy of sequencing results. Each sequencing reaction contained 50-100ng of purified PCR product (depending on the length of PCR products), 3.2μM of primer (2μl of 1.6μM/μl primer), 4μl of DTCS Quick Start Master Mix (Beckman Coulter, Singapore) and dH_2O to make a total volume of 20μl. The thermal cycling was carried out with the standard CEQ program of 30 cycles of 96°C for 20 second initially, followed by 50°C for 20 second, and then 60°C for 4 minute. Finally the PCR reactions were held at 4°C.

- *Ethanol precipitation of sequencing products*

The purpose of this step is to eliminate any unwanted chemical, protein or primer which could interfere with the analysis on the sequencing machine. Unwanted PCR reagents were removed from solution by ethanol precipitation in the presence of sodium acetate. The precipitation was performed in 0.5ml tubes for each sample. Four microlitre of Stop solution (3M NaOAc and 100mM EDTA) and 1μl of 20mg/ml glycogen was aliquoted into 0.5ml tubes before adding 20μl of each sequencing reaction. Sixty microlitre of cold 95% (v/v) ethanol (kept at –20°C) was added and mixed thoroughly. The mixture was immediately centrifuged at 14,000 rpm for 15 minutes at -4°C and the supernatant was discarded. The collected pellet was rinsed twice with 200μl of 70% (v/v) ethanol (kept at –20°C). For each rinse, samples were centrifuged immediately at 14,000 rpm for a minimum of 2 minutes and the supernatant was discarded. The pellet was dried under vacuum for 40 minutes, then

resuspended in 40µl of Sample Loading Solution (supplied in DTCS Quick Start kit). The resuspended samples were then ready for analysis using the CEQ8000 sequencer. The sequencing results were recorded and analysed by CEQuence Investigator software.

2.1.8.2 BigDye Terminator v3.1 Cycle Sequence Kit

Similar to the process using CEQ Quick Start Kit, sequencing using BigDye Terminator v3.1 Cycle Sequence Kit has the following steps:

- *Template preparation* (as for CEQ, above)
- *Preparation of the DNA sequencing reaction*

According to the manufacturer, the BigDye method can sequence a fragment from 500bp-1000bp. Like other sequencing methods, each fragment of DNA of interest was sequenced from both strands to improve the accuracy of the results. Each sequencing reaction contained 5-20ng of purified PCR product, 3.2µM of primer, 2µl of BigDye Terminator (Applied Biosystem, Hitachi), 1µl of ABI buffer and dH_2O to make a total volume of 10µl. The thermal cycling was carried out with the standard ABI program of 25 cycles of 96°C for 10 second, followed by 50°C for 5 second, and then 60°Cfor 4 minutes. Finally the PCR reactions were held at 4°C.

- *Ethanol precipitation of sequencing products*

The first steps of precipitation of sequencing products were similar to CEQ. The only modification being: after the pellet was dried under vacuum for 40 minutes it was resuspended in 40µl of HiDi formamide solution (supplied with BigDye kit). The resuspended samples were then ready for analysis using the 3130xl Genetic

Analyzer (Applied Biosystem, Hitachi). The sequencing results were recorded and analysed by Sequencing Analysing 5.3 software.

2.2 Development of rapid diagnostic tests

2.2.1 Isolates

An existing archive collection of RIF/INH resistant *M.tuberculosis* isolates was used to develop and evaluate rapid molecular tests for drug resistance. Consecutive *M.tuberculosis* isolates routinely identified as resistant to either INH or RIF at PNT microbiology laboratory were collected for this study. One hundred and thirty one isolates were collected in total between January and March 2005. DST testing is not routine for new TB patients in the Vietnamese NTP and is only performed on request by the treating clinician, usually after treatment failure (smear positive after 5 months of treatment or more) or relapse/re-treatment cases. Information on individual reasons for DST was not available. Resistant isolates were transferred to HTD and were sequenced prior to this thesis in *rpoB*, *katG*, *inhA* and *ahpC* genes (Caws, Duy et al. 2006). In these 131 resistant isolates, 104 isolates are RIF resistant, 129 isolates are INH resistant.

All 104 isolates identified as RIF resistant (102 of which were MDR, 98.1%) which had been sequenced in the RRDR of *rpoB* gene, as previously described (Caws, Duy et al. 2006) were used to develop and evaluate the Multiplex Allele Specific PCR (MAS-PCR) for RIF resistance.

Only the first 100/129 consecutive INH resistant isolates which had been sequenced in *katG*, *inhA* and *ahpC* genes (Caws, Duy et al. 2006) were used to develop and evaluate the MAS-PCR for INH resistance.

In addition to the resistant isolates, 50 fully sensitive isolates were used as a control group for the analysis. These were archived isolates from TBM patients in a clinical trial of adjunctive dexamethasone at HTD with DST results available from previous research (Thwaites, Nguyen et al. 2004). Both drug susceptible and resistant isolate collections were used to evaluate the tests developed in this thesis by comparing the novel MAS-PCR tests with the commercial MTBDR and MTBDR*plus* LPA tests (Hain lifesciences, Germany) using the phenotypic DST results as the gold standard.

2.2.2 MAS-PCR for RIF resistance (MAS-RIF)

Multiplex PCR was used to detect point mutations in the *M.tuberculosis* genome. For RIF resistance, three separated MAS-RIF tests were used for each isolates that need to be tested. Each of three MAS-RIF reactions had the same outer primers RPOBF and RPOBR to amplify the RRDR of *rpoB* and one of the three inner primers RIF2R, RIF3F and RIF4R, which targeted to wild type at each hot spot codons 516, 526 and 531, respectively (Table 2.3).

Each MAS-RIF reaction contained 0.75 Unit of Taq DNA polymerase (Bioline), 10mM Tris pH 8.3, 50mM KCl, 0.2mM of each dNTP (Roche) and 125 - 400nM of primers (Table 2.3) and 2mM $MgCl_2$. Fifteen nano-grams of genomic DNA

was added. All of the reactions were amplified under the same thermocycling program: 95°C for 1 min, following by 35 cycles of 95°C for 10s, 68°C for 20s, 72°C for 20s and a final step of 72°C for 5 mins. A negative control (distilled water) and a positive control for each of the 3 target codons were included with each batch processed. PCR products were analysed by electrophoresis on 1% agarose gel at 150V for 45 minutes.

Table 2.3: Primers used for MAS-PCR for detection of RIF and INH resistance

Primer	Sequence (5'-3')	Ta (°C)	Conc (nM)	Target in *rpoB* gene	Product length (bp)
RPOBF	GGG AGC GGA TGA CCA CCC A	68	125	Flank RRDR	350
RPOBR[1]	GCG GTA CGG CGT TTC GAT GAA C		125		
RIF2R	GAC AGC GGG TTG TTC TGG T		300	Codon 516	139[+]
RIF3F	CCG CTG TCG GGG TAG ACC CA		400	Codon 526	219[++]
RIF4R	CCG CCG GGC CCC ATC GCC G		300	Codon 531	184[+]
HSP65F1	GCG GTA GAG GTG ACA TTG GG	62	150	HSP65	656
HSP65R2	TTA CCG GCT CCG ATG ACC T		150		
KatG2F	GGT AAG GAC ACG ATC ATC AGT		300	*katG* 315	329
katGP6 [2]	CGG TGG ATC AGC TTG TAC CAG		200		
TB92 [3]	CCT CGC TGC CCA GAA AGG GA		150	*inhA* -15	174
inhARmut [4]	AGT CAC CCC GAC AAC CTA TTA		150		

[+] amplicon generated with primer RPOBF

[++] amplicon generated with primer RPOBR

[1]: (Kapur, Li et al. 1994) [2]: (Marttila, Soini et al. 1996)
[3]: (Telenti, Honore et al. 1997) [4]: (Herrera-Leon, Molina et al. 2005)

2.2.3 MAS-PCR for INH resistance (MAS-INH)

The reaction was performed in a single tube with three pairs of primers. Each PCR reaction contained 1 Unit of Taq DNA polymerase (Bioline), 10mM Tris pH 8.3,

50mM KCl, 0.2mM of each dNTPs (Roche) and 150 - 250nM of primers (Table 2.3) and 2mM $MgCl_2$. Fifteen nanograms of genomic DNA was added. All of the reactions were amplified under the same thermocycling program: an initial denaturation step at 95°C for 2 mins, followed by 35 cycles of 95°C for 20s, 62°C for 20-60s (60s in the first 10 cycles, 20s in the latter 25 cycles), 72°C for 40s and a final step of 72°C for 5 mins to complete all reactions.

Positive and negative controls (distilled water) were included in every batch of PCR to ensure that the reaction was performed correctly and to detect cross-contamination during processing. PCR products were analysed by electrophoresis on 1% agarose gel at 150V for 30 minutes.

2.2.4 GenoType MTBDR and MTBDR*plus*

The MDRTB and MTBDR*plus* kits are commercial LPAs of Hain Lifesciences company (Nehren, Germany). These tests were performed according to the manufacturer protocol. Briefly, 15ng of sample DNA was used as a template to amplify *rpoB* and *katG* (and *inhA* for MTBDR*plus*) target sequences with biotin-labeled primers. Amplification products were analyzed by reverse hybridization to membrane-bound probes. Colorimetric detection gave purple bands, read by eye. A negative control (water) was included with each run.

The shaking waterbath was prewarmed to 45°C (±1°C). Solutions hybridization buffer (HYB) and the stringent wash solution were placed in the waterbath to warm to 37°C – 45°C so that the reagents were free from precipitates.

The remaining reagents except the conjugate concentrate (CON-C) and substrate concentrate (SUB-C)) were warmed to room temperature.

Diluted conjugate solution was prepared by diluting 1:100 CON-C with conjugate denaturate (CON-D) (10ul CON-C plus 990ul CON-D for each tested strip) in the required volume. The solution was mixed well and brought to room temperature. This solution was prepared before each use.

Diluted substrate solution was prepared with 1:100 of SUB-C diluted into SUB-D (10µl SUB-C plus 990µl SUB-D for each strip). The solution was mixed well and warmed to room temperature. Diluted SUB-C solution was stable for 4 weeks at room temperature and was stored in the dark.

Hybridization reaction

The strips were taken out of the tube using tweezers and labelled with a pencil underneath the coloured marker. Twenty microlitres of denaturation (DEN) solution was dispensed in a corner of each tray used. Twenty microlitres of amplified sample (PCR product) was added and mixed well by pipetting up and down. The mixture was left at room temperature for 5 minutes to denature all of the amplified products. Prewarmed HYB buffer (1ml) was added to each well and the tray gently shaken until the solution had a homogenous color. Each strip was placed in a well with the coated side (the side with color marker) facing upward, ensuring the strip was completely submerged in solution. The tray was then incubated in a shaking waterbath for 30 minutes at 45°C at 60rpm. The tray was submerged in the water at least one third of its height. After 30 minutes of incubation, HYB buffer was aspirated completely with

a pasteur pipette. One milliliter of prewarmed stringent solution was added to each strip and the tray was incubated for 15 minutes at 45°C in a shaking waterbath.

The following steps were performed at room temperature. The washing stringent solution was discarded completely by pouring out the solution, then turning the tray upside down and gently striking it on absorbent paper. The strip was washed again with 1ml rinse (RIN) solution for 1 minute on a shaking platform.

One milliliter of diluted conjugate solution was added to each strip and incubated for 30 minutes on a shaking platform before washing twice with 1ml of RIN solution and once with 1ml of distilled water for 1 minute on the shaking platform.

One milliliter of diluted substrate solution was added to each strip and incubated in the dark without shaking for 10 minutes. The colour development reaction was stopped by briefly rinsing twice with distilled water. Strips were removed from the tray and dried between two layers of absorbent paper. The strips were pasted on the result-report sheet to analyse the results and stored protected from light.

2.3 The association of *katG*315 and Beijing lineage with higher MIC to INH

2.3.1 INH resistant isolates for MIC test

In the archive collection (described in section 2.2.1) of *M.tuberculosis* phenotypically resistant to INH, 90 isolates representing the three main lineages of *M.tuberculosis* found in Vietnam were selected: Beijing, Indo-Oceanic and Euro-

American, 10 isolates of each genotype were selected which carried either a *katG*315 mutation or a mutation in the *inhA* promoter or no identified mutation at either the *katG* and *inhA regions.*

2.3.2 MIC test for INH

This technique was developed by modifying the automatic DST of the BACTEC960 system (Becton Dickinson, USA).

The BACTEC MGIT SIRE kit is a commercial phenotypic DST test which can be performed on either isolates or primary specimens. Based on the growth of *M.tuberculosis* in drug containing tubes (STR, INH, RIF, EMB) in comparison with control tube (without the drug), the system reports the drug susceptibility profile of tested isolates (section 2.1.5). Using the same reporting system, MIC was tested using 4 drug-containing tubes with varying concentrations of only one drug and a control for comparison of the growth. MIC was defined based on the lowest concentration of drug which inhibited the growth of *M.tuberculosis.*

This technique was chosen in order to standardize the results through objective result reporting.

Preparation of INH stock

Stock of INH (Sigma, USA) was prepared to a final concentration of 0.1μg/ml, 0.5μg/ml, 1μg/ml, 2μg/ml, 4μg/ml, 8μg/ml, 16μg/ml and 32μg/ml in the MGIT media.

INH was diluted in ELGA water, filtered to sterilize, and 320μl aliquots stored at -20°C until use.

Clean sterile tubes were arranged and labeled in appropriate order, set one included: Growth control (GC), 0.1μg/ml, 0.5μg/ml, 1.0μg/ml, 2.0μg/ml of INH tubes; and set two included: GC, 4.0μg/ml, 8.0μg/ml, 16μg/ml, 32μg/ml of INH tubes. All isolates were tested with set one; only isolates which showed resistance to all INH concentrations in set one were tested with the higher concentrations in set two. The prepared INH solutions were thawed to room temperature before each use. One hundred microlitre of appropriated INH stock solution were added to the labeled MGIT tubes. Then 800μl of OADC (supplied with SIRE kit) was added into each prepared MGIT tube.

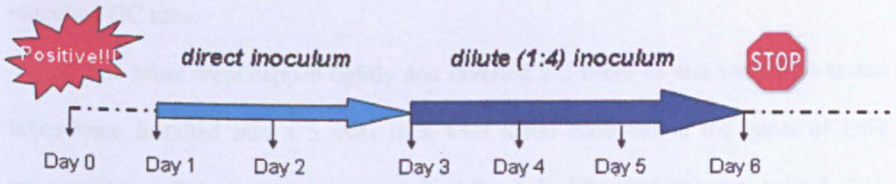


Figure 2.2: The process of inoculation dependent on the testing day after the test isolate became positive in MGIT.

Dilution and inoculation:

Isolates in the log phase were used for inoculation, having the number of post-positivity day greater than 1 and less than 6 reported by BACTEC960 (Figure 2.2). The positive tubes were vortexed 30 seconds to mix well and left to stand for about 15 – 20 minutes.

Isolates positive at day 1 or 2 were used directly for inoculation. Those with longer positive days (from day 3 to 5) were diluted 1:5 by transferring 1ml organism suspension (avoiding clumps) to the prepared 4ml saline tube. The dilution was pipetted several times to mix and then used for inoculation. The purpose of this step was to bring the bacilli to a concentration between 0.8 x $10^5$to 3.2 x 10^5 CFU/ml. Five hundred microlitre of this inoculum was used in drug contained tubes (4 tubes).

In this 1% proportion test of INH resistance, the control tubes should contain less bacilli, 1/100 the concentration of inoculation used in drug contain tubes. Thus the inoculation for GC tube was diluted by adding 100μl normal inoculum suspension into 9.9ml of sterile saline. This dilution was vortexed to mix and 500μl was used to inoculate GC tube.

The tubes were capped tightly and inverted 2-3 times to mix well. Five tested tubes were installed into a 5 slots rack with SIRE code bar in the order of INH concentration: GC, 0.1μg/ml, 0.5μg/ml, 1.0μg/ml, 2.0μg/ml for set 1; and GC, 4.0μg/ml, 8.0μg/ml, 16μg/ml, 32μg/ml for set 2. The bar code of the rack was scanned and put into 5 indicated consecutive slots in BACTEC960 machine.

Result interpretation

The BACTEC system reports a drug susceptibility profile for the test organism based on the growth index in drug-containing media compared with the drug-free control tube. At the time the growth index of the GC tube reaches 400 units, the growth index of any drug-containing tubes is determined. If a drug-containing tube has a growth index of 400 or above, the isolate is reported as resistant. Results were

reported between 3 and 14 days post-inoculation. If a result is reported in less than 3 days, a contamination event is likely to have occurred and if the growth control has not reached positivity within 14 days, an error has occurred with the inoculum concentration, or the bacilli are not viable. The MIC was categorized in 9 groups: susceptible to INH, resistant at 0.1μg/ml, 0.5μg/ml, 1.0μg/ml, 2.0μg/ml, 4.0μg/ml, 8.0μg/ml, 16μg/ml or 32μg/ml of INH.

2.3.3 Spoligotyping

Spoligotyping is one of the earliest typing techniques for *M.tuberculosis*. This is a PCR-based method, which depends on the amplification of a highly polymorphic direct repeat (DR) locus in *M.tuberculosis* genome. The numbers of DR elements vary among different strains of *M.tuberculosis* and the region may also contain one or more IS*6110* elements. Standard spoligotyping is used for detecting the presence or absence 43 variable spacers of known sequences in DR region which intersperse with the non-variable repeat sequence by hybridizing the PCR amplified spacer DNA to the set of 43 oligo-nucleotides embedded on a membrane (Figure 2.3). Evolution of the DR region is unidirectional through loss of segments of DNA in the DR region. Re-acquision of spacers does not occur, although convergent evolution of spoligotypes is theoretically possible, there is little evidence that it has occurred. Spoligotyping was performed following the international standard protocol (Kamerbeek, Schouls et al. 1997), using the spoligotype membrane (Isogen Bioscience B.V., Maarsen, The Netherlands), incubated with streptavidin-peroxidase, and detected with the ECL system (Amersham, United Kingdom). PCR to amplify the

spacers between DRs of *M.tuberculosis* strains was done with primers: DRa: biotin-5'GGTTTTGGGTCTGACGAC and DRb: 5'CCGAGAGGGGACGGAAAC. The PCR reaction contained 15ug of genomic DNA, 10mM Tris pH 8.3, 50mM KCl, 2mM $MgCl_2$, 200 µM dNTPs, 300nM of primer DRa and DRb and 0.5unit of DNA polymerase. The thermal cycling was carried out with an initial denaturing step of 94°C for 3 minutes, followed by 20 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°Cfor 30 seconds and a final step at 72°C for 3 minutes before holding at 4°C (Kamerbeek, Schouls et al. 1997).

The PCR products were then denatured at 99°C for 10 minutes, cooled on ice immediately and transferred to the miniblotter for hybridization for 60 minutes at 60°C. Any unbound PCR products were washed away and finally, bound products were detected by streptavidin-peroxidase and ECL development.

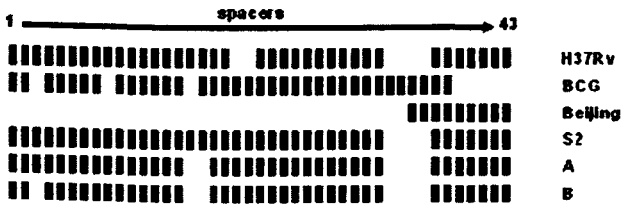


Figure 2.3: Representative spoligotype patterns obtained using 43 spacers in *M.tuberculosis*. Spoligotypes of H37Rv, BCG, a Beijing strain and 3 other clinical isolates are shown.

Note: this technique was performed by Vo Sy Kiet.

2.3.4 Statistical analysis for MIC

Log-transformed MIC values were compared using analysis of variance (ANOVA). If there was a significant difference on ANOVA, pairwise comparisons of the groups was performed. As the global ANOVA acts as a 'gatekeeper', no multiplicity adjustment for the pairwise comparisons is necessary. As a sensitivity analysis, the Kruskal-Wallis test was used instead of parametric ANOVA. Two-way ANOVA was used for pairwise comparisons.

All statistical analysis were performed using R, version 2.10.1 (R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org.) with Dr Marcel Wolbers (Head of biostatistics, OUCRU).

2.4 The influence of INH resistance on the outcome of TBM

2.4.1 Archive samples for the analysis

Patients enrolled into two consecutive studies of HIV-associated TBM who had *M.tuberculosis* isolated from the CSF were included in the analysis.

The first study was an observational cohort study of 58 HIV-associated TBM admitted to the HTD, HCM city between November 2004 and September 2005 (Torok, Chau et al. 2008).

The second study was a randomized, double-blind, placebo-controlled trial of immediate versus delayed ART in adults with HIV-associated TBM (PI: Estee Torok, trial registration number: ISRCTN63659091). Two hundred and fifty three HIV-

infected patients with TBM who were antiretroviral naïve were randomly assigned to receive immediate or deferred (2 months) initiation of ART between September 2005 and December 2008. TBM patients randomization was stratified according to the MRC grade I, II or III of TBM at presentation. The primary trial endpoint was 9-month mortality. The trial was conducted at two sites in HCM city: HTD and PNT hospitals (Torok, Yen et al. 2011).

Both studies were approved by the Oxford Tropical Research Ethics Committee (OXTREC), the Institutional Review Boards of the participating hospitals and the Health Services of HCM city.

2.4.2 Anti-tuberculous therapy

According to PNT and Vietnamese NTP guidelines, TBM patients were given 4 first-line antituberculous drugs daily: INH (5mg/kg), RIF (10mg/kg), PZA (25mg/kg), and EMB (20mg/kg). After 3 months, the patient continued to receive RIF and INH at the same dosage for a further 6 months.

Intramuscular STR (20mg/kg) was added for patients previously treated for TB, or if drug resistance was suspected due to clinical deterioration. Additional drugs or second-line antituberculous agents were not given as they were not routinely available in Vietnam.

All patients were treated with adjunctive dexamethasone. A reducing dose of dexamethasone was administered on study entry, according to the grade of TBM at presentation (Torok, Yen et al. 2011).

2.4.3 Anti-retroviral therapy

Antiretroviral treatment was not generally available in Vietnam at the time of the observational study and therefore only 7/58 patients in the first cohort study received ART. All patients who survived to hospital discharge were referred to the National HIV Programme for HIV treatment and care, according to local procedures (Torok, Chau et al. 2008).

In the RCT, ART or identical placebo tablets were commenced as soon as possible after study entry. The antiretroviral regimen was zidovudine 300mg, lamivudine 150mg (as a fixed dose combination tablet) and efavirenz 800mg (if taken with RIF) or efavirenz 600mg (if taken without RIF) taken daily. After 2 months all patients were receiving ART (Torok, Yen et al. 2011).

2.4.4 TBM sample processing

CSF samples were transferred to the laboratories at HTD and PNT for initial processing. Samples were stored securely in freezers at HTD and PNT prior to transfer to the OUCRU for further investigations and long term storage (archived samples).

2.4.5 Statistical analysis of DK study

We visualized survival by anti-TB drug resistance group or *M.tuberculosis* lineage, respectively, with Kaplan-Meier curves. Formal comparisons of 9-month survival between groups were based on the log-rank test.

Comparison of resistance was based on three prospectively defined resistance groups: fully susceptible or STR mono-resistance; INH resistance with or without STR-resistance; and MDR. We also performed exploratory analyses to determine if ignoring SM-resistance in the grouping was consistent with the study data.

In addition, we compared the three groups with a Cox regression model with indicator variables for the different groups and adjustment for MRC TBM stage at presentation, CD4 cell count (log-transformed), and the cohort group (observational cohort study, RCT – immediate ART, RCT – deferred ART). The proportional hazards assumption of Cox regression was tested based on scaled Schoenfeld residuals. If it was violated for a covariate, we alternatively fitted separate (time-dependent) regression coefficients for the intensive TB treatment phase (first 3 months) and thereafter.

Analyses were performed both with and without inclusion of patients receiving STR treatment. CD4 cell counts were missing for 18 subjects and TBM grade for one patient of the 186 participants included in the analysis and the adjusted Cox regression analyses were based on multiple imputation of missing covariates.

All statistical analyses were performed with the statistical software R version 2.11.1 (R_Development_Core_Team 2010) and the companion R package mice 2.8 (White, Royston et al. 2011).

2.5 The correlation of acetylator phenotype and NAT2 genotype

2.5.1 Healthy volunteers for caffeine study

The study was approved by OXTREC (reference number: 09 09). Participants had not taken any medication for at least one month prior to participation and were in good general health. Staffs at OUCRU and health care workers at HTD were invited to enroll in this healthy volunteer study through a presentation at the weekly academic meeting and information session.

Data collection

All volunteers were provided with a participant information sheet, given the opportunity to ask any questions, and if willing to participate in the study asked to sign a consent form and complete a questionnaire (Figure 2.4).

Section B - *to be completed on caffeine metabolism day*

ILLNESS HISTORY – SECTION B	
1. Have you been sick in the last 2 weeks?	Yes ○ No ○
2. Have you taken any prescription drugs in the last 2 weeks?	Yes ○ No ○
3. Have you drunk coffee, Coke, Pepsi, Red-Bull, Sting, tea or chocolate in the last 3 days?	Yes ○ No ○
- Coffee	1...2...3...>3... cup(s)
- Coke / Pepsi	1...2...3...>3... can(s)
- Red-Bull / Sting	1...2...3...>3... can(s)
- Tea	1...2...3...>3... cup(s)/bottle(s)
- Chocolate	1...2...3...>3... cup(s)/bar(s)
4. Do you drink coffee? If yes, how many cup of coffee a day do you drink?	Yes ○ No ○
5. Do you drink alcohol?	Yes ○ No ○
6. Do you smoke? If yes, how many cigarettes a day do you smoke?	Yes ○ No ○

Figure 2.4: Questionnaire for caffeine metabolism study

Sample collection

Each volunteer was asked to provide an initial baseline urine sample, take two tablets of caffeine (100mg total dose) and provide another urine sample after 6 hours. Five ml of blood was drawn into EDTA tubes (Beckton Dickinson, USA). Samples were stored in a fridge for no longer than 24 hours before DNA extraction or kept in freezer -20°C for further analysis.

2.5.2 Extraction of human chromosomal DNA from whole blood

Nucleon Genomic DNA extraction kit (GE Healthcare, Amersham Biosciences) was used for DNA extraction. Blood was collected in sodium EDTA tubes (Becton Dickinson, USA) to prevent clotting.

The extraction process was prepared by dispensing 20ml reagent A (10mM Tris-HCl, 320mM sucrose, 5mM $MgCl_2$, 1% (v/v) Triton X-100, pH 8.0) into 50ml Falcon tubes. Whole blood sample (5ml) was transferred into the falcon with a pasteur pipette, vortexed and left to stand at room temperature for 10 minutes. The Falcon tube was centrifuged at 1300rpm for 5 minutes before discarding the supernatant. Two milliliters of reagent B (protein lysis buffer) was added to the pellet, vortexed for 30 seconds and incubated at 37°C for 20 minutes. The solution was transferred to a 15ml tube with addition of 500µl of 5M sodium perchlorate solution. The tube was tightly capped and mixed by inverting 7-10 times. Three milliliter of chloroform was added and mixed by inverting the capped tube until opaque and left to stand for 5 minutes. Three hundred milliliters of Nucleon resin was gently added to the mixture without remixing the phases. The tube was capped and centrifuged at 2400rpm for 3 min.

After the centrifugation, the solution was divided in to three layers, 3ml of the top aqueous layer contain soluble DNA was carefully pipetted to a fresh 15ml tube. Two milliliters of isopropanol was added to the new tube to precipitate the DNA. The tube was mixed well by inverting several times and left at –20°C for at least 30 minutes before being centrifuged at 2400rpm for 10 minutes. The supernatant was

removed by pouring-out gently. The remaining pellet was washed by adding 2 ml of cold 70% ethanol, centrifuging for 5 minute at 2400rpm and removing the ethanol solution.

The tube was left to stand with the lid open at room temperature until the last traces of liquid had evaporated. Three hundred microliters of ELGA water or TE buffer was added to the tube, mixed by hand and left at room temperature for 2 hours or placed at 4°C overnight for the optimal resolution of the DNA. All extracted DNA were stored at –20°C for later analysis.

Human DNA were used for genotyping NAT2 by sequengcing. The DNA also used to develop PCR-RFLP assay.

2.5.3 Measuring caffeine metabolism in healthy volunteers

Reagents

1- methyluric acid (1U), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U), 7-beta hydroxyethyltheophylline (β-HT) (Internal Standard IS) were purchased from Sigma (Sigma-Aldrich, Singapore), 5-acetylamino-6-formylamino-3-methyluracil (AAMU) from SynFine Research (Richmond Hill, Canada). All other solvents and chemicals were purchased from Merck, Vietnam.

Caffeine metabolites are quite complex that have more than one enzyme involve in the process. In order to measure the clearance process of caffeine by NAT2, we used the ratio of AAMU/(AAMU+1X+1U) to tell how fast of NAT2 activities in comparison with other enzyme (mostly CYP1A2) (Figure 2.5).

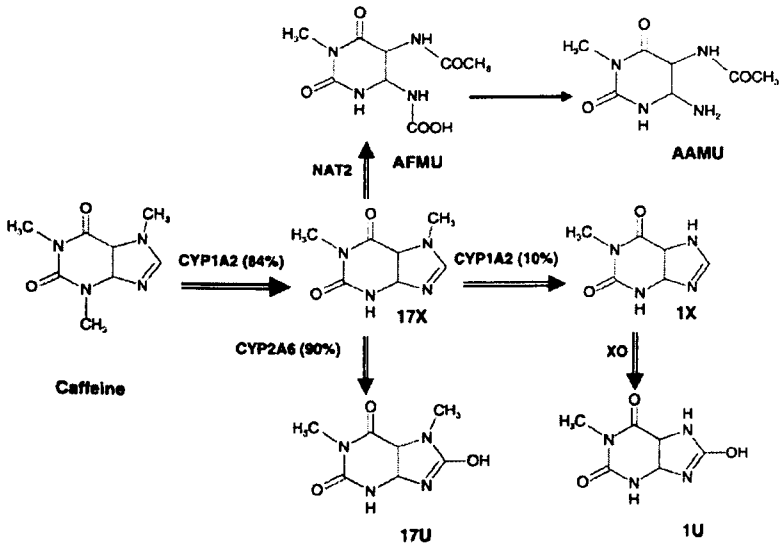


Figure 2.5: Caffeine metabolites

HPLC performance

The chromatographic apparatus (Merk, Vietnam) was composed of Hitachi modules: two L-2130 pumps, one L-2200 refrigerated autosampler, one column oven L-2350, and one diode array detector L-2455. The software EZ-ChromElite (EZChrom Elite for Hitachi Version 318b) was used to pilot the HPLC instrument and to process the data throughout the method validation and the sample analysis.

The caffeine metabolites were separated on a Ascentis RP-Amide (250 mm x 4.6 mm ID, 5µm) column (Supelco, USA) equipped with an Ascentis RP-Amide guard column (20 mm x 2.1 mm ID, 5µm). This configuration allowed performance of the extractionless analysis of the caffeine metabolites from urine with the best selectivity, accuracy and precision. The mobile phase consisted of 20 mM acetic acid

(HAc), acetonitrile (ACN) and was delivered at a flow rate of 1 $ml.min^{-1}$. For all caffeine metabolites and internal standard, a stepped gradient elution was developed using HAc-ACN (100:0) at 0 min → (96:4) at 2 min → (88:12) at 13 minutes maintained for 3 additional minutes. The chromatographic separation was performed at 55°C and monitored simultaneously at 264 nm for AAMU, 1X, I.S and 284 nm for 1U and 17 U.

Stock solutions of metabolites at a concentration of 5 mM were prepared separately in different solutions: AAMU, 1 X in 5.0 mM NaOH solution; 17U in NaOH 2.5 mM, 1U in water-methanol (70-30 v/v). All metabolites needed the addition of 20 µl NaOH 1M to be dissolved completely. The stock solution was neutralized by adding 20 µl HCl 1M. All stock solutions were stored at -20°C and thawed prior to use. Sets of working solutions containing all metabolites (AAMU, 1X. 1U, 17U) were diluted in water. These standard mixtures were diluted 10 times to achieve 5, 12.5, 25, 50, 125, 250 µM in blank urine (obtained from one healthy volunteer after 3 days xanthine-free diet) and were used for the calibration.

For quality control samples, working solutions were diluted 10 times in a blank urine to yield final concentration of 20µM, 100µM and 200 µM, corresponding to quality control at low (QCL), medium (QCM) and high concentration (QCH), respectively.

β- hydroxyethyltheophylline (β-HT) was used as an internal standard (IS). A stock solution of β-HT at 1 mg/ml was prepared in water-methanol (70-30 v/v). Prior

to spiking the samples, a solution at 10 µg/ml was freshly prepared by dilution in water.

One hundred microlitres of urine sample was added to 200 µl of internal standard. After 20 s vortex mixing, 100 µl of NaOH 0.1N was added to the mixture to fully convert AFMU into AAMU. After 10 min of incubation at room temperature, 100 µl of HCl 0.1N was added to neutralize the excess of base. Finally an aliquot of 10 µl of sample was injected onto HPLC column.

These experiments were conducted by Hoang Thi Thu, of the OUCRU pharmacology group.

2.5.4 Statistical analysis

R-package "mclust" was used for normal mixture modeling via the EM algorithm. The number of components in the best-fitting mixture was chosen by the Bayesian information criterion (BIC). We considered both models with equal variance (denoted as "E" in the BIC plot) in each group and those with unequal variance (denoted as "V" in the BIC plot). Of note, this approach is statistically better justified than methods based on probit plots.

The statistical tests were performed by Mann-Whitney test with Prism 5 for Windows, version 5.02.

2.5.5 PCR-RFLP for NAT2 genotyping

PCR was done to amplify 628bp fragment of the second exon in human NAT2 gene with two primers NAT2Fa (5'-CCAGTTAACAAATACAGTAC-3') and NAT2R (5'-GAGTTGGGTGATACATACAC-3'). Each 50ul of PCR mix contained 50-100ng of genomic DNA (section 2.5.2), 10mM Tris pH 8.3, 50mM KCl, 2mM $MgCl_2$, 200 µM dNTPs, 200nM of each primer and 1 Unit of Taq DNA polymerase.

The amplified PCR run under the following thermocycling program: 95°C for 1min, following by 30 cycles of 95°C for 10 sec, 65°C for 20 sec, 72°C for 40 sec and a final step of 72°C for 5min. A negative control (distilled water) was included in every run.

After the PCR finished, 5ul of PCR product was used to check for the present of PCR product by electrophoresis on 1% agarose gel at 150V for 10 minutes.

Then 15ul of PCR amplicons were aliquoted into three separate PCR tubes, 5U of the respective restriction enzyme was added to each small Eppendorf tubes: *Tsp*RI, *Taq*I and *Bam*HI (New England Biolabs, USA) and the tubes were then incubated at the appropriate temperature: 65°C, 65°C and 37°C, respectively, for 3 hours. The products were electrophoresed on a 1.5% agarose gel for 2 hours before visualise under UV light.

Chapter 3

Development of rapid diagnostic tests for MDR and INH resistance

3.1 Background

The rise of MDR TB, which is defined as *M.tuberculosis* resistant to at least INH and RIF, threatens the success of TB control programs in many regions of the world (WHO 1997; Zignol, Hosseini et al. 2006). It has been demonstrated that MDR TB can successfully transmit within a community (van Doorn, de Haas et al. 2006; Sun, Lee et al. 2007) and therefore early detection of infectious cases is vital to interrupt chains of transmission (Blower and Chou 2004; Cohen and Murray 2004). MDR TB is difficult to treat, with treatment durations of 18 months or longer, using more toxic and less potent second line drugs (Caminero 2006; Resch, Salomon et al. 2006). Further, the cost of treating a MDR patient is many times the cost for a TB patient infected with a fully susceptible strain even with the help of the Green Light Committee, which has negotiated significant reductions in second line drug costs for government programs (Gupta, Irwin et al. 2004; Resch, Salomon et al. 2006).

Besides, detecting drug resistance, particularly MDR TB, before instigation of inappropriate chemotherapy reduces morbidity, mortality, economic costs and unnecessary treatment with ineffective drugs. Conventional, phenotypic DST only return results after several weeks of culture in MGIT or LJ media.

Two commercial molecular LPAs (Baltussen, Floyd et al. 2005) for MDR TB are currently available: the INNO-LiPA RIF TB test (Innogenetics, Zwijnaarde, Belgium) (Morgan, Kalantri et al. 2005) and the MTBDR-*Plus* kits (Hain Lifescience, Nehren, Germany) (Hillemann, Rusch-Gerdes et al. 2007). The MTBDR-*Plus* LPA has replaced the MTBDR assay. These kits perform well in research settings (De Beenhouwer, Lhiang et al. 1995; Cavusoglu, Turhan et al. 2006; Makinen, Marttila et al. 2006; Somoskovi, Dormandy et al. 2006). The newly developed Xpert MTB/RIF test for use with the GeneXpert system (Cepheid, USA) is a cartridge-based real time PCR test. The Xpert test requires minimal sample processing by automatically performing the extraction, purification, concentration, amplification and identification of *M.tuberculosis* genes directly from clinical samples (Boehme, Nabeta et al. 2010). The Green Light Committtee and FIND have negotiated preferential pricing of these tests for high-burden or developing country settings. However, at over 5USD per test strip (MTBDR*plus* test,) or around 17USD per test cartridge (Xpert MTB/RIF,), the tests are currently too expensive to be used routinely on all TB patients in developing countries like Vietnam. Scale-up of the use of these tests has focused on high-risk groups for MDR TB, such as failure and relapse patients. It is necessary to develop low-cost techniques which are accurate, simple and rapid for the identification of drug resistant TB to allow early identification and treatment of MDR TB.

It has been shown that RIF resistance is a surrogate marker of MDR, because RIF mono-resistance is rare in most settings, including Vietnam, (Van Rie, Warren et al. 2001; Johansen, Lundgren et al. 2003; Caws, Duy et al. 2006) therefore a

molecular technique for the rapid detection of RIF resistance can be used to screen for MDR TB. RIF mono-resistance occurs more frequently in HIV positive TB cases (Sandman, Schluger et al. 1999) and therefore in areas of high HIV prevalence, RIF resistance may not be applicable as a surrogate marker of MDR TB and confirmation of both RIF and INH resistance may be necessary to confirm MDR TB.

Resistance to RIF is conferred by single-step mutation in the 81bp RRDR of the *rpoB* gene which encodes the beta subunit of the RNA polymerase. Single base substitutions, insertions or deletions in the *rpoB* gene can lead to RIF resistance (Gillespie 2002).

Studies in the literature have used various molecular mutation detection techniques to identify MDR TB, such as real-time PCR (Sajduda, Brzostek et al. 2004; Wada, Maeda et al. 2004), Single Strain Conformation Polymorphism (SSCP) (Bobadilla-del-Valle, Ponce-de-Leon et al. 2001) or sequencing to detect mutated nucleotides (Kapur, Li et al. 1994; Cavusoglu, Hilmioglu et al. 2002). However, these techniques require expensive equipment and technical expertise.

Multiplex allele specific (MAS) PCR is the most inexpensive method that has been described (Mokrousov, Otten et al. 2002). In a previous study in Vietnam, we have demonstrated that the mutations in RRDR of the *rpoB* gene are located mostly at three hot spot codons 531 (43%), 526 (31%) and 516 (15%) (Figure 3.1), concurring with prevalence reported worldwide (Van Rie, Warren et al. 2001; Gillespie 2002). To minimise post-amplification processing, we used 3 MAS PCR reactions for each

isolate, with two outer primers as positive control for each sample and one inner primer to detect the mutation.

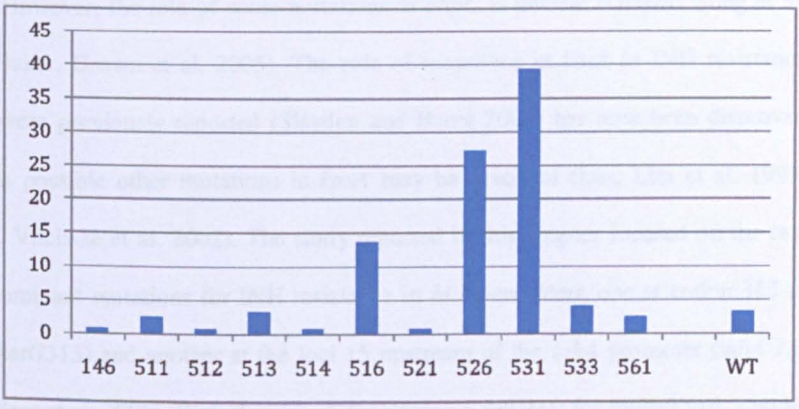


Figure 3.1: The distribution of mutations in *rpoB* gene in *M.tuberculosis* in our RIF resistance sample collection (Caws, Duy et al. 2006).

Although detection and treatment of MDR TB has become a priority of international TB control efforts in the last decade, a focus on RIF resistance alone is inadequate since patients with other forms of first-line drug resistance are also at higher risk of treatment failure and INH resistance is a precursor to MDR TB. The prevalence of INH resistance is always higher in comparison with MDR. In Vietnam particularly, the prevalence of INH resistance is extremely high, around 25% in new smear positive TB patients in HCM city (Quy, Buu et al. 2006). Methods for the detection of INH resistance which can be applied in resource limited, high-burden settings are urgently needed.

There are various single mutations related to INH resistance which are mostly located in *katG, inhA* and *ahpC* as described in the literature (Marttila, Soini et al. 1996; Telenti, Honore et al. 1997; Mokrousov, Otten et al. 2002; Silva, Senna et al. 2003). However, the role of some mutations in *ahpC* is unclear (Deretic, Song et al. 1997; Baker, Brown et al. 2005). The role of mutations in *kasA* in INH resistance which were previously reported (Slayden and Barry 2002) has now been disproven but it is possible other mutations in *kasA* may be involved (Lee, Lim et al. 1999; Larsen, Vilcheze et al. 2002). The study reported in this chapter focused on the two most dominant mutations for INH resistance in *M.tuberculosis*, one at codon 315 in *katG* (*katG*315) and another at the loci 15 upstream of the *inhA* promoter (*inhA-15*) (Guo, Seet et al. 2006). With the aim of developing a new test for routine use, a novel multiplex-PCR was developed, which is specific for *M.tuberculosis* and INH resistance conferring mutations at *katG315* and *inhA-15*.

The distribution of mutations responsible for resistance in *M.tuberculosis* varies geographically but the reasons for this are not known. A previous investigation of 104 RIF resistant isolates and 100 consecutive INH resistant isolates showed the frequency of these mutations in the Vietnamese *M.tuberculosis* population (Caws, Duy et al. 2006), and was used to develop and to evaluate the tests.

The sensitivity and specificity of these two multiplex PCRs were compared with the commercial reverse-hybridisation MTBDR test. Fifty fully susceptible *M.tuberculosis* isolates were used as controls. The MTBDR test detects both RIF and

INH resistance in one strip. Phenotypic DST results were considered the gold-standard.

AIMS:

1) Develop MAS-PCR test for RIF resistance.
2) Develop MAS-PCR test for INH resistance.
3) Evaluate these novel tests in comparison with commercial LPA using an archive collection of *M.tuberculosis* strains with well characterised DST profiles.

3.2 Results

3.2.1 Development of MAS-PCR to detect RIF resistance

Based on a previous study of the mutation prevalence among 104 clinical isolates resistant to RIF collected in HCM city, Vietnam (Caws, Duy et al. 2006), 88.5% of mutations for RIF resistance were located in three codons 531, 526 and 516 in the *rpoB* gene. Therefore a test was developed to analyse these 3 hot spot codons.

Six primers were designed to target these three codons in both directions on *rpoB* template of H37Rv. These primers were designed with Primer Express version 2.0 software (Applied Biosystems Inc, USA) to anneal to wild type DNA only but not to mutated strains. After serial tests on DNA of clinical isolates which had been sequenced and stratified as wild type and mutated isolates, three inner primers were selected (Figure 3.2).

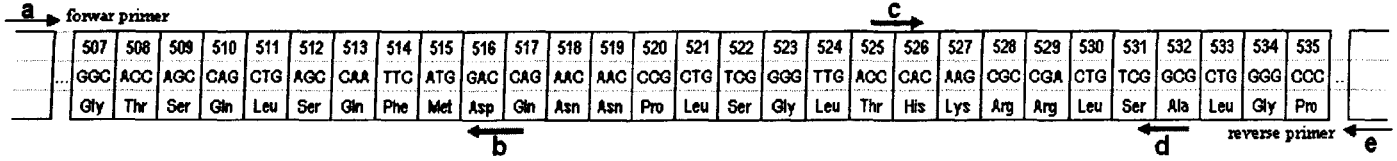


Figure 3.2: Location of primers for MAS-RIF in the RRDR of the H37Rv *rpoB* gene.

a, e: outer primers amplifying 350bp product.

b,c,d: inner primers to detect wild type at codon 516, 526 or 531

A multiplex PCR was designed with the following principle:

For RIF resistance, three different multiplex PCRs were used in order to identify mutation at the three hot spot codons. Each MAS- PCR was optimized individually for the best annealing temperature, magnesium and primer concentration (Figure 3.3, 3.4. 3.5).

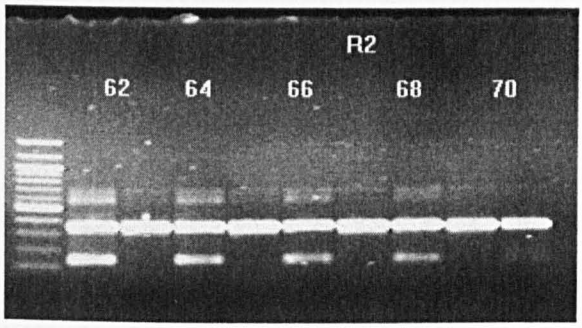


Figure 3.3: Gradient of temperature in MAS-RIF with RIF2R primer to identify mutation strain (strain 765) from wild-type strain (H37Rv) at codon 516

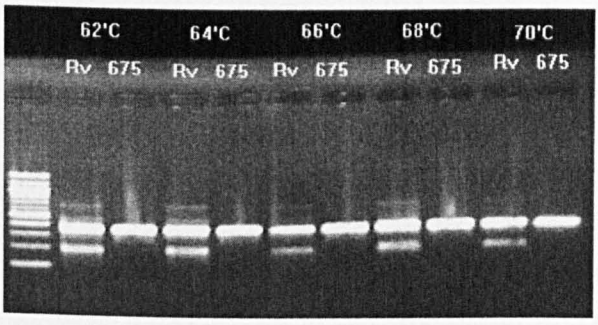


Figure 3.4: Gradient of temperature in MAS-RIF with RIF4R primer to identify mutation strain (strain 675) from wild-type strain (H37Rv) at codon 531

With the final PCR condition that we found as described in method chapter, section 2.2.2, the MAS-PCR can work at a wider range of temperature (Figure 3.5). For this reason, we could being able to use a same PCR programme for three MAS-RIF reactions.

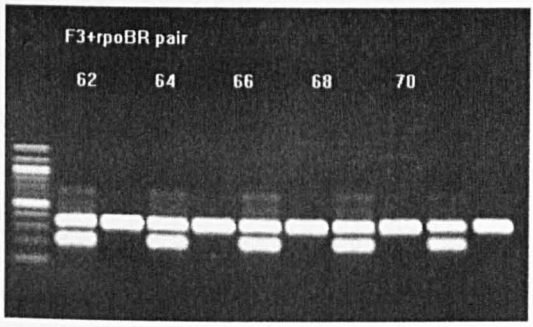


Figure 3.5: Gradient of temperature in MAS-RIF with RIF3F primer to identify mutation strain (strain 681) from wild-type strain (H37Rv) at codon 526

After individual optimization all three multiplex PCRs were combined for the final optimization using a single PCR program, in order to allow the test to be performed in the single PCR programme, improving work-flow and practicality (Figure 3.6).

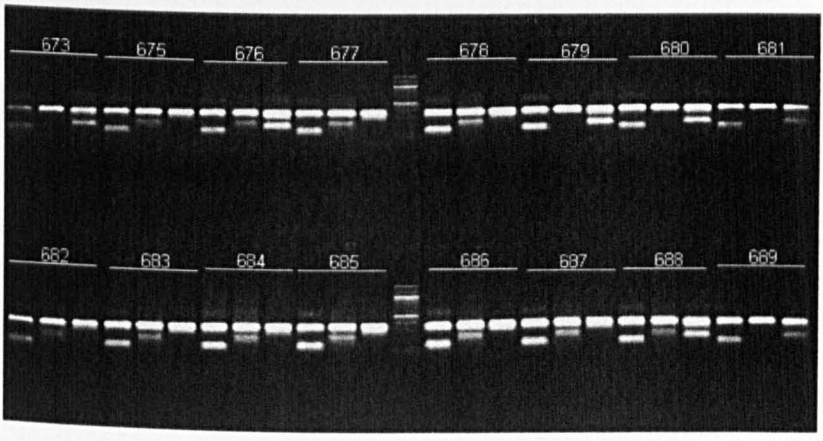


Figure 3.6: Results of MAS-RIF PCR on archived strains of *M.tuberculosis* resistant to RIF. Each strain was tested with three consecutive reaction for mutations at codon 516, 526 and 531.

Each of three MAS-RIF reactions was performed with the same outer primers to amplify the RRDR of *rpoB*, and one of three inner primers targeted to wild type at hot spot codons 516, 526 and 531 (Figure 3.2).

The 350bp fragment of the *rpoB* gene was amplified using outer primers RPOBF (5'-GGGAGCGGATGACCACCCA-3') and RPOBR (5'-GCGGTACG-GCGTTTCGATGAAC-3'). The inner primers used to detect mutations at codon 516, 526 and 531 were RIF2R (5'-GACAGCGGGTTGTTCTGGT-3'), RIF3F (5'-CCGCTGTCGGGGTAGACCCA-3') and RIF4R (5'-CCGCCGGGCCCCATCG-CCG-3'), respectively (Method chapter, section 2.2.2). The primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc., CA, USA).

The results were read with 2 bands for wild type isolates and only one band for mutant isolates (Figure 3.7).

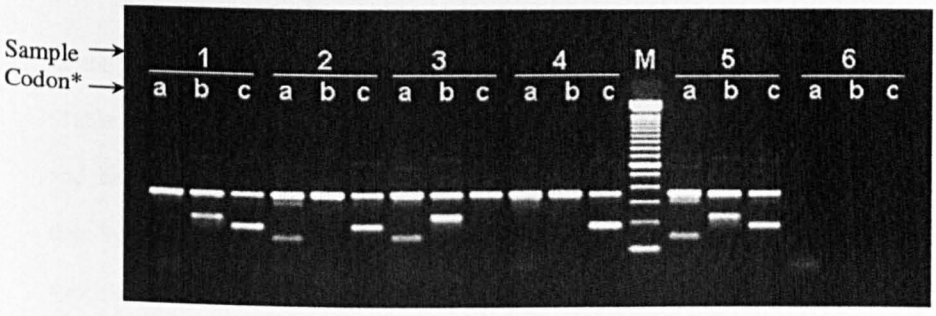


Figure 3.7: Amplicon profiles in MAS-RIF of isolates carrying mutations at each site.

*(a) : codon 516; (b) codon 526; (c) codon 531. Sample: **(1)** mutation at codon 516; **(2)** mutation at codon 526; **(3)** mutation at codon 531; **(4)** codon 516 and 526 mutated; **(5)** wild type control sample; and **(6)** negative control; (M) 100bp ladder.

3.2.2 Accuracy of MAS-RIF compared with MTBDR LPA

The sensitivity and specificity of this MAS-RIF were calculated in comparison with the commercial MTBDR assay (Hain Lifesciences, Germany) using conventional 1% phenotypic DST as the gold standard. Any discrepant results were evaluated by analysis of sequencing data to determine if mutations were present which had not been detected by the molecular assays.

The results of the MAS-RIF, sequencing and commercial MTBDR tests are summarized in Table 3.1. Sequencing showed that among 104 RIF resistant isolates, 92 isolates (88.5%) had mutations located at the 3 target codons: 531 (43%), 526 (31%), 516 (15%). Eight (7.7%) had mutations at other codons in the RRDR of the *rpoB* gene. Four (3.8%) had no mutation in the regions which were sequenced.

Using the MTBDR test, 97 isolates (93.3%) were detected as resistant to RIF. Of the 7 resistant isolates returning a susceptible result, 4 had no mutations in RRDR. (Table 3.1), 2 had mutations outside the location of the primers (L533P and V146F) and the remaining isolate had three nucleotides inserted at the end of a probe (514::F), thus hybridisation still occurred, returning a wild type result. The sensitivity and the specificity of MTBDR for RIF resistance was 93.3% [95% CI 86.6-97.3%] and 100% [95% CI 92.9-100%], respectively. In this selected sample set, the positive predictive value (PPV) was 100% [95% CI 96.2-100%], and a negative predictive value (NPV) was 87.7% [95% CI 80.0-93.6].

Table 3.1: Comparison between MAS-RIF test and commercial MTBDR LPA in 104 RIF resistant isolates.

Mutation	Number of isolates	MTBDR test		MAS-RIF test	
		Res	Sens	Res	Sens
S531L	46	46	0	45	1
H526D/F/L/R/S/Y	28	28	0	28	0
D516V	9	9	0	8	1
D516V, H526Q	1	1	0	1	0
D516A, H526N	1	1	0	1	0
L511P, H526N	1	1	0	1	0
Q513K, H526D	1	1	0	1	0
D516G, 522::SGL	1	1	0	1	0
D516A, L533P	1	1	0	1	0
Q513K/L	3	3	0	0	3
D516G, L533P	2	2	0	0	2
L511P, D516G	1	1	0	0	1
L511P, S512G	1	1	0	0	1
L533P	2	1	1	0	2
514::F	1	0	1	0	1
V146F	1	0	1	0	1
WT	4	0	4	0	4
Total	**104**	**97 (93.27%)**	**7 (6.7%)**	**87(83.65%)**	**17 (16.3%)**

The MAS-RIF test detected 87/104 (83.7%) of RIF resistant isolates. Of the 17 discrepant results, 4 of the isolates carried no mutations in any sequenced region, eight had mutations in the *rpoB* gene but outside the three target codons (Q513K/L x3, L533P x2, 514::F x1, V146F x1 and L511P/S512G x1), two showed evidence of a mixed population of resistant and susceptible isolates on sequencing with double peaks at a single allele (Figure 3.8), which would be defined as wild type by the MAS-RIF test due to the generation of the wild-type band. The remaining three isolates had a D516G mutation which primer RIF2R hybridises weakly with, generating a weak wild-type band and returning a false-susceptible result.

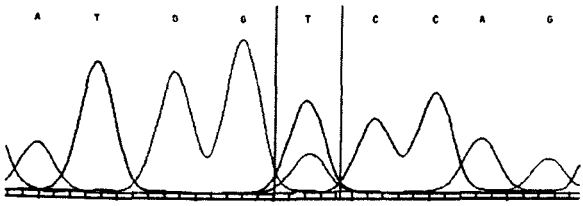


Figure 3.8: Double peak in the sequencing trace from sample DQ786 showing a heterogeneous population of susceptible strain (wild-type, D516) and resistant mutant (D516V).

The specificity of the MAS-RIF test was 100% [95% CI 92.9-100%] with no sensitive isolates returning a false-positive resistant result (Table 3.2). Sensitivity was 83.7% [95% C.I. 75.1-90.2%]. The PPV and NPV for RIF resistance in this sample set were 100% [95% CI 85.8-100%], and 74.6% [95% C.I. 65.3-83.1%], respectively.

Table 3.2: Sensitivity of MAS-RIF and MTBDR with reference to 1% conventional phenotypic DST as gold-standard.

Conventional DST		MAS-RIF		MTBDR	
		Resistant	Sensitive	Resistant	Sensitive
RIF	Resistant (104)	87 (83.7%)	17	97 (93.3%)	7 (6.7%)
	Sensitive (50)	0	50 (100%)	0	50 (100%)
INH	Resistant (132)	n/a	n/a	109 (82.6%)	23 (17.4%)
	Sensitive (22)	n/a	n/a	0	22 (100%)

3.2.3 Development of MAS-PCR to detect INH resistance

The majority of INH-resistant *M.tuberculosis* isolates carry mutations at either *katG*315 or *inhA*-15, although the proportion of each mutation varies geographically (Guo, Seet et al. 2006). A previous investigation of 100 consecutive INH resistant isolates showed that 90% of INH resistant isolates in Vietnam have at least one of these two mutations (Caws, Duy et al. 2006)

A multiplex PCR to detect INH resistance (MAS-INH) was designed with three primer pairs named as HSP65F1 - HSP65R2, katG2F - katGP6, TB92–inhARmut (Table 2.3). One pair targets *hsp65* which is specific for *M.tuberculosis* and serves as the control. A second primer pair targets the *inhA* promoter region, with the reverse primer designed to detect mutation C-15T in this region. The third primer pair is located inside the *katG* gene where the forward primer will yield a PCR product if the isolate is wild type (Figure 3.9).

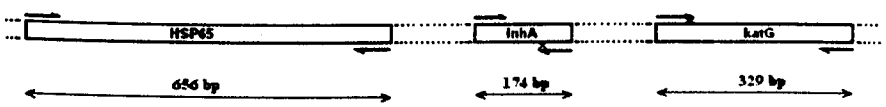


Figure 3.9: The principle of the MAS-INH test.

Three primer pairs located on three different regions of the *M.tuberculosis* genome. The small arrows show the position of the primers. The cross over the arrow head shows the loci at which a mismatch occurs and returns a disappearing band when the tested isolate carries any mutations at this position.

All primers were designed using Primer Express version 2.0 software (Applied Biosystems, USA). The specificity of the primers to *M.tuberculosis* was tested *in-silico* and in the laboratory with some common enterobacteria: *Chryseomonas luteola, Klebsiella spp., Klebsiella oxytoca, Klebsiella pneumoniae, E. coli, Enterobacter cloacae, Citrobacter freundii, Pantoea spp.* and atypical mycobacteria *M.avium-intracellulare.* (Figure 3.10)

Figure 3.10: Specificity of MAS-INH tested with different bacterial species.

Two clinical *M.tuberculosis* (lanes 1,2); BCG (lane 3); *M.avium-intracellulare* (lane 4); *Chryseomonas luteola* (5); *Citrobacter freundii* (6); *E. coli* (7); *Enterobacter cloacae* (8); *Klebsiella oxytoca* (9); *Klebsiella pneumoniae* (10); *Klebsiella spp.* (11); *Pantoea spp.* (12); negative sample (13).

Serial dilution of genomic DNA showed 0.15ng of DNA of genomic DNA is required to obtain reliable identification of mutations (Figure 3.11).

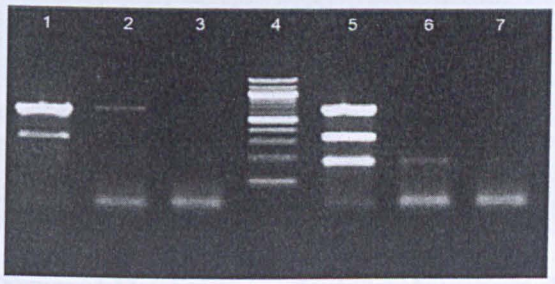


Figure 3.11: Results of MAS-INH test using different genomic DNA concentrations

Lanes 1,5: 0.15ng Lanes 2,6: 0.015ng

Lanes 3, 7: 0.0015ng Lane 4, ladder 100bp.

Lanes 1, 2, 3: DNA from clinical isolate 694

Lanes 5, 6, 7: DNA from clinical isolate 721.

After optimization, the protocol for MAS-INH was established and described in Method chapter, section 2.2.3

This PCR yielded three bands after electrophoresis on agarose 2% for 40min at 140V. The largest band of 656bp represents *hsp65* of *M.tuberculosis* and serves as a control for the presence of *M.tuberculosis* in the sample. The second band of 329bp represents isolates with a wild type *katG*315, thus the isolates without this band carry a *katG*315 mutation, determining INH resistance. The lowest band of 174bp represents isolates carrying mutation *inhA-15* in the promoter, which also confers INH resistance (Figure 3.12).

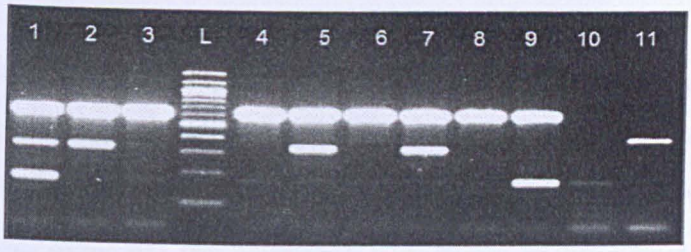


Figure 3.12: Agarose gel electrophoresis of PCR amplicons from multiplex PCR for INH resistance.

Lane 1: Isolate with mutation at *inhA-15*;
Lanes 2, 5, 7: Isolates have wild-type at *katG315*
Lanes 3, 4, 6, 8: Isolates with mutation at *katG315*;
Lane 9: Isolate with mutation at both *katG315* and *inhA -15*
Lane 10: H37Rv (15*pg*) Lane 11: *M. avium intracellulare*
L: 100bp ladder.

3.2.4 Accuracy of MAS-INH in comparison with MTBDR and MTBDR*plus* LPAs.

All sensitivities and specificities were calculated in comparison with the gold standard of conventional 1% phenotypic DST. Discrepant results were evaluated by analyzing sequencing data.

The results of MAS-INH, commercial MTBDR test and sequencing are summarized in Table 3.3. The first 100 consecutive INH resistant isolates were sequenced in the *katG* gene, among which 21 isolates were wild type and were therefore further sequenced in the *inhA*, and *oxyR-ahpC* regions (Caws, Duy et al. 2006). In general, 78 isolates carried mutations at *katG315*. Ten isolates which did not

have any mutation in the *katG* region carried a C -15 T mutation in the *inhA* promoter. One isolate did not give PCR product but had a *M.tuberculosis* specific spoligotype. This sample was retested by PCR with alternative primer sets (Table 3.4) covering an expanded region flanking the *katG* gene, but still no PCR product was obtained and this isolate was thus categorised as containing an unconfirmed *katG* whole-gene deletion.

Table 3.3: Comparison of MAS-INH test with sequencing and the commercial tests MTBDR and MTBDR*plus* in 100 phenotypically INH resistant clinical isolates

No. of isolates	Sequencing *katG* gene	Sequencing *inhA* gene	MTBDR mut at *katG*	MTBDR*plus*		MAS-INH	
				katG	*inhA*	*katG*	*inhA*
71	S315T	N/A	71	71	0	71	0
2	S315 I	N/A	2	2	0	2	0
2	S315N	N/A	2	2	0	2	0
1	S315G	N/A	1	1	0	1	0
1	S315T T308T	N/A	1	1	0	1	0
1	Del†	N/A	0	1	0	1	0
1	S315T	N/A	1	1	1	1	1
10	WT	C -15T	0	0	10	0	10
1	WT	WT*	0	0	1	0	1
10	WT	WT	0	0	0	0	0
Total 100	**78%**	**10%**	**78%**	**79%**	**12%**	**79%**	**12%**
				90%		**90%**	

(*: two peaks were detected in sequencing results

†: deletion was defined by PCR)

Table 3.4: Primers used to amplify the region flanking suspected *katG* whole-gene deletion.

Primer	Sequence	PCR length
P1_Marttila	AAC GGC TTC CTG TTG GAC GAG	2458bp
P10_Marttila	GGG TCT GAC AAA TCG CGC CG	
katGwgF1	GTC CTC TAT ACC GGA CTA CGC	2899bp
katGwgR1	TCG CAC ATC CAG CAC ATT TC	
katGwgF2	TAC TGG GGT CTA TGT CCT GA	2571bp
katGwgR2	ATG AGC ATT ATC CCG TAC AC	

The MTBDR test detected all 78 mutants out of 100 phenotypically INH resistant isolates. All 50 phenotypically INH susceptible isolates had a susceptible result on MAS-PCR. Thus, the specificity and the sensitivity of the test for INH resistance were 100% [95% CI 92.9-100%], and 78% [95% CI 68.6-85.7%], respectively.

The MTBDR*plus* assay detected all 90 mutants out of 100 INH phenotypically resistant isolates. All 50 INH susceptible isolates gave a susceptible result; the sensitivity and specificity of the MTBDR*plus* assay for INH resistance is therefore 90% [95% CI 82.4-95.1%] and 100% [95% CI 92.9-100%], respectively.

The MAS-INH PCR test detected 79% (79/100) INH resistant isolates based on mutations in *katG*315. In addition, this test also detected 12% (12/100) INH resistant isolates based on mutations at *inhA-15*. One of these isolates was found carrying both mutations in *katG* and *inhA* genes. Another isolate showed a discrepant result between MAS-INH and sequencing, the former test found only a mutation in *inhA*, when a double peak at this upstream position was found by sequencing, as a result of heterogeneous infection. For the MAS-INH PCR test, the overall sensitivity

and specificity were 90% [95% CI 82.4-95.1%] and 100% [95% CI 92.9-100%], respectively (Table 3.5).

Table 3.5: Number of isolates detected by MAS-INH test and MTBDR*plus* in comparison with phenotypic DST.

Phenotypic test		MAS-INH		MTBDRplus	
		Resistant	Sensitive	Resistant	Sensitive
INH	Resistant (100)	90 (90%)	10	90 (90%)	10
	Sensitive (50)	0	50 (100%)	0	50 (100%)
RIF	Resistant (78)	N/A	N/A	74 (%)	4
	Sensitive (72)	N/A	N/A	0	72 (100%)

3.3 Discussion

Neither molecular test was 100% sensitive for the detection of drug resistant TB, however both the MAS-PCR assays and the LPAs are 100% specific and therefore useful as rapid detection tests for positive identification of RIF resistance and by proxy for MDR TB (for MAS-RIF) or identification of INH resistance (for MAS-INH). A negative result from either test cannot be used to "rule-out" drug resistant TB.

Commercial LPAs, such as MTBDR and MTBDR*plus*, have been endorsed for use on smear positive sputum by WHO for use in high-burden settings, following effective demonstration projects in South Africa (WHO 2008). The MTBDR*plus* test strips are one of the technologies in the FIND portfolio, which aims to evaluate novel

diagnostic technologies for poverty-related diseases and promote uptake of those that are effective through public-private partnerships. However, the cost of these tests is around 5USD/test strip under preferential pricing for NTP of high burden countries. Wide-scale application of the test, with associated labour and infrastructure costs is not currently sustainable on a large scale for routine use of this kit in most developing settings, such as Vietnam. Therefore the need for cheaper, widely applicable routine screening tests remains.

3.3.1 MAS-RIF

In comparison with MAS-RIF, the commercial MTBDR test is more sensitive (93.3% vs. 83.7%) and allows the direct detection of INH resistant TB, directly confirming the diagnosis of MDR TB in the majority of cases (n=80/102, 78.4%). The MTBDR test used in this study was only able to detect INH resistance mutations at *katG*315. The new version of this test, MTBDR*plus* (Hillemann, Rusch-Gerdes et al. 2007), has additional probes for mutations at *inhA*-15 (the 2nd most common INH resistance mutation) and for additional regions of the *rpoB* RRDR. Sensitivity for MDR TB detection should therefore be higher with the MTBDR*plus* test. The MAS-PCR test evaluated here offers several advantages over the Hain test LPA in a high volume setting. Firstly, the cost for a batch of 10 samples is around 16USD using reagents from local suppliers without bulk discounts (1.6USD per sample), excluding salary and capital equipment costs. The equipment required is basic PCR equipment- a thermocycler, gel electrophoresis equipment and UV trans-illuminator, which is now available in many reference TB laboratories. There is minimal post-amplification processing involved, considerably less hands-on time and the MAS-PCR does not

require adherence to a strict time protocol for post-amplification processing, which increases flexibility to incorporate the test into a routine diagnostic laboratory schedule. After DNA extraction, the MAS-PCR can be completed in two hours, whereas the MTBDR test requires 6 hours of post-amplification processing (including PCR, electrophoresis and hybridisation).

The performance of the MAS-PCR test in routine use as an MDR detection tool should be evaluated both on cultures and directly on clinical samples. However, molecular tests are only appropriate diagnostic tools in laboratories which have sufficient resources and expertise to implement quality control and assurance, particularly regarding contamination.

There were 13 discrepant cases between sequencing and *rpoB* MAS-PCR. Eight of these isolates have mutations outside the three hot spot codons 516, 526 and 531. Three had a D516G mutation, which is not detected by this test. The two remaining isolates probably contained a heterogenous population of sensitive and resistant isolates as they had a double peak at a resistance allele on the sequencing trace (Figure 3.8). These isolates were defined as wild type by the MAS-PCR test due to generation of the wild-type band. The MTBDR test is able to detect the resistant population in these isolates, as hybridisation with the resistant band will occur. It is likely that for clinical purposes these isolates should be treated as resistant because a resistant population is emerging. However it is possible that the patient may be successfully treated on a standard regimen, particularly if the organism remains INH sensitive, therefore the clinical implications of such results remain unclear.

Molecular tests will not have 100% accuracy until all the relevant mutations have been described. Thus, a sensitive result returned by a molecular test cannot yet be used to exclude RIF resistance. Ideally DST should be performed on every patient at the time of diagnosis, however in high volume developing settings where this is not feasible priority should be given in cases where the index of suspicion is high, such as HIV positive patients, MDR contact cases and re-treatment/ relapse cases, where a susceptible result returned by molecular screening methods should be confirmed by DST or other phenotypic methods. However, with high specificity (100%), molecular techniques allow the early detection of MDR cases, potentially reducing morbidity, mortality and interrupting chains of transmission, when combined with effective MDR treatment programs.

3.3.2 MAS-INH

The sensitivity and specificity of both MTBDR*plus* and the MAS-INH test were 90% and 100% in identify INH resistance, respectively against conventional phenotypic DST.

The convenience of this MAS-INH test is that it can detect the two most common mutations in one reaction. One pair of primers targets *hsp65* gene, which is specific for *M.tuberculosis*. This primer pair was used as a control band of the reaction as well as the presentation of the pathogen in samples. This primer pair showed specific for *M.tuberculosis* when it yielded no specific product on other bacteria, such as *M.avium* and *M.fortuitum*.

The MAS-INH detected 79% (79/100) INH resistant isolates based on mutations in *katG315* and detected another 12% (12/100) INH resistance based on mutations at *inhA-15* promoter. One of these isolates was found carrying both mutations in *katG* and *inhA* genes. Thus, the total detection rate rose up to 90%, much higher than 78% in commercial MTBDR test.

One study recently looking at other regions in *katG* identified many mutations at codon 4 of *katG* (Siqueira, Freitas et al. 2009). This indicates that the resistant isolates which do not carry any mutation at *katG*315 nor *inhA-15* may carry mutations elsewhere in *katG*, outside the investigated area.

The multiplex-PCR test is a simple single PCR test which will give the result of INH resistance after 2 hours. In addition, because of the considerable difference between the lengths of PCR products in each sample, the electrophoresis needs only 20-40 minutes to report a clear result. The performance is simple and can be done in any general molecular laboratory with appropriate quality control measures.

Several MAS-PCR tests have been described and evaluated (Mokrousov, Otten et al. 2002; Herrera-Leon, Molina et al. 2005). However, the techniques either did not detect mutation -15 C→T in *inhA* promoter, or detected only mutation G944C at codon *katG*315. Thus, detection rate of mutations in INH resistance of these tests would be lower than this multiplex-PCR. The MAS-PCR described here will additionally detect other rarer mutations at *katG*315, thus increasing the sensitivity (+6% in this study).

This multiplex-PCR test requires 0.15ng of chromosomal DNA to amplify and identify the mutations correctly. Increasing the number of cycles in the thermocycling program will increase the sensitivity of the test, but face a higher risk of contamination in the laboratory.

This test could potentially be used to screen for INH resistance in *M.tuberculosis* isolates and, if used in combination with a MAS-PCR for RIF resistance, to identify MDR TB. WHO recommends adding EMB to the continuation phase for TB treatment in regions with a high prevalence of INH resistant TB (2HRZE/4HRE) (WHO 2009) but the recommendation is based on expert opinion only. Cattamanchi *et al.* compared the difference in outcome of fully sensitive and INH mono-resistance showed no difference in two groups (Cattamanchi, Dantes et al. 2009), however, the comparison was biased with different regimens and longer periods of treatment in INH mono resistant cases. The WHO drug resistance data showed that the prevalence of INH resistance is extremely high in relapse cases (50%) (WHO 2009), suggesting that INH resistance could probably reduce treatment success. The new WHO treatment guideline recommends that countries with high prevalence of INH resistance should used EMB in addition to RIF and INH in continuous phase (even with weak evidence). The daily dose in continuous phase could help by reducing the risk of INH resistance development during TB treatment (WHO 2009). The most effective regimen for the treatment of INH-resistant TB is still unknown. Patients carrying INH resistant strains are likely to have a higher risk of developing MDR on standard NTP treatment regimens and should be monitored more intensively for treatment failure and acquired MDR TB.

To sum up, two multiplex-PCRs were successfully developed that can differentiate wild type and mutated isolates. By combining the MAS-PCR for INH test described here (Tho, Lan et al. 2011) with MAS-PCR for RIF resistance (Tho, Ha et al. 2008), MDR TB can be accurately detected in *M.tuberculosis* isolates.

Chapter 4

The association of *katG*315 mutations and Beijing lineage with higher MIC to INH

4.1 Background

INH is a key drug in TB treatment with excellent bactericidal activity which is responsible for the rapid reduction in bacterial load during the first few days of treatment on standard first line therapy.

From a pharmacological perspective, the effectiveness of INH is described by the ratio of C_{max}:MIC, thus INH activity is highly dependent on the MIC of the *M.tuberculosis* isolate to INH (Srivastava and Gumbo 2011).

The MIC is defined as the lowest concentration of antibiotic which inhibits the growth of a microorganism. Mutations for INH resistance which increase the MIC arise spontaneously in *M.tuberculosis* with a frequency of approximately 1 in 10^6 to 1 in 10^8 and are selected under drug pressure. The two most commonly occurring INH resistance mutations are *katG315* and *inhA-15*, as described in the introduction (section 1.18.2). There are a number of mutation types at *katG* gene which confer different level of resistance to INH in *M.tuberculosis*. While mutation of *katG* (normally at codon 315) confers resistance to INH at a relatively high MIC, deletion of the whole *katG* gene (normally rare with <1% of INH resistant clinical isolates) confers complete resistance to INH since the catalase peroxidase enzyme (encoded by *katG*) that is required for activation of the pro-drug is removed. Moreover, mutation in the *inhA* promoter has been shown to confer an intermediate level of resistance to

INH, approximately 1-2ug/ml (Lavender, Globan et al. 2005). The relative proportions of *katG* and *inhA* mutations among INH resistant isolates appears to vary geographically and the reasons for this are still unclear (Lavender, Globan et al. 2005; Caws, Duy et al. 2006; Dalla Costa, Ribeiro et al. 2009). In Vietnam, we have described a very high prevalence of the *katG315* mutants among phenotypically INH resistant isolates from patients with pulmonary TB (79%).

This dominant mutation in the INH resistant *M.tuberculosis* population may suggest that the *katG315* has a relatively low fitness cost in this setting, which has been shown in a number of *in vitro* and epidemiological studies (van Soolingen, de Haas et al. 2000; Pym, Saint-Joanis et al. 2002).

In HCM city, the Beijing lineage of *M.tuberculosis* is responsible for 40% of TBM cases in HIV-uninfected patients and is associated with MDR TB (Caws, Thwaites et al. 2006). It has been hypothesized that this lineage is 'hypervirulent' and may have a mechanism or intrinsic property that renders its less susceptible to chemotherapy (de Jong, Hill et al. 2008; Hu, Ma et al. 2010). Many studies have shown that the Beijing lineage is associated with the *katG315* mutation for INH resistance, in preference to the *inhA* promoter mutation (Hu, Hoffner et al. 2010). We hypothesized that the Beijing lineage may either preferentially develop the *katG315* mutation or retain the mutation which increased frequency once it is acquired, due to a lower relative fitness cost of this mutation in the Beijing lineage backbone. As a result, the Beijing lineage could be associated with high MIC for INH resistance compared to INH-resistant strains of other lineages. The association of the Beijing lineage and high MIC to INH, if demonstrated, could help to explain the high

prevalence of this specific lineage in Vietnamese TBM patients and the high prevalence of primary INH resistance in this setting.

The relationships between mutations conferring INH resistance and lineages of *M.tuberculosis* to MIC for INH were investigated in this chapter.

Aims:

1) Determine the association between MIC to INH and specific mutations conferring INH resistance.
2) Determine if the lineage backbone of an INH resistant *M.tuberculosis* isolate affects the MIC independently of the mutation.

4.2 Results

Seventy two archived isolates of INH resistant *M.tuberculosis* from pulmonary TB and TBM patients admitted to the HTD from Feb 2001 to Jan 2005 were analysed in this study.

Seventy two sequenced isolates were taken from previous studies (see materials and methods section 2.3.1), of which 33 isolates carried a *katG315* mutation, 17 isolates had a mutation at *inhA-15* and 22 isolates had no mutation at the sequenced regions (*katG*, *inhA* promoter, *oxyR-aphC* intergenic region). In this sample set, 32 isolates were Beijing lineage, 25 isolates were Indo-Oceanic lineage (East-African-Indian by spoligotyping) and 15 isolates were Euro-American lineage strains.

All samples were tested for MIC to INH at concentrations of 0.1μg/ml, 0.25μg/ml, 0.5μg/ml, 1.0μg/ml, 2.0μg/ml, 4.0μg/ml, 8.0μg/ml, 16μg/ml and 32μg/ml of INH. The MIC technique was developed by modifying the automatic DST of the BACTEC960 system as described in the methods, chapter 2, section 2.3.2.

In general, *katG315* mutated isolates had a median [interquartile range- IQR] MIC to INH of 2.0 [1.0-4.0]μg/ml, *inhA-15* mutated isolates had a median [IQR] MIC to INH of 0.25 [0.1-0.5]μg/ml and INH resistant isolates without any identified mutation had a median [IQR] MIC to INH of 0.1 [0-0.1]μg/ml (Figure 4.1).

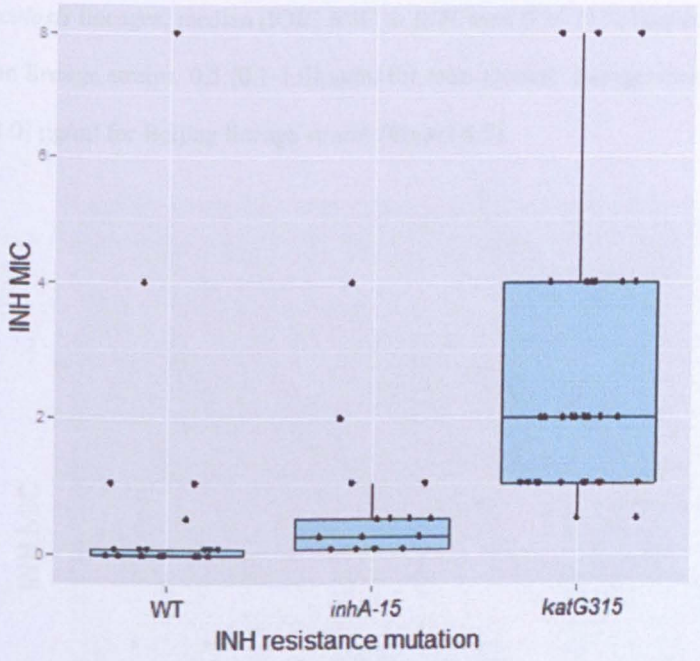


Figure 4.1: Comparison of the median INH MIC among *M.tuberculosis* isolates resistant to INH, by INH-resistance mutation: *katG*315, *inhA-15* and wild-type (WT).

Box plots show the median and IQR of MIC associated with each INH resistance mutation.

When the INH MIC of the isolates were stratified according to the three major *M.tuberculosis* lineages, median [IQR] MIC to INH were 0.25 [0-2.0]µg/ml for Euro-American lineage strains, 0.5 [0.1-1.0]µg/ml for Indo-Oceanic lineage strains and 1.0 [0.625-4.0] µg/ml for Beijing lineage strains (Figure 4.2).

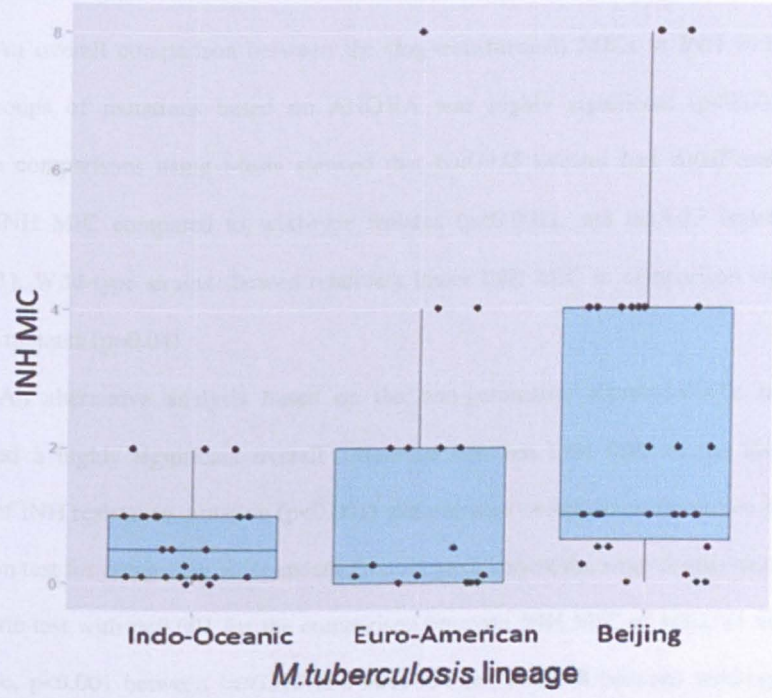


Figure 4.2: Comparison of the median INH MIC among phenotypically INH-resistant isolates of the three major *M.tuberculosi* lineages (Beijing, Indo-Oceanic and Euro-American).

Box plots show the median and IQR of MIC associated with each lineage.

The MIC test was performed with serial 2-fold dilutions of INH concentration, therefore most of the MIC results were under 1μg/ml of INH. For all statistical analyses, log-transformed MIC data were analyzed which showed a more symmetrical distribution and a better homogeneity of variance between subgroups than untransformed values.

An overall comparison between the (log-transformed) MICs to INH in the three groups of mutations based on ANOVA was highly significant ($p<0.001$). Pairwise comparisons using t-tests showed that *katG315* isolates had significantly higher INH MIC compared to wild-type isolates ($p<0.001$), and *inhA-15* isolates ($p<0.001$). Wild-type strains showed relatively lower INH MIC in comparison with *inhA-15* mutants ($p=0.04$).

An alternative analysis based on the non-parametric Kruskal-Wallis test confirmed a highly significant overall difference between INH MIC of the three groups of INH resistance mutation ($p<0.001$) and pairwise comparisons (based on the Wilcoxon test for comparing independent groups) gave almost the same results as the parametric test with $p<0.001$ for the comparison between INH MIC of *katG315* and wild-type, $p<0.001$ between *katG315* and *inhA-15*, and $p=0.008$ between wild-type and *inhA-15* mutated isolates (Figure 4.3).

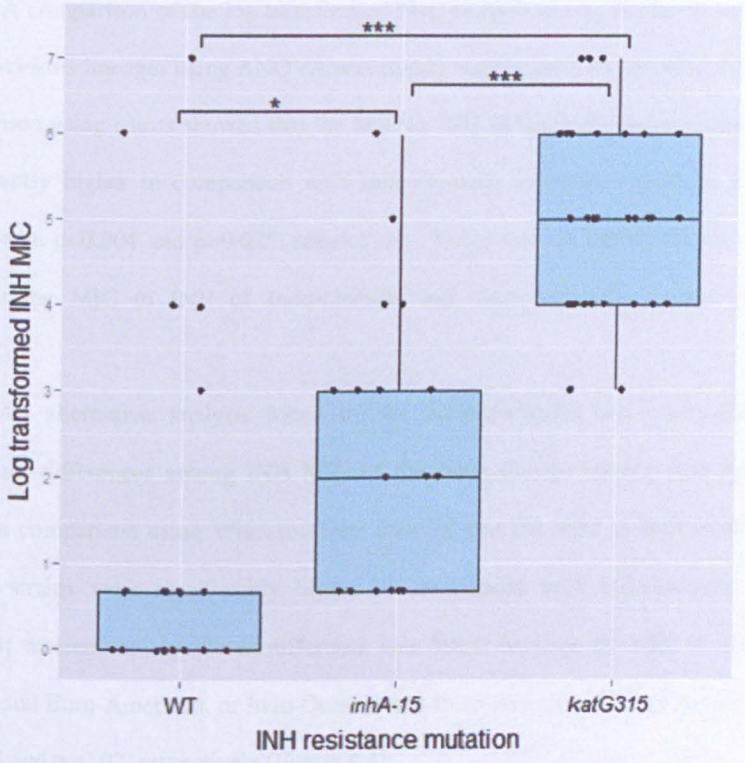


Figure 4.3: Comparison of the median of log transformed INH MIC among INH-resistant *M.tuberculosis* isolates with different INH resistance mutations.

Box plots show the median and IQR of MIC associated with each INH resistance mutation.

Differences between the MIC for each mutation type were calculated pairwise by Wilcoxon test.

*** significant at p=0.008, *** significant at p<0.001**

A comparison of the log transformed MIC to INH among strains of the three *M.tuberculosis* lineages using ANOVA was highly significant with p=0.009. Pairwise comparison using t-tests showed that the MIC to INH in Beijing lineage strains were significantly higher in comparison with Indo-Oceanic and Euro-American lineage strains with p=0.004 and p=0.025, respectively. There was no significant difference between the MIC to INH of Indo-Oceanic and Euro-American lineage strains (p=0.73).

An alternative analysis based on the Kruskal-Wallis test confirmed the significant differences among INH MIC of the three lineage groups with p=0.02. Pairwise comparison using Wilcoxon tests showed that the MIC to INH in Beijing lineage strains were significantly higher in comparison with Indo-Oceanic with p=0.004; whereas no significant difference was found between the MIC to INH of Beijing and Euro-American, or Indo-Oceanic and Euro-American lineage strains with p=0.088 and p=0.92, respectively (Figure 4.4).

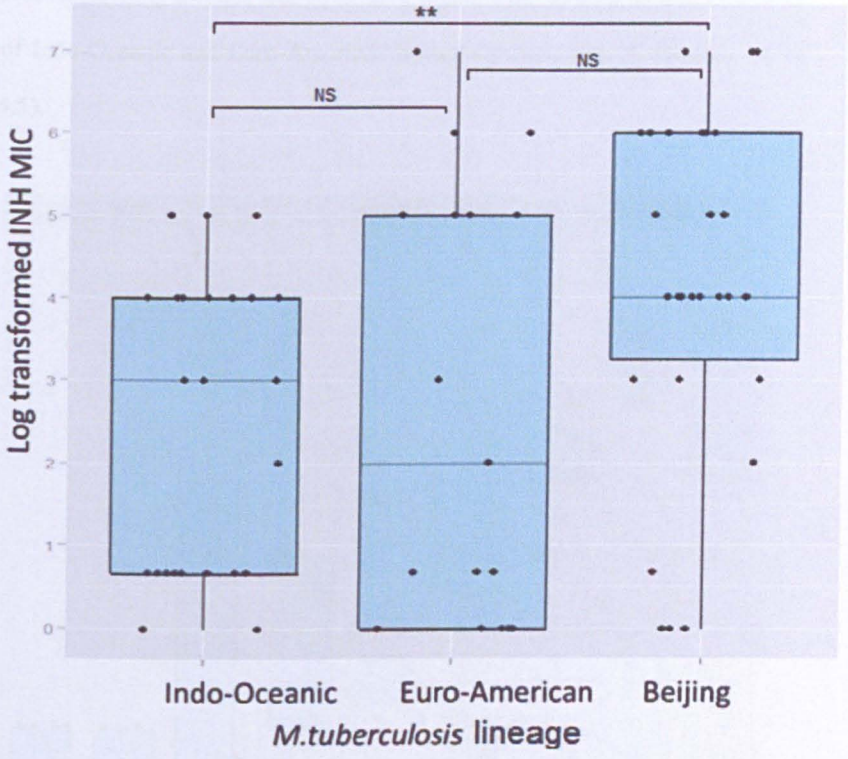


Figure 4.4: Comparison of the median of log transformed INH MIC among three major *M.tuberculosis* lineages.

Box plots show the median and IQR of MIC for each lineage.

Comparisons between lineages were calculated pairwise by Wilcoxon test.

**** significant at p=0.004, NS: no significant**

When comparing the INH MIC by both mutation and strain lineage, the results clearly showed that isolates carrying *katG315* mutation always confer high MIC to

INH. Moreover, Beijing isolates tended to have higher MIC in comparison with other isolates of Indo-Oceanic and Euro-American lineages irrespective of mutation status (Figure 4.5).

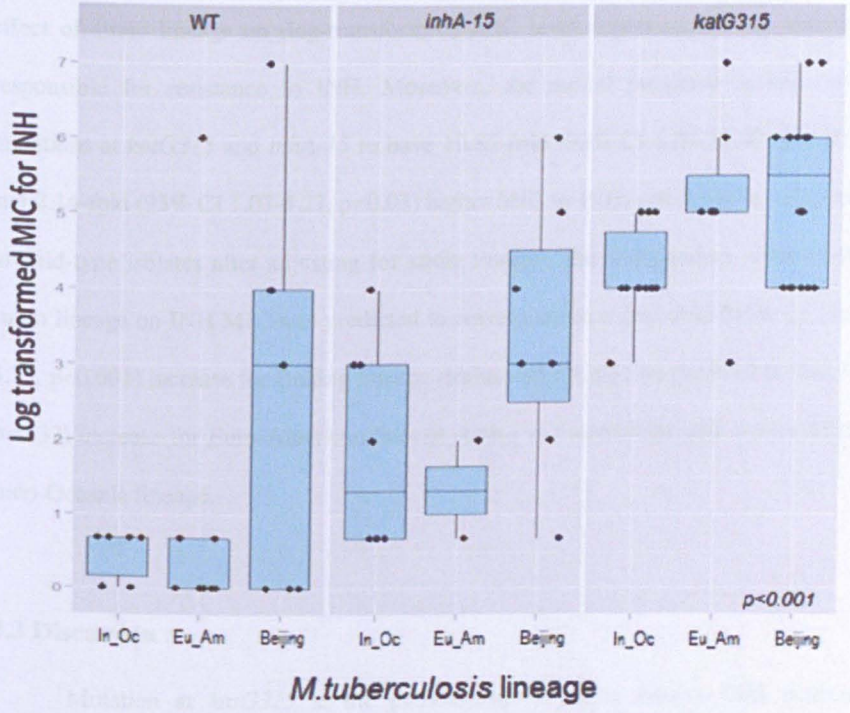


Figure 4.5: Comparison of the medians of log transformed INH MIC with INH-resistance mutation grouped by *M.tuberculosis* strain lineage.

Comparison by two-way ANOVA for mutation and lineage showed statistically significant differences, p<0.001.

In_Oc: Indo Oceanic; Eu_Am: Euro-American

A two-way ANOVA of MIC including both strain lineage (p=0.003) and resistance mutation (p<0.001) showed highly significant independent effects of both factors on MIC level. However, there was no evidence of an interaction between the lineage backbone and resistance mutation (p=0.49), i.e. no evidence of a different effect of strain lineage on (log-transformed) MIC level depending on the mutation responsible for resistance to INH. Moreover, the model predicted isolates with mutations at *katG315* and *inhA-15* to have 10.85-fold (95% CI 6.04-19.48, p<0.001) and 2.14-fold (95% CI 1.07-4.27, p=0.03) higher MIC to INH, respectively, compared to wild-type isolates after adjusting for strain lineage. The independent effect of the strain lineage on INH MIC was predicted to correspond to a 2.68-fold (95% CI 1.52-4.73, p<0.001) increase for Beijing lineage strains and a 1.40-fold (95% CI 0.72-2.74, p=0.32) increase for Euro-American lineage strains in comparison with strains of the Indo-Oceanic lineage.

4.3 Discussion

Mutation at *katG315* is the predominant mutation among INH resistant *M.tuberculosis* isolates worldwide (Caws, Duy et al. 2006; Lipin, Stepanshina et al. 2007; Dalla Costa, Ribeiro et al. 2009). It has been demonstrated that this mutation has a low or no fitness cost (Pym, Saint-Joanis et al. 2002) and confers a high level of resistance to INH. *M.tuberculosis* isolates carrying *katG315* mutations can be successfully transmitted within the community and are also often associated with resistance to other first-line drugs and MDR TB. (van Soolingen, de Haas et al. 2000).

Our results showed that different mutations result in different levels of phenotypic resistance to INH *in vitro*. The results from this study showed that *katG315* mutated isolates possessed the median [IQR] of INH MIC of 2.0 (1.0-4.0) μg/ml; whereas *inhA-15* mutated strains had a resistance to INH only at concentrations around 0.1-0.5μg/ml. The data are in accordance with a study conducted in South America (Dalla Costa, Ribeiro et al. 2009), despite the difference in proportion of *katG* mutation among INH resistant isolates in different geographical areas, such as Brazil (81.3%) and Peru (82.4%) (Dalla Costa, Ribeiro et al. 2009), Russia (95%) (Lipin, Stepanshina et al. 2007).

Some studies have shown that patients with INH resistant TB can be treated with a high dose of INH dose (Katiyar, Bihari et al. 2008; Van Deun, Maug et al. 2010). However it is possible that this high dose regimen could be effective only if the isolate that the patient is infected with is resistant to only a low dose of INH and further studies are required to clarify which patients may benefit from high dose INH. There are many reports in the literature which show that mutations at the *inhA* promoter confer resistance to low levels of INH (Zhang and Telenti 2000; Lavender, Globan et al. 2005), thus patients infected with these isolates are the most likely to benefit from INH high-dose therapy. However, isolates with a mutation at *inhA-15* do not always have a low-level of INH resistance as shown in a study by Vijdea *et al.* (Vijdea, Stegger et al. 2008). In the study reported here, this phenomena was also observed with some isolates carrying an *inhA-15* mutation without any other identified resistance mechanism were resistant to INH at a high level. Interestingly, these isolates belonged to the Beijing lineage; and the abnormally high INH resistant

phenotype of these isolates may be explained by an as yet unidentified mechanism intrinsic to the Beijing lineage backbone. Whole genome sequencing studies of *M.tuberculosis* isolates with different resistance profiles and different lineage backbones will help to unravel the underlying mechanisms. Literature review showed that a *katG* whole gene deletion leads to a high level of INH resistance (Vijdea, Stegger et al. 2008). However, *katG* whole gene deletion a very rare mutation in the INH resistant *M.tuberculosis* population (less than 1%), probably due to the fitness cost of this mutation, (Caws, Duy et al. 2006; Lipin, Stepanshina et al. 2007; Dalla Costa, Ribeiro et al. 2009).

Some isolates in our collection of phenotypically INH resistant *M.tuberculosis* did not show any mutation in either *katG* or *inhA* promoter regions studied. Other mechanisms of INH resistance (eg. mutations in other genes such as *kasA, nat*) were not investigated in the scope of this thesis. Efflux pump systems may also play a role in INH resistance for these isolates and those with levels of resistance to INH not explained by a single *inhA* promoter mutation (Siddiqi, Das et al. 2004).

An alternative explanation for these wild-type INH resistant isolates could be the emergence of an INH-resistant mutation during the treatment leading to a mixed wild-type/mutant population at the time of sample collection. Re-infection, or super-infection by a second isolate which is resistant during treatment appears to be is rare among HIV negative patients (Glynn, Yates et al. 2004). During isolation and archiving, it is possible the mutated sub-population with a lower relative fitness would be selected out and the final isolate contained only the wild-type sub-strain. This population selection would lead to a final isolate susceptible to INH (10 isolates in

this study). This explanation is supported by the fact that many wild-type isolates in this study had MIC <0.1μg/ml, or categorised as INH susceptible when re-tested on MGIT MIC. Alternatively, the original classification of these isolates as INH resistant by 1% LJ proportion method may have been an error, or there may be differences in determination of resistance by liquid and solid methods which resulted in the discrepancy.

Genotyping of *M.tuberculosis* discriminates lineages base on a limited number of characteristics. However, the divergent characteristics of these lineages have not yet been fully described and there is growing evidence of different virulence determinants between the lineages, including the ability to maintain mutations (Hu, Hoffner et al. 2010) or differences in disease severity, and associations with drug resistance (Caws, Thwaites et al. 2006; Lipin, Stepanshina et al. 2007). This study contributes to the evidence that there are, as yet unidentified mechanisms intrinsic to the lineage backbone which influence the acquisition of drug resistance.

The Beijing lineage has attracted a lot of attention from researchers all over the world. It has been reported as a lineage with 'hyper-virulence' and associated with drug resistance and MDR (Lipin, Stepanshina et al. 2007; Dalla Costa, Ribeiro et al. 2009; Niemann, Diel et al. 2010). One study in China showed that there was an association of Beijing lineage with extra-pulmonary TB; however, the number of extra-pulmonary patients infected with Beijing lineage in that study was relatively small (n=8) and the results was not statistically significant (p=0.0617) (Kong, Cave et al. 2007). In a previous study at HTD reported by Thwaites *et al.*, there was no significant association of INH resistance (non-MDR), HIV infection and pathogen

lineage with in-hospital mortality in TBM (Thwaites, Chau et al. 2002). The reason for this may be that the number of each analysis groups was too small (9 INH resistant, 11 HIV positive, 16 Beijing lineage infected patients) to be able to show significant association with mortality. A later larger study with a revealed that HIV infection increased the relative risk of death RR= 2.91 (95% CI, 2.14-3.96) in TBM patients (Thwaites, Duc Bang et al. 2005). Overall data on disease phenotype differences of the Beijing lineage have been conflicting and inconclusive.

Other studies at HTD have shown that Beijing lineage was also significantly associated with MDR in HIV-associated TBM patients (Caws, Thwaites et al. 2006) and that the Euro-American lineage strains were less likely to disseminate than Indo-Oceanic and Beijing lineage strains. Another study in China showed that the Beijing lineages associated with MDR belonged to a subgroup of Beijing lineages (Hu, Ma et al. 2010). In our study, two Beijing lineage isolates without mutation at *katG315* or *inhA-15* had very high MICs to INH. These isolates had Beijing, thus could belong to this subgroup of Beijing lineage. Further research is needed to identify and investigate this subgroup of Beijing lineage.

In this study, we did not have a large number of Euro-American lineage strains in the comparison. However, the difference between Beijing and Euro-American lineages were significant with p=0.025. This result was supported by other studies which have described a selective transmission advantage of MDR strains of the Beijing lineage over Euro-American lineages (Kong, Cave et al. 2007; Niemann, Diel et al. 2010).

The majority of the Indo-Oceanic lineage strains in this study are a subgroup of the EAI lineage (EAI4_VNM by spoligotype classification) which has only been reported in Vietnamese patients. Data from this study showed that the INH resistant strains of the Indo-Oceanic lineage and Euro-American lineage share a similar level of INH resistance with the median [IQR] of INH MIC of 0.5 (0.1-1.0) μg/ml and 0.25 (0-2.0) μg/ml, respectively ($p=0.7$ by ANOVA and $p=0.9$ by Kruskal-Wallis test).

The results also showed a significant difference between Beijing and Indo-Oceanic lineage strains ($p=0.004$). The effect of the Beijing lineage backbone on to the increase of INH MIC was predicted by the model as 2.68 (95% CI 1.52-4.73, $p<0.001$) fold higher in comparison with the Indo-Oceanic lineage backbone.

Collectively this data and the data in the literature (Lipin, Stepanshina et al. 2007), show that as yet unidentified lineage factors influence the MIC to INH and by extension may have an influence on the effectiveness of INH in TB treatment. The lineage backbone of *M.tuberculosis* strains may also be important for resistance to other anti-TB drugs and should be investigated.

Chapter 5

The influence of INH resistance on the outcome of HIV-associated TBM

5.1 Background

HIV infected individuals are more likely to develop severe disseminated forms of TB disease (Schutte, Van der Meyden et al. 2000). TBM is the most severe form of TB infection with high mortality and morbidity, though TBM occurs in only approximately 1% of clinical TB cases, (Garg 1999; Murthy 2010). TBM is rare in developed settings with a low TB prevalence but is seen more frequently in settings with a high prevalence of TB or both TB and HIV (Verdon, Chevret et al. 1996).

Chapter 4 showed that both the mutation and lineage of *M.tuberculosis* exert an independent effect on MIC to INH. These results suggest that some *M.tuberculosis* lineages could be more resistant to TB treatment at standard doses. Additionally, some studies suggest that BCG vaccination does not offer equal protection against all *M.tuberculosis* lineages, such as Beijing (Abebe and Bjune 2006; Tsenova, Harbacheuski et al. 2007).

Resistance to one or more first-line anti-tuberculous drugs is becoming an increasingly common clinical problem, and the consequences of MDR TB, resistant to both INH and RIF, are well described in pulmonary TB. MDR TBM is fatal without the benefit of second line drugs (Thwaites, Lan et al. 2005) but the impact on outcome in TBM of INH resistance without resistance to RIF is uncertain. An earlier study at HTD suggested that INH resistance, HIV infection and *M.tuberculosis* lineage were

not associated with in-hospital mortality in TBM patients (Thwaites, Chau et al. 2002). However, the lack of a significant effect was probably due to small sample size (n=160). A subsequent study showed that HIV coinfection increases the risk of treatment failure in TBM and reduces the survival rate with a relative risk of death 2.91 [95% CI, 2.14-3.96] in comparison with HIV uninfected TBM patients (Thwaites, Duc Bang et al. 2005). MDR TBM also showed 100% mortality without second-line drugs in another similar study in HIV coinfected TBM patients at HTD (Torok, Chau et al. 2008). However, there are limited data describing the influence of drug resistance (except MDR and XDR) or more specifically different resistance mutations of the bacilli which are known to influence the MIC level, on treatment outcome for TBM patients. This study was therefore conducted to investigate bacterial factors associated with outcome from TBM.

AIMS

1) Determine the impact of INH resistance without RIF resistance on outcome among TBM patients treated with a standardized first-line drug regimen.
2) Determine if specific mutations conferring INH resistance are associated with increased mortality.
3) Determine if specific *M.tuberculosis* lineages are associated with increased mortality for TBM.
4) Examine the influence of prior BCG vaccination on the infecting *M.tuberculosis* lineage among patients with HIV-associated TBM.

The study was conducted with a collection of *M.tuberculosis* isolates from two studies conducted in HCM city. The first was an observational cohort study of 58 patients with HIV-associated TBM (Torok, Chau et al. 2008) and the second was a RCT in which 253 patients with HIV-associated TBM were randomized to receive either immediate or delayed (8 weeks) ART for HIV-associated TBM (Torok, Yen et al. 2011) (see details in chapter 2, section 2.4.1) in collaboration with the HTD and PNT.

5.2 Results

The primary analysis of both studies has been reported elsewhere (Torok, Chau et al. 2008; Torok, Yen et al. 2011). We included all patients from these studies who had *M.tuberculosis* isolated from the CSF available for analysis of drug resistance: 46/58 (79%) from the cohort study and 141/253 (56%) from the RCT. We further excluded one duplicate patient from the cohort study who was subsequently enrolled into the randomised trial leading to a total sample size of 186 patients. Baseline characteristics of these patients are reported in Table 5.1.

Table 5.1: Baseline characteristics of the 186 patients with a CSF culture for *M.tuberculosis.*

	Prospective descriptive study N=45	Immediate ART N = 77	Deferred ART N = 64
Age in years, median (IQR)	27 (23-31)	28 (25 – 34)	29 (25 – 33)
Male sex, number (%)	41 (91.1%)	71 (92.2%)	57 (89.1%)
Weight in kilograms, median (IQR)	50 (45-51)	45 (40-51)	46 (40-50)
Previous TB, number (%)	8/42 (19.1%)	8/76 (10.5%)	6/63 (9.5%)
TBM grade I II III	 11 (24.4%) 14 (31.1%) 20 (44.4%)	 25 (32.9%) 29 (38.2%) 22(29.0%)	 18 (28.1%) 25 (39.1%) 21 (32.8%)
CD4 T-lymphocyte count, cells/μl, median (IQR)*	32 (12-59)	28 (16-108)	32 (14-78)
CSF ZN stain positive, number (%)	32 (71.1%)	38 (49.4%)	37 (57.8%)

* Missing for 18/186 patients (8, 4, and 6 in the three groups, respectively).

Of the included 186 patients, 112 patients died during the nine months of follow-up of the study and 55 survived; the remaining 19 patients were lost to follow-up and censored at the time they were last known to be alive. The overall nine-month mortality in the study population was 63.9% (95% CI 55.8 to 70.4%); it was higher in the patients from the cohort study (75.6%) but similar for the patients from the immediate and deferred ART group of the RCT (61.3% vs. 58.6%). Thirty-five patients (4 from the cohort, 31 from the RCT) received STR in addition to the standard treatment regimen.

5.2.1 Anti-tuberculous drug resistance

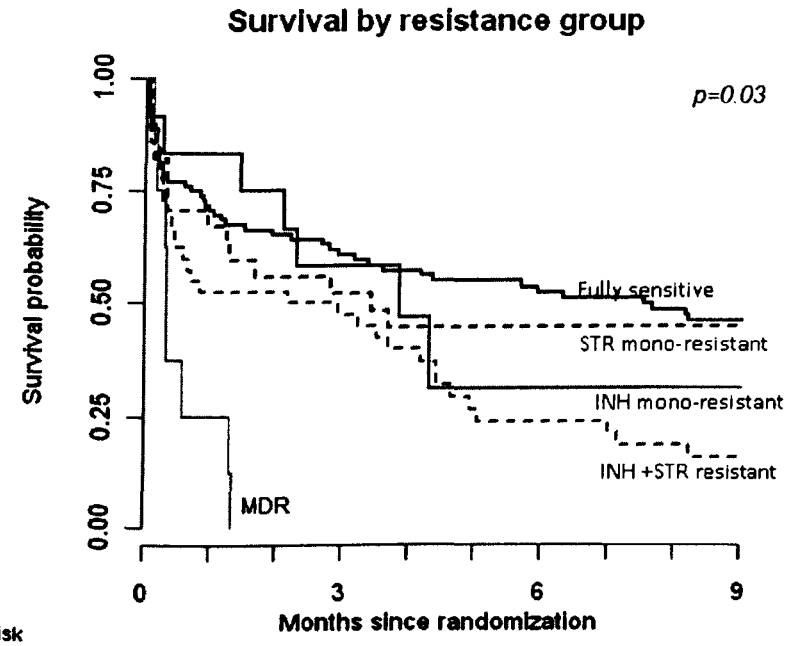
Overall, 98 patients were infected with an *M.tuberculosis* isolate which was sensitive to all five drugs (INH, RIF, STR, EMB and PZA), 28 isolates were STR-resistant only, 12 INH-resistant only, 40 resistant to both STR and INH, and 8 MDR. Of the MDR isolates, two were resistant to INH and RIF, five were resistant to INH, RIF and STR, and a single isolate was resistant to INH, RIF, STR and EMB). No isolates were resistant to PZA (Table 5.2).

Table 5.2: DST profiles of CSF *M.tuberculosis* isolates from 186 patients with HIV-associated TBM for RIF, INH, STR, PZA and EMB.

Drug susceptibility profile	No (%)
Fully susceptible	98 (52.7%)
STR resistant	28 (15.1%)
INH resistant	12 (6.5%)
INH, STR resistant	40 (21.5%)
INH, RIF resistant	2 (1.1%)
INH, RIF, STR resistant	5 (2.7%)
INH, RIF, STR, EMB resistant	1 (0.5%)
PZA resistant	0 (0%)
Total	186

Of thirty-five patients who received STR, eighteen isolates were sensitive to all drugs, three were resistant to STR, three were resistant to INH, ten were resistant to INH and STR, and one was resistant to INH, STR, and RIF.

Kaplan-Meier survival curves for patients in five resistance categories (fully sensitive, STR mono-resistant, INH mono-resistant, INH + STR resistant, and MDR) is displayed in Figure 5.1. Overall log-rank test showed significant difference between these groups, p=0.03.



No. at risk				
Fully sensitive	98	52	41	35
STR mono-resistant	28	14	12	12
INH mono-resistant	12	6	2	2
INH+STR resistant	40	19	9	6
MDR	8	0	0	0

Figure 5.1: Kaplan-Meier survival curves of HIV-associated TBM patients infected with *M.tuberculosis* stratified by 5 drug resistance categories.

The difference among survival time for 5 drug resistance categories was statistically significant overall (log-rank test, p=0.03).

Based on the weak effect of STR in TBM treatment that the additional STR resistance might not affect the outcome; we pre-defined resistance pattern groups: group 1: fully susceptible or STR mono-resistant (n= 126), group 2: INH resistant +/- STR resistant (n= 52), group 3: multidrug resistant (n=8), as showed in Figure 5.2

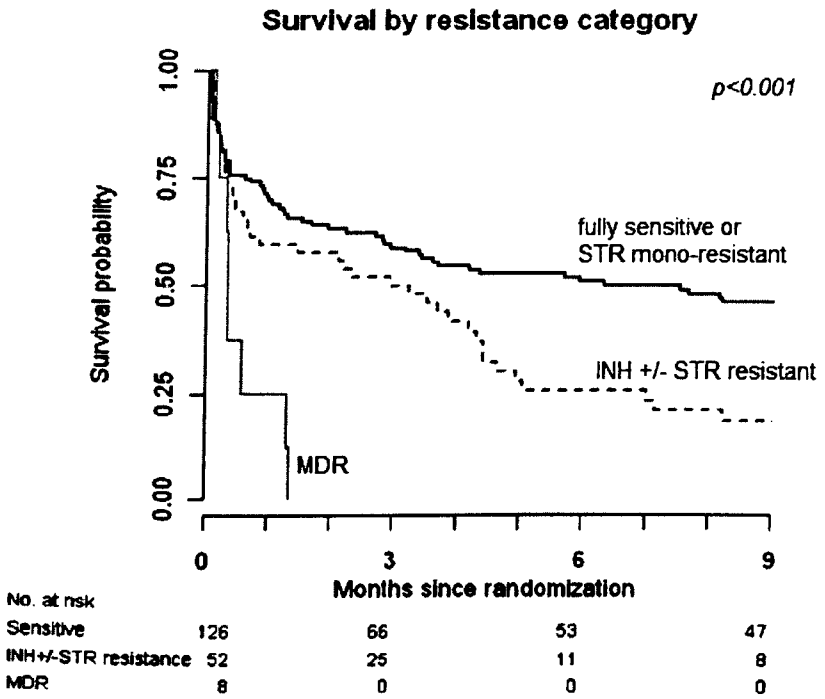


Figure 5.2: Kaplan-Meier survival curves of HIV-associated TBM patients infected with *M.tuberculosis*, stratified by 3 drug resistance categories.

The difference among survival time for 3 drug resistance categories was statistically significant (log-rank test, p<0.001)

There was a significant difference between survival curves of patients with different antituberculous drug resistance patterns base on three drug resistance categories (log rank test $p<0.001$). Patients with fully susceptible or STR mono-resistant isolates had the highest survival rates, whereas those with MDR TBM had the lowest survival, with all MDR TBM cases dying within 41 days of study enrolment. For patients with INH-sensitive isolates median survival was 192 days with a nine-month mortality of 54.2% [95% CI 44.1-62.5%]. For patients with INH resistant (non-MDR) isolates median survival was 89 days with a nine-month mortality of 81.5% [95% CI 66.0-90.0%] (Figure 5.2).

After adjusting for the effect of TBM grade at presentation, baseline CD4 cell count and cohort group in a multiple Cox regression analysis, the drug resistance pattern remained an independent predictor of mortality. The adjusted hazard ratio for MDR patients in comparison with fully susceptible or STR-monoresistant isolates was 5.21 [95% CI 2.38-11.42], $p<0.0001$ compared with HR= 1.78 [95% CI 1.18-2.66], $p=0.005$ for patients with INH resistant isolates (+/-STR resistance). The addition of STR-resistance as an additional covariate did not significantly improve the fit (hazard ratio 1.02 [95% CI 0.66-1.59], $p= 0.91$). There was also no evidence of an interaction between drug resistance status and TBM grade ($p=0.29$), CD4 cell count ($p=0.63$), or study group ($p=0.61$). However, there was evidence of a time-dependent effect of INH resistance (+/-STR resistance) on survival with $p=0.03$. Fitting separate INH resistance effects for the first three months (during the intensive TB treatment phase) and subsequently, gave adjusted hazard ratios (in comparison with fully susceptible or mono-resistant isolates) of 1.45 [95% CI 0.87-2.40], $p= 0.15$ for the first three months

and 5.05 [95% CI 2.23-11.44], p=0.0001 thereafter. Exploratory analyses showed that the model with a cut-point at three months for modeling INH resistance lead to a better fit (in terms of the likelihood) than models with a cut-point at other months.

Exclusion of 35 patients who received streptomycin did not substantially alter the adjusted hazard ratio for MDR patients (HR=4.86 [95% CI 2.11-11.22), p=0.0002) or INH-resistant patients (HR=1.90 [95% CI 1.20-3.00], p=0.006).

5.2.2 *M.tuberculosis* INH resistance mutation

Characterisation of the mutations responsible for INH resistance in the 60 isolates phenotypically resistant to INH using the MAS-PCR technique developed in chapter 3 showed that 59/60 (98.3%) isolates carried a *katG315* mutation. Therefore it was not possible to further examine the effect of specific mutations for INH resistance on outcome, as originally planned.

5.2.3 *M.tuberculosis* lineage

M.tuberculosis genotype data were available for 122 isolates (HTD patients only). Of these, 18 were of the Indo-Oceanic lineage (15%), 22 (18%) of the Euro-American lineage and 82 (67%) East Asian/Beijing lineage (Table 5.3).

Kaplan-Meier survival curves of the patients in the three lineages are displayed in Figure 5.3.

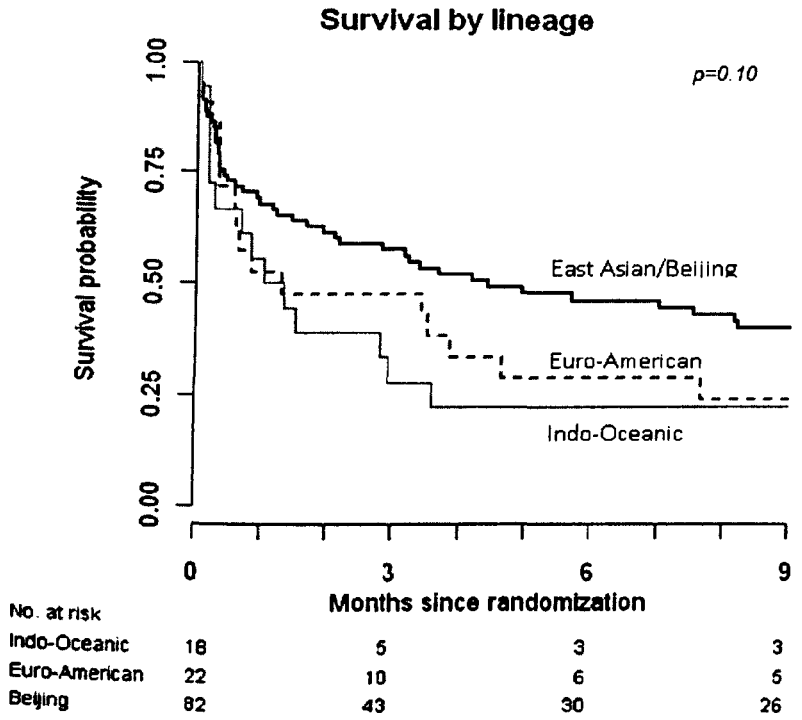


Figure 5.3: Kaplan-Meier survival curves of HIV-associated TBM patients infected with *M.tuberculosis* by lineage of the *M.tuberculosis* strain for all patients.

The survival time among different lineage groups was not statistically significant overall (log rank test, p=0.10)

Table 5.3: *M.tuberculosis* lineage and drug susceptibility profiles of 122 patients with HIV-associated TBM.

M.tuberculosis lineage	Drug susceptibility profile					
	Fully susceptible n (%)	STR mono-resistant n (%)	INH mono-resistant n (%)	STR/INH -resistant n (%)	MDR n (%)	Total n
Beijing	39 (47.6)	16 (19.5)	6 (7.3)	18 (22.0)	3 (3.7)	82
Euro-American	9 (40.9)	5 (22.7)	1 (4.6)	5 (22.7)	2 (9.1)	22
Indo-Oceanic	14 (77.8)	0	1 (5.6)	1 (5.6)	2 (11.1)	18
Total	62 (50.8)	21 (17.2)	8 (6.6)	24 (19.7)	7 (5.7)	122

In the crude analysis (n=122), *M.tuberculosis* lineage had no significant effect on outcome (log rank test p=0.10, Figure 5.3). Any impact of lineage on outcome is likely to be confounded by drug resistance phenotype. There were only two INH-resistant and two MDR-resistant Indo-Oceanic isolates (Table 5.3) and we therefore further analysed the impact of lineage on outcome including only patients infected with fully susceptible or STR-monoresistant strains (n=83, Figure 5.4).

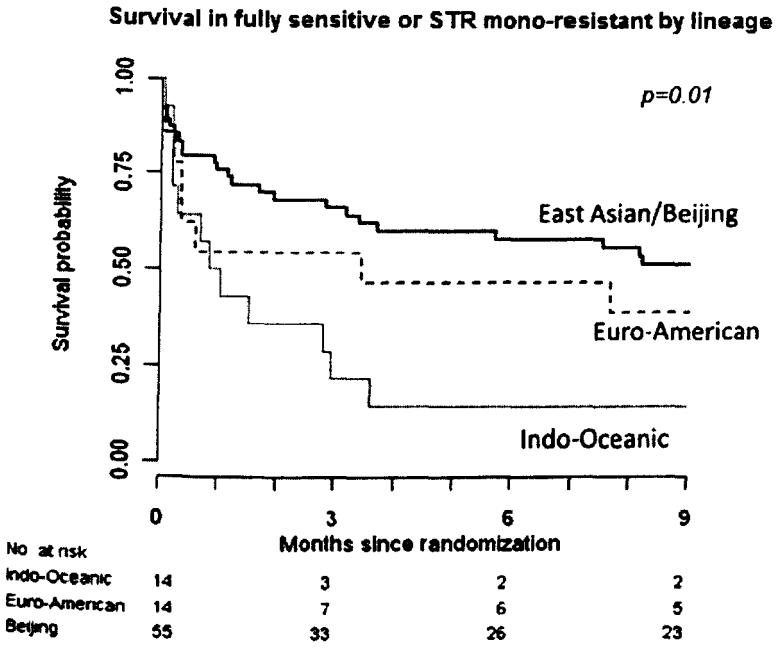


Figure 5.4: Kaplan-Meier survival curves of HIV-associated TBM patients infected with *M.tuberculosis* by the lineage for patients infected with strains susceptible to all drugs or STR mono-resistant strains.

The survival time among different lineages was statistically significant, log rank test, p=0.01.

In patients with fully susceptible or STR mono-resistant isolates (n=83) there was a significant difference between the survival curves of patients infected with different *M.tuberculosis* lineages (log rank test p=0.01). A Cox regression analysis adjusted for CD4 count, TBM grade at presentation and cohort group showed lower mortality in the East Asian/Beijing lineage compared to Indo-Oceanic lineage strains with an adjusted hazard ratio of 0.29 [95% CI 0.14-0.61], p=0.001. There were no

significant differences between East Asian/Beijing versus Euro-American strains HR=0.64 [95% CI 0.27-1.52], p=0.31 or between Euro-American versus Indo-Oceanic strains; HR=0.45[95% CI 0.17-1.18], p=0.11. Adjusted pairwise comparisons in patients with only fully susceptible isolates after exclusion of patients receiving streptomycin (n=62 patients remaining) were HR=0.27 [95% CI 0.12-0.60], p=0.001 for East Asian/Beijing lineage compared to Indo-Oceanic strains, HR=0.74 [95% CI 0.28-1.92], p=0.53 for East Asian/Beijing lineage versus Euro-American strains, and HR=0.37 [95% CI 0.13-1.05], p=0.06 for Euro-American versus Indo-Oceanic strains.

5.2.4 BCG vaccination

Previous BCG vaccination (assessed by visible scar) was recorded in patients recruited to the RCT and data were available for 74/81 (91%) of patients with *M.tuberculosis* genotype data. In these patients, we also analysed the influence of previous BCG vaccination on the lineage of the infecting strain and duration of illness prior to presentation. There was a significant association between prior BCG vaccination and lineage (Fisher's exact test p=0.01). Patients with a prior BCG vaccination were more likely to be infected with a Euro-American strain. Beijing genotype was less common among those with BCG vaccination (Table 5.4).

Table 5.4: Association between prior BCG vaccination *M.tuberculosis* lineage isolated from the CSF for 74 patients with HIV-associated TBM.

	M.tuberculosis lineage			
	East Asian/Beijing n (%)	Euro-American n (%)	Indo-Oceanic n (%)	Total n
BCG vaccinated	11 (22.9)	10 (62.5)	4 (40.0)	25
Not BCG vaccinated	37 (77.1)	6 (37.5)	6 (60.0)	49
Total	48 (100)	16 (100)	10 (100)	74

There was no evidence for an association between lineage and duration of illness prior to presentation (Kruskal-Wallis test p=0.76) or for duration of illness and BCG vaccination (Mann-Witney test p=0.42). There was also no evidence for an association between BCG vaccination and survival in univariate analyses (p=0.95) or after adjustment for lineage and resistance category (p=0.61).

5.3 Discussion

The effect of MDR on the outcome of TBM is well documented; however the role of INH mono-resistance in TBM is less clear. A recent retrospective study in the United States showed that INH resistance, HIV positive status and CSF culture positive are associated with increased mortality among TBM patients (Vinnard, Winston et al. 2010). These results are consistent with the data presented in this chapter. The retrospective analysis by Vinnard *et al.* was not able to examine standardized mortality endpoints or treatment regimens. In our collection of culture positive samples from HIV-associated TBM patients, INH resistance without concurrent RIF resistance had a significant impact on outcome among HIV-associated

TBM patients treated with standard first-line anti-TB therapy (adjusted HR= 1.78 [95% CI 1.18; 2.66], p=0.005).

Analyses modeling a time-varying effect of INH-resistance and Kaplan-Meier survival curves (Figure 5.1b) showed that the detrimental effect of INH-resistance was most pronounced from month 3 onwards, when treatment with PZA and EMB is stopped and patients continue on INH and RIF only. This suggests that these patients effectively on RIF monotherapy during the continuation phase, a RIF resistant sub-population was able to emerge once EMB and PZA were discontinued, resulting in treatment failure and increased mortality (time adjusted for the continuous treatment period, HR=5.05 [95% CI 2.23-11.44], p=0.0001). In this situation, extension of the duration of the intensive phase of treatment alone beyond three months may not be sufficient to achieve complete sterilization of the CSF and it is likely that an alternative or additional bactericidal agent will be required. Of the currently available drug classes for TB, a FQN is likely to represent the best option since the later generation FQNs (levofloxacin, gatifloxacin and moxifloxacin) have good CSF penetration, are well tolerated and have comparable early bactericidal activity to INH. Levofloxacin for the treatment of TBM is currently being explored in a treatment trial at OUCRU (trial registration number: ISRCTN61649292) (Heemskerk, Day et al. 2011). Ongoing multicenter RCTs in pulmonary TB are testing whether FQs can reduce the standard regimen for pulmonary TB to four months. The Remox TB study (Trial registration number: NCT00834383) is testing the replacement of INH and ETH with moxifloxacin and has completed recruitment, with reporting expected in 2014. It has been shown that rifapentine and rifabutin can be used as substitution for

RIF, which have less drug-drug interaction with ART (Loeliger, Suthar et al. 2012), and a longer halve-life which thus could extend the intermittent time between each dose (Sirgel, Fourie et al. 2005). Rifabutin penetrates the CSF (Nau, Sorgel et al. 2010) and has been used successfully to treat a case of HIV-associated *M.avium* meningo encephalitis (Malessa, Diener et al. 1994); thus may be alternative to RIF in HIV-associated TBM treatment. Novel agents such as PA-824 and TMC207 also show promise in the treatment of pulmonary TB and should be evaluated in TBM (Ma, Lienhardt et al. 2010). The TB alliance has announced a trial of PZA, moxifloxacin and PA-824 in combination for the treatment of both drug sensitive and drug resistant pulmonary TB and novel drug combinations may also be effective for the treatment of drug resistant TBM and trials should be considered if regimens prove effective in pulmonary TB. Since the morbidity and mortality of TBM is significantly higher in HIV infected cases than HIV uninfected cases (Katrak, Shembalkar et al. 2000), a smaller trial size would be required to demonstrate efficacy in HIV-associated TBM, however, mortality of 25% in HIV-uninfected TBM cases remains unacceptable and both groups should be included in future trials of novel anti-tuberculous drug regimens.

Only 35 patients in this study were given STR in addition to the standard treatment regimen. The inclusion /exclusion of 35 patients who received STR as an additional drug in the therapy did not significantly alter the findings. This shows that STR did not have a detectable effect on TBM outcome. STR resistance is most prevalent form of mono resistance (15.1%) in this study, however the standard treatment regimen did not include STR therefore resistance to STR is unlikely to have

an effect on outcome. The small number of patients who received STR and were infected with an isolate resistant to STR meant that it was not possible to examine the impact of STR resistance on outcome. HIV-uninfected TBM patients infected with STR resistant *M.tuberculosis* had a slightly lower mortality than patients infected with fully susceptible *M.tuberculosis* in a previous study at HTD (16.7% vs 28.7%), however this difference was not significant (Thwaites, Lan et al. 2005). The role of STR resistance in outcome from TBM remains to be defined. In accordance with previous studies, this study showed that MDR-TBM was uniformly fatal in this HIV patient population treated with standard antituberculous therapy (Thwaites, Duc Bang et al. 2005; Heemskerk, Day et al. 2011). The rapid detection of drug resistance in TBM cases is extremely difficult due to the paucibacillary nature of the disease. Molecular diagnostic tests for *M.tuberculosis* are relatively expensive and are not yet routinely available in resource-limited settings. Molecular diagnostic tests, such as MAS-PCR, or commercial LPAs (Chapter 3), may present an alternative but they have poor sensitivity on paucibacillary samples and in-house tests are difficult to standardize (Greco, Rulli et al. 2009). The wide-spread ongoing implementation of GeneXpert MTB/RIF (Cepheid, US) in high-TB burden settings may enable rapid diagnosis of MDR TBM, but data are not yet available on the accuracy of the technique in TBM cases. Even if the diagnosis of MDR-TBM is confirmed, there is no data from RCTs to guide clinical management (Byrd and Davis 2007; Donald 2010). This is also true for patients with INH-resistant but RIF-susceptible TBM.

In pulmonary TB, recent meta-analyses have shown that outcomes are worse in patients infected with INH-resistant, RIF-sensitive *M.tuberculosis* but treatment

guidelines for INH-resistant pulmonary TB vary and none are based on high quality evidence from RCTs (Lew, Pai et al. 2008; Menzies, Benedetti et al. 2009). The WHO recommends adding EMB to the continuation phase in regions with a high prevalence of INH resistance (WHO 2009), or to use a RZE continuation phase for 6-9 months in cases of confirmed INH resistance, with the note that 'a FQN may strengthen the regimen for patients with extensive disease' (WHO 2008), the American Thoracic Society recommends adding a fluoroquinolone in "extensive cavitatory disease" and the British Thoracic Society recommends an extended continuation phase of 7 months, with EMB and RIF. These recommendations probably should not be extrapolated to the treatment of TBM due to the need for high CSF penetration of bactericidal agents and further studies are required to improve the early detection of drug-resistant bacteria in patients with TBM and to determine the optimal treatment for both INH-resistant and MDR TBM cases.

This study did not quantify *M.tuberculosis* in CSF. The impact of drug resistance may be greater in patients with higher bacterial loads, particularly to a single agent such as INH mono-resistance, since a larger population of *M.tuberculosis* will have a higher chance of containing bacilli with spontaneous resistance mutations. We also did not examine the impact of NAT2 status on outcomes. The majority of Asian patients are fast acetylators for INH (Kita, Tanigawara et al. 2001; Cho, Koh et al. 2007) and this may result in lower CSF INH concentrations and consequently increase the impact of INH resistance. As INH penetrates the blood:brain barrier into the CSF very well while RIF penetrates poorly (approximately 20% of serum concentrations, the role of NAT2 acetylator status in the outcome of these HIV-

associated TBM patients might be greater than on pulmonary TB patients (Donald, Parkin et al. 2007). The next study in outcome of TBM patients should also examine the role of NAT2 status on the concentration of INH in the CSF. Further study is required to demonstrate the role of NAT2 in both pulmonary and disseminated TB disease, including TBM.

The multivariate regression model suggests the lineage of the infecting *M.tuberculosis* strain influenced outcome of patients infected with fully-susceptible strains. Interestingly, patients infected with the modern Beijing lineage strains had a lower mortality risk than those with the ancient Indo-Oceanic strains. This finding contradicts previous reports in the literature of increased mortality in patients infected with Beijing lineage strains (Sun, Lee et al. 2007; de Jong, Hill et al. 2008; Parwati, van Crevel et al. 2010); however, none of these studies were done in HIV-associated TBM patients. A previous study at OUCRU showed no impact of *M.tuberculosis* lineage on mortality for 160 TBM patients, who were HIV-uninfected, although a higher proportion of patients infected with Beijing lineage strains were dead or severely disabled at nine months, this difference did not reach significance (34.3% vs. 25.6% for Euro-American and Indo-Oceanic strains combined, p=0.236) (Thwaites, Caws et al. 2008). The number of patients examined in this study is relatively small and this finding needs to be confirmed in a larger study.

The higher event rate for mortality among HIV-associated TBM in this population may have strengthened the power of the study to detect a difference between the lineages of *M.tuberculosis*. Alternatively, the difference may be due to variable pathogenesis with HIV co-infection. The 'modern' Beijing genotype and

Euro-American lineages have a pro-inflammatory profile, inducing more TNF-α and IL-1β *in-vitro* and also show higher growth rates and propensity to disseminate in a mouse model, compared to the 'ancient' Indo-Oceanic strains (Krishnan, Malaga et al. 2011). There is increasing evidence of differences in virulence between the major lineages of *M.tuberculosis* in different host populations (Gagneux, DeRiemer et al. 2006; Gagneux and Small 2007; Caws, Thwaites et al. 2008; de Jong, Hill et al. 2008; Thwaites, Caws et al. 2008; Nahid, Bliven et al. 2010). The mechanism responsible for these differences remains unclear but may be due to differential immune modulation by strains of the major lineages (Wang, Peyron et al. 2010; Portevin, Gagneux et al. 2011); it is possible that the pro-inflammatory effect, while detrimental in immune-competent patients and resulting in increased propensity to disseminate conversely reduces the severity of TBM in the severely immune-compromised.

A previous study at OUCRU has shown an association between the Beijing genotype and HIV-associated TBM in this setting (Caws, Thwaites et al. 2006) and in this thesis we have demonstrated the association of Beijing genotype with higher MIC to INH (chapter 4) which implies that there are significant lineage-associated variations in virulence relating to drug resistance and pathogenesis. The data presented in this chapter shows HIV-associated TBM patients infected with a Beijing lineage do not have a higher mortality but in fact a lower mortality. This result demonstrates that the bacterial lineage is one factor independently affecting the outcome of TBM.

HIV-infected TBM patients who have been previously BCG vaccinated were more likely to be infected with a Euro-American strain of *M.tuberculosis* and less

likely to be infected with a Beijing strain. This is an unexpected finding as it has been previously hypothesised that the success of the Beijing strain is attributable to a lower protective efficacy of BCG against Beijing lineage strains and that Beijing lineage could be emerging under selection pressure following wide-spread application of BCG vaccination, although the evidence to support this theory remains weak (Abebe and Bjune 2006; Tsenova, Harbacheuski et al. 2007; Parwati, van Crevel et al.). The patients in this study were severely immune suppressed which may alter the protective dynamics of the immune response to BCG. Improved understanding of the differential immune evasion mechanisms of *M.tuberculosis* lineages is vital to the development of a globally effective vaccine (Gagneux and Small 2007).

In conclusion, this study demonstrates that INH resistance, MDR and bacterial lineage play an important role in outcome from TBM. Methods to improve the rapid diagnosis of drug-resistant TBM and clinical trials to determine optimal antimicrobial treatment strategies are urgently required.

Chapter 6

The correlation of acetylator phenotype and NAT2 genotype in Vietnamese Kinh population

6.1 Background

The efficacy of INH is dependent upon drug concentration and therefore it is explained by the ratio of C_{max}:MIC (Jayaram, Shandil et al. 2004). The drug can be taken intermittently without compromising the efficacy, unlike other time-dependent antibiotics, such as β lactam group and vancomycin which must maintain a consistent serum concentration for efficacy (Rybak 2006). The C_{max} value can be changed actively by altering the dose of INH administered. Inter-individual variation has been shown by their age, gender, dietary habits (Schaaf, Parkin et al. 2005). However the most significant factor in inter-individual variation in INH serum C_{max} is due to variations in genotype of the NAT2 genotype (Donald, Parkin et al. 2007).

Individuals can be grouped into one of three acetylator types: homozygous fast-fast (FF), heterozygous fast-slow (FS) and slow-slow (SS) acetylator types, which may also be defined as fast, intermediate or slow, respectively. People with fast acetylator status eliminate INH relatively quickly. An individual with fast acetylator status will have lower serum concentrations of INH comparing with others using the same dose (Kinzig-Schippers, Tomalik-Scharte et al. 2005; Donald, Parkin et al. 2007), which may decrease the efficacy of INH. A study by Donald showed when all TB patients received the standard dose of INH (5mg/kg), 100% SS acetylators, 96.3% FS acetylators and only 33% FF acetylators reached the optimal EBA_{90} of INH with

the AUC of 10.52 μg/ml/hour INH in the sera (Donald, Parkin et al. 2007). Therefore, fast acetylators status decreases the efficacy of INH in TB patients. In contrast, individuals with a slow acetylator phenotype take a longer time for the drug clearance process. An individual who metabolizes INH slowly has been shown to accumulate INH in the body, and thus could be at increased risk of hepatotoxicity (Higuchi, Tahara et al. 2007).

The distribution of fast and slow acetylators varies in different ethnic populations. The majority of Asian populations are predominantly fast acetylators and the Vietnamese Kinh are likely to be predominantly fast acetylators as are the Chinese (Zhao, Seow et al. 2000), Japanese and Thai ethnic groups (Kukongviriyapan, Prawan et al. 2003), in contrast to the Caucasian and the African American population of the United States, where the majority are slow (Walker, Ginsberg et al. 2009). The high prevalence of fast acetylators may play an important role in the development of *M.tuberculosis* resistant to INH or the outcome of TB treatment with standard regimens through its effects on the INH clearance process.

NAT2 is a human acetylator enzyme that is predominantly found in liver and extrahepatic tissues, known to detoxify chemicals in mice (Sugamori, Wong et al. 2003). The enzyme is encoded by the *NAT2* gene which defines the acetylator phenotype of each individual. NAT2 is known to be responsible for the INH elimination process in humans and affects the serum concentration (Donald, Parkin et al. 2007); therefore, it may have an impact on the efficacy of INH and INH-based therapies.

A TB drug resistance survey in HCM city-Vietnam showed that a quarter of new smear-positive TB patients had INH resistant TB (Quy, Buu et al. 2006). The finding is consistent with WHO data which ranks Vietnam as a country with a high prevalence of INH resistance (more than 20% INH resistance among new TB cases) (WHO 2008). If there is a predominance of NAT2 fast acetylator phenotypes in the population, it may influence the *M.tuberculosis* drug resistance pattern in this region; however, the correlation between NAT2 genotype and acetylator phenotype has not been established in the Vietnamese population and this data is necessary for further investigation of the role of NAT2 genotypes in TB therapy.

INH and caffeine share the same pathway of NAT2 metabolism; as a result, caffeine metabolism is a surrogate indicator that has been used to determine the acetylator phenotypes of healthy volunteers (Conte, Golden et al. 2002). When acetylator phenotypes are defined, genotype data can be used to predict INH clearance activities. Caffeine can be administered at a dosage equivalent to a large cup of coffee (100mg) which is considered safe and consumed regularly by the majority of western adults. Metabolites of caffeine such as 1-methyluric acid (1U), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U), 1,7-dimethylxanthine (17X), 5-acetylamino-6-formylamino-3-methyluracil (AFMU) are excreted in urine. AFMU is spontaneously degraded to 5-acetylamino-6-amino-3-methyluracil (AAMU). The ratio AAMU/(AAMU+1X+1U) was shown to be more stable than AFMU(AFMU+1X+1U) (Nyeki, Buclin et al. 2003); thus was used to study NAT2 phenotype in Vietnamese healthy volunteers.

The aim of this study was:

1) To determine the correlation between the NAT2 phenotype and NAT2 genotype in Vietnamese healthy volunteers via a caffeine assay.
2) Develop a rapid test to genotype the NAT2 gene (RFLP method) which can be used in place of sequencing and is feasible in most laboratories equipped with a PCR machine.

6.2 Results

Thirty seven healthy volunteers consented to participate in the study, of which 12 were males and 25 were females. All volunteers were non-smokers and the majority did not drink tea or coffee on a regular basis (65%). All volunteers were asked not to consume tea, coffee, chocolate or other caffeine products 36 hours prior to the caffeine administration and until after urine sample collection.

6.2.1 Acetylator phenotype results

The HPLC method provided a rapid extraction less procedure to determine and quantify accurately 1U, 1X, 17U, and AAMU. The retention times for AAMU, 1U, 1X, 17U and 7-beta hydroxyethyl theophylline (used as the internal standard) were 4.8 mins, 9.3 mins, 10.2 mins, 13.1 mins and 15.3 mins, respectively.

Calibration curves of AAMU, 1U, 1X, 17U were linear over the concentration range (5 μM to 250 μM). The correlation coefficients (goodness of fit, R^2) were greater than 0.99 for all the metabolites. The intra-day and inter-day (mean relative standard deviation, SD) for the 4 metabolites was less than 3.1% and less than 5.9%

respectively. The accuracy ranged from -6.4% to +4.4% for all the metabolites for all the occasions (repeatability and intermediate precision). The limit of detection was estimated at 2 μM for AAMU, 1X, and 1 μM for 1 X, 17U. The lower limit of quantification was set at 5 μM for all metabolites.

The subjects were classified according to the estimated value of the ratio AAMU/(AAMU+1X+1U). The best fitting model classified 13/37 (35.1%) individuals in the slow acetylator group and 24/37 (64.9%) in the fast acetylator group. The frequency distribution of NAT2 activities had a cut-off value of 0.3 with the ratio AAMU/(AAMU+1X+1U) (Figure 6.1).

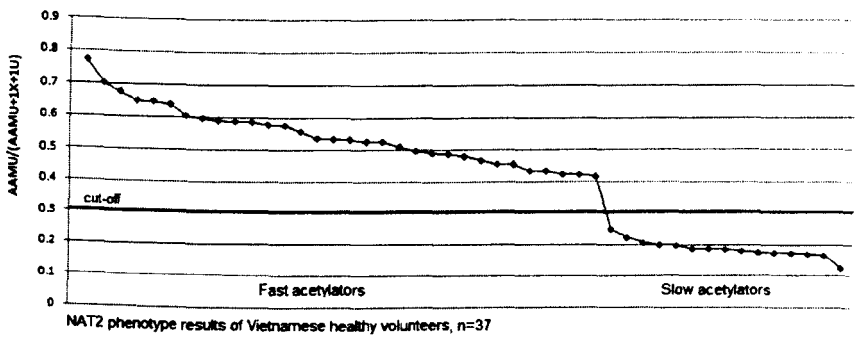


Figure 6.1: Acetylator phenotype results of 37 Vietnamese healthy volunteers showed a clear cut-off between the ratio of fast and slow acetylators.

Individuals with metabolic ratios <0.3 were classified as slow acetylators and those with the ratios >0.3 as fast acetylators. The mean metabolic ratio (±SD) was 0.19±0.03 and 0.54±0.09 for slow acetylator and fast acetylator group, respectively. The NAT2 fast and slow activities were compared between males and females. The median values of the NAT2 ratio were not different between males and females of

slow acetylators with 0.19 and 0.18, respectively (p-value >0.05). Similarly there was no significant difference in NAT2 ratio between males and females of fast acetylators, with the median 0.55 and 0.58, respectively (p-value > 0.05).

6.2.2 Genotype of NAT2

In this study, 24 of 37 people (64.9%) expressed the fast acetylator genotype. Among this fast acetylator group, the most frequent genotype was NAT2*4/4, accounting for 29.7% (11/37), followed by NAT2*4/6A with 24.3% (9/37) and NAT2*4/7B with 8.1% (3/37). In the slow acetylator group, genotypes NAT2*6A/6A and NAT2*6A/7B were at higher frequency with 18.9% (7/37) and 8.1% (3/37), respectively (Table 6.1).

Table 6.1: Genotype and the associated phenotype results from caffeine metabolism analysis among 37 healthy Vietnamese Kinh volunteers.

Acetylator Status	Genotype			Phenotype	
	NAT2 combination	N	%	AAMU/(AAMU+1X+1U) Mean - range	
Slow	*6A/6A	7	18.9%	0.19	0.17-0.25
	*6A/7B[a]	3	8.1%	0.19	0.17-0.22
	*7B/7B	1	2.7%	0.12	N/A
	*5B/6A[b]	1	2.7%	0.20	N/A
	*5B/7B[c]	1	2.7%	0.18	N/A
	Total	**13**	**35.1%**		
Fast	*4/4	11	29.7%	0.63	0.53-0.77
	*4/13A	1	2.7%	0.53	N/A
	*4/6A	9	24.3%	0.48	0.42-0.58
	*4/7B	3	8.1%	0.49	0.47-0.50
	Total	**24**	**64.9%**		

[a] Could be misclassified as *13A/6J (fast acetylator) without phenotype result

[b] alternative genotype of *5B/6A are *5A/6C and *5G/6B

[c] alternative genotype of *5B/7B are *5A/7C and *5G/7A

[b,c] Genotype *5B/6A and *5B/7B was defined base on the popularity of haplotypes *6A and *7B in Vietnamese population

When stratify according to NAT2 haplotypes, majority of Vietnamese healthy volunteers carry haplotype *4, accounting for 47.3% (35/74), following by haplotype *6A with 36.5% (27/74) and *7B with 12.2% (9/74). The others haplotypes take only a small portion in this population of the study (Table 6.2)

Combining caffeine phenotypic and NAT2 genotypic results, we had a correlation between these two data. Phenotype results were fit with genotype (Figure 6.2)

Table 6.2: Distribution of NAT2 haplotype in 37 healthy Vietnamese Kinh volunteers

Phenotype	*Haplotype*	*N*	*Frequency (%)*
Fast	*4	35	47.3
	*13A	1	1.3
Slow	*6A	27	36.5
	*7B	9	12.2
	*5B	2	2.7

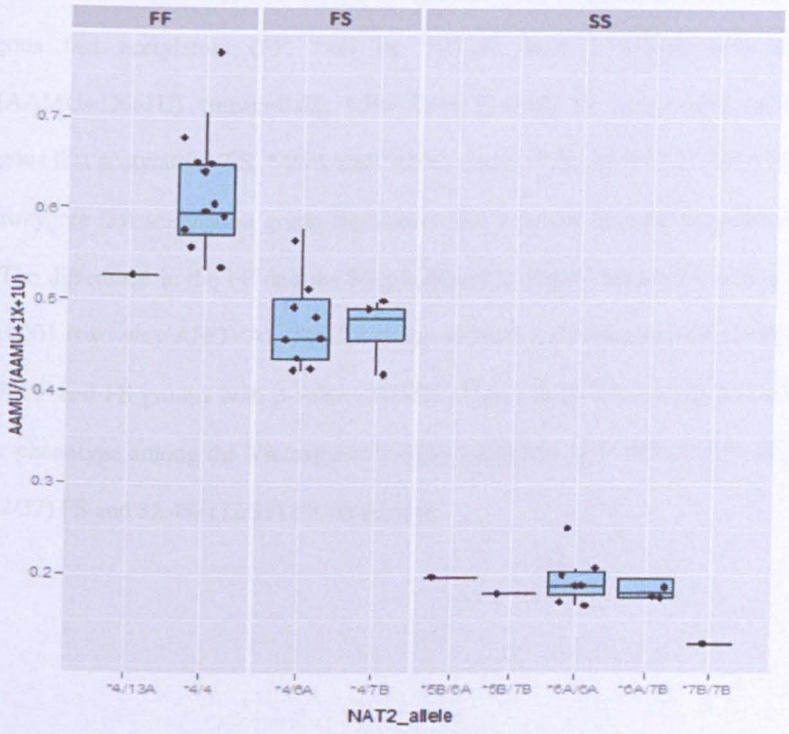


Figure 6.2: Acetylator phenotype results and the associated NAT2 allele combinations in 37 Vietnamese healthy volunteers divided by FF, FS and SS acetylators.

Box plots show the median and IQR of the ratio associated with NAT2 genotype.

The caffeine phenotype study also showed that individuals who are homozygous fast acetylators (FF, *4/4 or *4/13A) have a higher ratio of AAMU/(AAMU+1X+1U) (mean=0.62, 95% CI 0.57-0.66) in comparison with heterozygous fast acetylators (FS, *4/6A and *4/7B) (mean=0.48, 95% CI 0.46-0.51). The heterozygote fast acetylation group represents 32.4% in our sampled population (12/37). The difference in the FF and the FS phenotype is highly significant with p-value <0.0001 (two-way ANOVA). The SS group showed a significantly difference from both FF and FS groups with p-value <0.0001 (Figure 6.3). The distribution of acetylator phenotype among the Vietnamese healthy volunteers is 35.1% (13/37) SS, 32.4% (12/37) FS and 32.4% (12/37) FF acetylators.

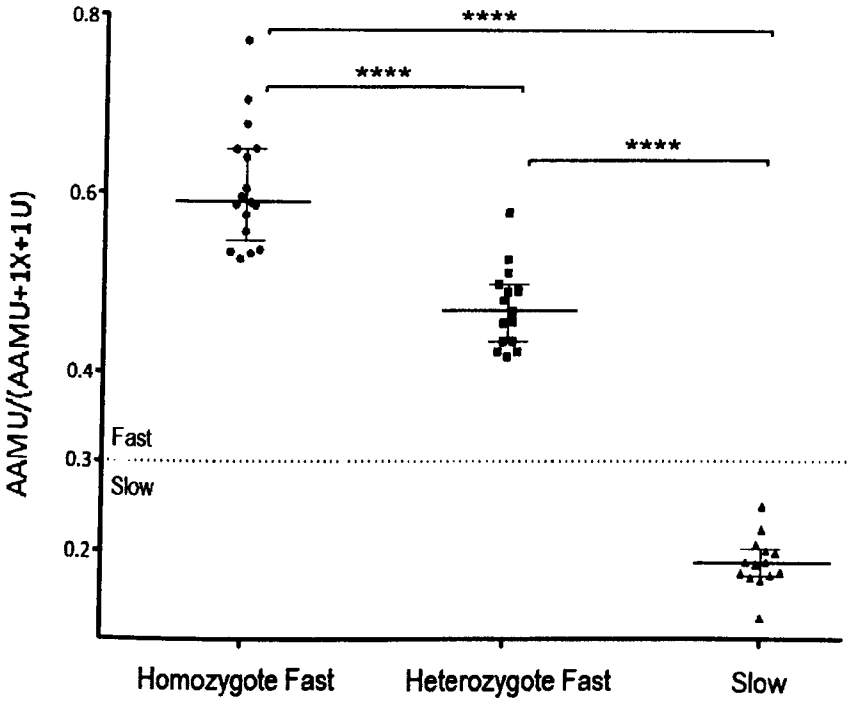


Figure 6.3: Acetylator status from the AAMU/(AAMU+1X+1U) ratio and the corresponding genotypes deduced from polymorphism at NAT2 allele.

Bars in the plots show mean and 95% CI of the ratio associated with each NAT2 genotype.

Differences between the ratio for each NAT2 genotype were calculated pairwise by two-way ANOVA and showed statistical significance $p<0.0001$.

****** significant at $p<0.0001$**

6.2.3 Development of PCR-RFLP to detect fast/slow acetylators

Analysis of the acetylator haplotype database showed that acetylator status was influenced when there are polymorphisms at nucleotide number T341C, G590A, G857A. By looking at these three SNPs, instead of seven SNPs as reported elsewhere (Rihs, John et al. 2007), we could define the acetylator phenotype status in this set of Vietnamese healthy volunteers. These SNPs lead to amino acid changes; and thus could have a potential impact in acetylator enzyme activities. This is concordant with other studies performed on Asian ethnic groups which focus only on three SNPs T341C, G590A, G857A (Kita, Tanigawara et al. 2001; Kukongviriyapan, Prawan et al. 2003); whereas studies in European (Caucasian) ethnic groups show an influence of a greater number of polymorphisms: G191A, C282T, T341C, C481T, G590A, A803G, G857A (Tanira, Simsek et al. 2003; Rihs, John et al. 2007) on the definition of fast/slow acetylators. NAT2 polymorphisms vary geographically (Garcia-Martin 2008; Sabbagh, Langaney et al. 2008); thus, a simplified test for NAT2 acetylator status will be region or ethnicity specific.

PCR RFLP is the simplest method that can detect polymorphism in different haplotype. However, none of the test in literature was designed for only the three polymorphisms at nucleotide number T341C, G590A, G857A. Some authors used an adapted technique from a PCR RFLP that was designed for six polymorphisms G191A, C282T, C481T, G590A, A803G and G857A. The unique T341C was detected only by MAS-PCR. It is because the cleavage site at position 341 was not available in wide type. So I had to use 341C mutation sequence template to design the

test. TspRI was the only available restriction enzyme for that site. Then I found that the cutting sites of from TspRI were close to each other which make it difficult to tell the different between wild type and T341C. A mismatch PCR was carefully designed to cover one cleavage site and make the PCR-RFLP simple, hand-on time short and very easy to interpret. The principle of PCR-RFLP was showed in Figure 6.4

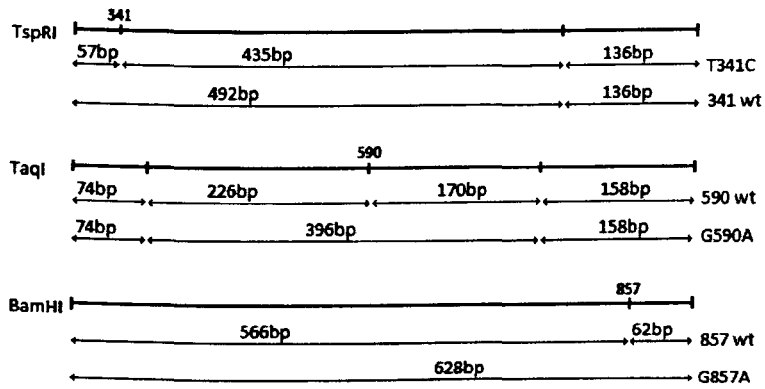


Figure 6.4: The principle of PCR-RFLP to detect SNPs at nucleotide 341, 590 and 857.

Bold lines are PCR products with restriction sites marked. The thin arrow lines show the length of restriction fragments.

The inner 628bp fragment of the second exon in human NAT2 gene was amplified with two primers NAT2Fa (5'-CCAGTTAACAAATACAGTAC-3') and NAT2R (5'-GAGTTGGGTGATACATACAC-3'). The PCR amplicons were aliquoted into three separate PCR tubes, 5U of the respective restriction enzyme was added to each: *Tsp*RI, *Taq*I and *Bam*HI (New England Biolabs, USA) and the tubes were then incubated at the appropriate temperature: 65°C, 65°C and 37°C, respectively, for 3 hours. The products were electrophoresed on a 1.5% agarose gel for two hours (Figure 6.5 and Figure 6.6).

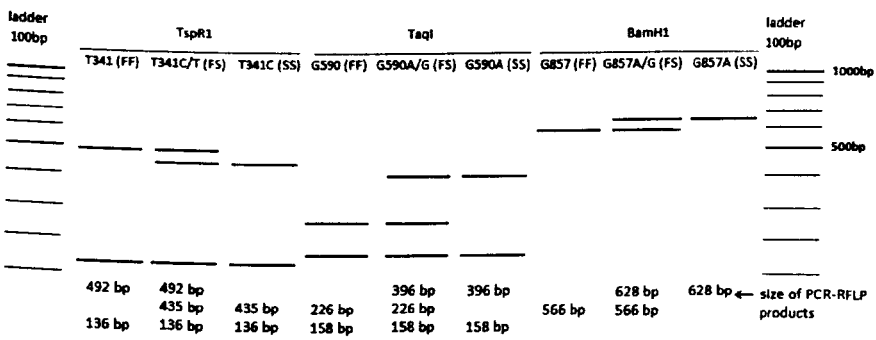


Figure 6.5: The representation of PCR-RFLP results for different SNP patterns in the NAT2 gene.

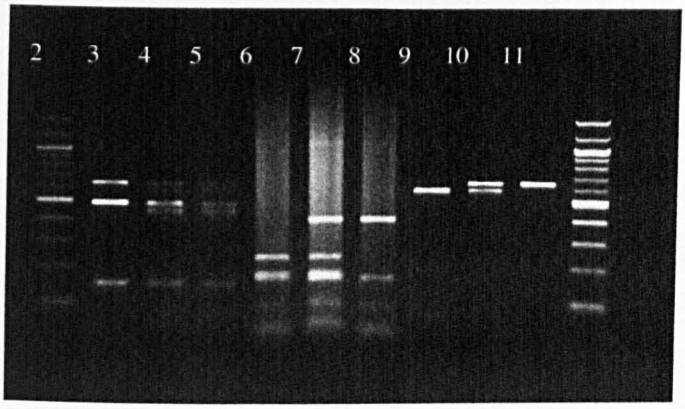


Figure 6.6: Results of PCR-RFLP on samples from individuals with the most frequently occurring NAT2 genotypes.

Lane 1, 11: 100bp ladder.
Lanes 2-4: digested with *Tsp*RI.
Lanes 5-7: digested with *Taq*I.
Lanes 8-10: digested with *Bam*HI.
Lane 2,5,8: wild type (FF acetylator).
Lane 3,4: F/S acetylator at SNP 341.
Lane 6: F/S acetylator at SNP 590.
Lane 7: SS acetylator at SNP 590.
Lane 9: F/S acetylator at SNP 857.
Lane 10: SS acetylator at SNP 857.

The PCR-RFLP test was repeated with samples from the initial 37 healthy volunteers and the results were 100% concordant with the sequencing and phenotypic results.

6.3 Discussion

The correlation between acetylator phenotype and NAT2 genotype has been clearly shown in this chapter, in accordance with studies in other ethnic populations.

A clearly defined difference between individuals with FF, FS, and SS acetylator status was shown by genotype results (sequencing) and phenotype results ($p<0.0001$). We found that Vietnamese healthy volunteers of Kinh ethnicity are predominantly fast acetylators (64.9%).

The determination of phenotypic acetylation analysis using AAMU/(AAMU+1X+1U) gave reproducible results. With the same cut-off value (ratio AAMU/ (AAMU+1X+1U)= 0.3) as reported in a previous study (Nyeki, Buclin et al. 2003), the NAT2 phenotypic results clearly classified fast and slow acetylator groups and the results were concordant with the NAT2 genotype results from sequencing.

The caffeine phenotype study showed that people who were homozygous fast acetylator had a higher ratio of AAMU/(AAMU+1X+1U) in comparison with heterozygous fast acetylator group. The heterozygote fast group is considered as the "intermediate" acetylator group (Kuznetsov, McDuffie et al. 2009), and represented 32.4% in our sampled population (14/37). The difference in the caffeine metabolites ratio between the homozygous fast (FF) and the heterozygous fast (FS) groups was highly significant with p-value <0.0001 (two-way ANOVA).

The prediction of the acetylator phenotype based only on NAT2 haplotype polymorphism database can lead to inaccurate prediction of phenotype status as shown with four samples (10.1%) in this study. This is due to the potential for inaccurate haplotype prediction. More than two polymorphisms found in one genotype will complicate the analysis. The phenotype of these individuals could be alternatively predicted as FS acetylation status using the genotype data, whereas the

measured phenotype was slow. Alternative haplotypes can be predicted from the database when they are taken as new NAT2 genotypes in studies without phenotype confirmation, such as haplotypes NAT2*5J and NAT2*6J (Tanira, Simsek et al. 2003; Sabbagh, Langaney et al. 2008) as shown in this study. Four SS acetylators (3 with *6A/7B and one with *5B/6A) could have been misclassified as genotypes NAT2*13A/6J and NAT2*12C/5J, (respectively) or FS acetylators. However, the phenotype of the four volunteers showed slow acetylators phenotype; thus should carry 2 copies of slow/slow acetylator in NAT2 gene (SS). This illustrates the need to determine the haplotype structure of individual populations before using genotype data alone to determine phenotypic acetylation status

For example, two genotypes were found with 5 polymorphisms in the NAT2 gene. We found that there was more than one combination of haplotypes which could fit to these genotypes, such as NAT2*5A/6C, NAT2*5B/6A, NAT2*5G/6B and NAT2*12C/5Jhave the same combination of polymorphism. Similarly, NAT2*5A/7C, NAT2*5B/7B and NAT2*5G/7A share another polymorphism combination. As NAT2*6A and NAT2*7B are the haplotypes with the highest prevalence in this population (36.5% and 12.2%, respectively), suggesting that the haplotype combination of these two polymorphisms should be NAT2*5B/6A and NAT2*5B/7B (Table 6.1). With the result from this study, knowing the prevalence of each NAT2 haplotype in the Vietnamese Kinh population allows the correct interpretation of the genotype results. Other studies have also shown that only mutations at nucleotide positions 191, 341, 590 and 857 result in a significant decrease of acetylator activity (Kita, Tanigawara et al. 2001; Patin, Barreiro et al.

2006; Rihs, John et al. 2007). This phenomenon was also observed in the present analysis which showed that any person carrying homozygous mutations at one of these loci or heterozygous mutations at two of these loci would have a slow acetylation phenotype. The prediction of acetylator phenotype should be based on the number of mutations at these nucleotide positions (191, 341, 590 and 857) in using the NAT2 haplotype database to predict the haplotype. As mutation at nucleotide 191 has never, to our knowledge, been reported in NAT2 genotypes of individuals of Asian ethnicity, including Vietnamese (Cavaco, Asimus et al. 2007), Japanese (Kita, Tanigawara et al. 2001), and Chinese (Garcia-Martin 2008; Dai, Leng et al. 2009), this suggests further study of NAT2 genotype in Vietnamese Kinh could focus only on 3 nucleotide sites (341, 590 and 857) to discriminate NAT2 genotypes, however a larger sample size should be examined to confirm this. Based on this analysis, we developed a PCR RFLP assay to detect fast/slow acetylators in Vietnamese Kinh (section 6.2.3).

Twenty four of 37 people (64.9%) expressed fast acetylator phenotype in this study. The predominance of fast acetylators in Vietnamese Kinh was similar to the results of other studies in Chinese (Zhao, Seow et al. 2000) and Japanese (Walker, Ginsberg et al. 2009).

Another study conducted in 72 Vietnamese in northern Vietnam also showed a similar pattern that fast acetylation genotypes were predominant in Vietnamese (Cavaco, Asimus et al. 2007). The study showed that 90% of participants carry at least one NAT2 fast allele, whereas only 64.9% volunteers in our study carry either fast alleles NAT2*4 or NAT2*13A. The reason for the discrepancy between that

study and the present analysis are not clear, however the study in northern Vietnamese did not confirm genotype predictions with phenotypic data. Alternatively there may be geographical variation (the two study sites are approximately 2,000km apart). The analysis of NAT2 alleles in the present study was not consistent with Hardy-Weinberg equilibrium and the proportion of slow acetylators was higher than it should be, suggesting that the sample size was not large enough to represent for the whole country. However, this is true with all other studies which have examined both genotype and phenotype of NAT2 (Kita, Tanigawara et al. 2001; Kinzig-Schippers, Tomalik-Scharte et al. 2005). Given these limitations, and the discrepancy with the report from northern Vietnam, the actual prevalence of fast acetylators in the Vietnamese Kinh population is still unknown and requires further investigation with a larger sample size.

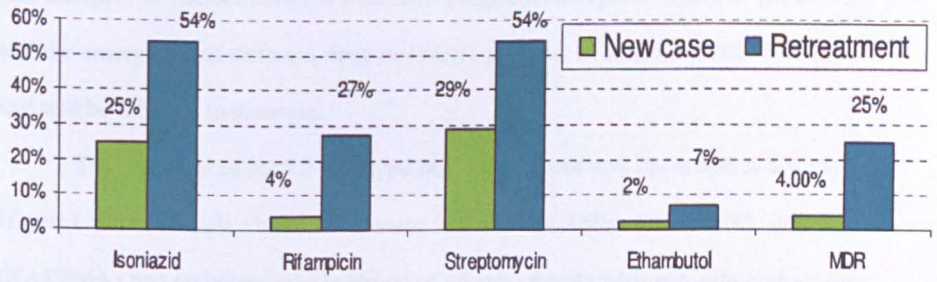


Figure 6.7: The prevalence of first-line drug resistance among *M.tuberculosis* isolates from pulmonary TB patients in HCMC (1998-2000) (Quy, Buu et al. 2006)

It is possible that the high prevalence of fast acetylators in the Vietnamese Kinh population could be a contributing factor to the high prevalence of INH resistant *M.tuberculosis* in this population (Figure 6.7) (Quy, Buu et al. 2006). Data from *M.tuberculosis* isolates analysed in HCM city showed that around only 10% of INH resistant isolates carry *inhA(C-15T)* mutation (Caws, Duy et al. 2006), which confers a low-level of INH resistance, as illustrated in chapter 4. With a standard administration dose of INH (5mg/kg), slow acetylators may accumulate a sufficient INH concentration in their sera (Kinzig-Schippers, Tomalik-Scharte et al. 2005) to effectively kill isolates with an *inhA* mutation but not those with a *katG315* mutation, which confers high-level resistance. This would favour the selection of INH resistant isolates with *katG*315 mutations in slow acetylators. Whereas, fast acetylators rapidly metabolise INH and these patients often do not reach the optimal EBA_{90} of INH concentrations in their sera (Donald, Parkin et al. 2007). Speculatively, INH resistant strains may be selected in fast acetylators in whom the efficacy of INH is decreased. The interplay of factors between treatment programs, acetylator status of populations and the emergence of different drug resistance patterns in *M.tuberculosis* is complex and will be difficult to untangle.

The influence of NAT2 genotype on TB treatment was illustrated in a study by Higuchi *et al.* which showed Japanese individuals with slow NAT2 genotypes (NAT2*6A) had an increased likelihood of adverse events with anti-tuberculous drug therapy (Higuchi, Tahara et al. 2007). A pharmokinetic study by Donald *et al.* showed that at normal INH dose for TB patients (5mg/kg), many F/F acetylators did not achieve a serum concentration of 2.19μg/ml after 2 hours, which is the concentration

for optimal early bactericidal activity of INH (Donald, Parkin et al. 2007). The high prevalence of fast acetylators in the Vietnamese Kinh population may have implications for the TB control programme. It is possible a higher dose of INH should be applied in F/F acetylators to ensure optimal early bactericidal activity, however the cost and risks of toxicity may outweigh the benefits. The most common adverse event from INH therapy is hepatotoxicity which has been described in all populations carefully (Ohno, Yamaguchi et al. 2000). A RCT in India suggested that high dose of INH is safe and can improve outcome in MDR TB patients (Katiyar, Bihari et al. 2008). Further studies should investigate the efficacy and toxicity of high-dose of INH in TB patients. It is possible that high dose INH will also be beneficial in TBM cases, where INH is one of the few drugs which has both high EBA and good CSF penetration.

WHO recommends that intermittent administration of chemotherapy should not be applied for patients with INH resistant TB (WHO 2009), and the drawbacks of intermittent INH therapy are likely to be exaggerated in populations with both a predominance of fast acetylators and high prevalence of INH resistant TB, such as Vietnam.

A simple technique which can determine acetylator status of TB patients would simplify studies of targeted high dose INH therapy. The novel PCR RFLP developed here could be used to rapidly determine NAT2 genotypes for stratified randomization to high or standard dose INH therapy. Further studies should focus on the influence of host acetylator status on INH resistance in TB patients and treatment

outcome. This could be a contributing factor to geographic variations in the prevalence of drug resistance

Only the correlation of caffeine metabolism and NAT2 genotype was investigated in this study. The correlation between phenotype of INH metabolism and NAT2 genotype is unlikely to be different since INH and caffeine share the same clearance pathway. However, further study should confirm accurate correlation of genotypes with INH metabolism phenotype in TB patients.

This study focused on caffeine metabolism as a surrogate marker for INH metabolism. TB therapy requires multiple drugs and further studies are also required to determine host genetic factors which influence the metabolism of other key drugs. Improved understanding of drug metabolism enables targeted therapy to minimize adverse events, optimize efficacy and improve drug adherence, ultimately reducing the potential for the emergence of drug resistance. However, although individualized therapies are not currently feasible in high-burden, resource limited settings where standardized regimens are applied, basic research is required to enable us to preserve efficacy of drugs such as INH, which has been the cornerstone of TB treatment for over fifty years.

Chapter 7

Discussion

INH has a critical role in drug therapy for TB and is likely to remain a key drug for decades to come. It has high early bactericidal activity, is active against latent TB and is well-tolerated and inexpensive. Despite this, research on drug resistant *M.tuberculosis* has largely focused on RIF resistance as the important determinant of MDR TB. Much remains unknown about both the mechanism of action of INH and the mechanisms of drug resistance for this drug. Globally, it is estimated 7% of *M.tuberculosis* strains are now resistant to INH and it is vital that we better understand the development, and transmission of INH resistant TB, as well as improving tools for rapid detection and optimizing treatment. This thesis has focused on several aspects of INH resistance in *M.tuberculosis* in Vietnam. Each chapter in this thesis addressed a different aspect of INH resistant TB to improve our understanding of some fundamental aspects of INH resistance and its impact on treatment outcomes.

A previous study showed that the rate of INH resistant *M.tuberculosis* was very high in HCM city, the biggest city in Vietnam, with 25% of primary TB patients infected with INH resistant strains (Quy, Buu et al. 2006). The resistance rate in previously treated TB patients was much higher with almost 50% of patients infected with INH resistant *M.tuberculosis* (Quy, Buu et al. 2006). These findings showed the urgent need for a feasible alternative to phenotypic DST for earlier detection of INH resistance and MDR *M.tuberculosis* in Vietnam. A previous study at OUCRU in 100

INH resistant isolates showed that the majority of the strains carried mutations at just two positions *katG*315 and *inhA*-15 (Caws, Duy et al. 2006). The first study in this thesis therefore developed MAS-PCR tests for RIF resistance (Tho, Ha et al. 2008) and INH resistance (Tho, Lan et al. 2011), and the results were comparable with the with commercial LPA MTBDR*plus*. The major drawback to these MAS-PCR tests was the need for DNA from an isolate of *M.tuberculosis*, meaning that they cannot yet be performed directly on clinical samples such as sputum. The Xpert MTB/RIF test is a close cartridge based system which has major advantages in the field in high burden settings but it remains expensive for wide-scale screening of all TB suspects and is currently only able to detect RIF resistance and is therefore being targeted to high-risk groups for MDR TB. The need for a rapid, robust and economically feasible screening test for INH resistant TB remains.

Several studies from other regions in the world have reported that the *katG*315 mutation confers a higher level of INH resistance than *inhA*-15 mutation (van Soolingen, de Haas et al. 2000; Vijdea, Stegger et al. 2008). Chapter 4 of this thesis showed a similar result that isolates carrying *katG*315 mutation had significantly higher MIC to INH in comparison with isolates carrying *inhA*-15. In addition with our previous finding that 90% of INH resistance isolates from Vietnam carry *katG315*, this result implies that majority of the INH resistant isolates have high MIC to INH and the application of high dose of INH to MDR patients might not be effective in Vietnamese population. No studies have as yet addressed the question of which host and pathogen characteristics are important in determining the benefit of high-dose INH.

There are a number of reports demonstrating differential virulence properties of different *M.tuberculosis* strains, both *in vitro* and *in vivo* such as CDC1551 outbreak strain from the USA (Tsenova, Ellison et al. 2005). Epidemiological studies have also shown a relationship between *M.tuberculosis* lineages and drug resistance patterns. In chapter 4, a strong association of Beijing lineage with a high MIC to INH was shown which was not accounted for solely by the mutation conferring resistance. This association may give INH resistant Beijing strains a selective advantage under programmatic treatment conditions. A report in 2003 suggested the Beijing lineage had a 'hypermutator' phenotype resulting from mutations in DNA repair genes (*mutT2, mutT4* (Ebrahimi-Rad, Bifani et al. 2003). However, the evidence for this theory remains weak and it is likely these mutations are simply phylogenetic mutations in Beijing lineage. The underlying mechanism of increase resistance in Beijing lineage may be an inherent resistance property or may be the result of the ability to acquire compensatory mutations more rapidly to mitigate the fitness cost of resistance mechanisms. It also remains unclear if this is an attribute of Beijing lineage strains, or a sub-group. The result in chapter 4 suggests that further study should be done to reveal the mechanism behind the increase resistance level of Beijing genotype. Whole genome sequencing of a diverse collection of Beijing lineage isolates now underway will help to address these questions. The study in chapter 5 showed that INH resistance has a strong impact on outcome from HIV-associated TBM. While the high mortality in all forms of TBM indicates that current treatment regimens are sub-optimal, this study highlights the increased risk in patients with non-MDR strains that are resistant to INH. Notably, the results of this study showed that

the adjusted hazard ratio for mortality of patients infected with INH resistant *M.tuberculosis* was 1.78 [95% CI 1.18-2.66], p=0.005 and that there was a time-dependent effect of INH resistance with an adjusted hazard ratio of 1.45 [95% CI 0.87-2.40], p= 0.15 for the first three months and 5.05 [95% CI 2.23-11.44], p=0.0001 thereafter, in comparison with patients infected with fully susceptible of STR mono-resistant strains.

The data clearly show that treatment with the standard first line regimen is inadequate in these patients and alternative regimens must be evaluated. While this study focused on HIV-associated TBM, a group with very high mortality, a retrospective study from the United States has also shown excess mortality in TBM patients with INH resistant, non-MDR TB. It is known from recent meta-analyses that treatment outcomes are also worse in pulmonary TB patients with INH resistant, non-MDR strains when treated with standard regimens (Lew, Pai et al. 2008; Cattamanchi, Dantes et al. 2009; Matthys, Rigouts et al. 2009). With the report that 25% of new TB cases in HCM city have INH resistant pathogen (Quy, Buu et al. 2006), this high prevalence of INH resistant *M. tuberculosis* is likely to have an impact on the success of NTP in Vietnam. Further study should investigate the impact of INH resistance in other groups of patients, especially pulmonary TB and determine which patients are at highest risk of treatment failure to target interventions.

IPT, which is used to treat latent TB, especially in HIV patients, could also have an impact on INH resistance prevalence. IPT is being scaled up in many regions of the world, including Viet Nam. Due to the need for the use of INH for a long period in the prevention therapy, the initial TB infection rate could be reduced, but the

long term consequences for the positive selection of INH resistant TB are not clear. Recently life-long IPT has been advocated in broader populations, including prisoners, miners and others who live in high TB transmission settings (WHO 2011). This could make the control of INH resistance more difficult. A RCT in South Africa is examining the impact of IPT on TB amongst gold miners (Registration number ISRCTN63327174) (Fielding, Grant et al. 2011). The result of this and similar studies help us understand the risk and benefits of IPT. Preliminary results reported in 2012 showed that IPT in gold miners failed to have a long-term impact on TB incidence; with a substantial reduction in TB incidence observed only during the therapy. (Churchyard, Fielding et al. 2012).

It is likely that other factors besides resistance also play a role in outcome from HIV-associated TBM. The study in chapter 5 showed that pathogen lineage is also a determinant of outcome, but the mechanism again remains unelucidated. Many patients with HIV-associated TBM in HCM city also have other viral infections in the CNS (Torok, Chau et al. 2008) and we have not investigated the impact of these on outcome here. The prevalence of HBV infection in Vietnamese population is high with around 8.8-16.4% patients positive with HBsAg or in active HBV form (Nguyen, McLaws et al. 2007; Duong, Nguyen et al. 2009). This study has demonstrated that multiple pathogen factors are independent and significant determinants of mortality in HIV-associated TBM and highlights the urgent need for RCT to treat drug-resistant forms of TBM.

In chapter 6, I investigated the association of NAT2 genotype with NAT2 phenotype and the prevalence of fast acetylators in the Vietnamese Kinh population.

The strong correlation result between genotype and phenotype of NAT2 in Vietnamese Kinh healthy volunteers paves the way for further studies on the effect of acetylator status on the outcome of TB patients treated with INH. The study showed the majority of Vietnamese Kinh population are fast acetylators, which may reduce the serum concentrations of INH achieved with standard dosage. The result of this study implied that under DOTS therapy, many Vietnamese might not reach the appropriate INH concentration to achieve optimal EBA. High dose of INH might improve the efficacy of the drug in this particular population; however increased toxicity may be a problem, particularly in slow-acetylator patients with pre-existing liver damage or hepatotropic viral co-infections (Van Deun, Maug et al. 2010). As a result, the development and application of a NAT2 PCR-RFLP test presented here provides a useful tool that makes further clinical trials to investigate targeted application of INH dosage dependent on NAT2 genotype become feasible. Further studies should focus on the effect of acetylator status on serum INH level, EBA and treatment outcome and determine if targeted therapy which adjusts INH dosage dependent on acetylator status is beneficial.

Overall, the studies presented in this thesis underline the importance of INH resistance in TB therapy and highlight the need for further research developing diagnostic tools and optimizing treatment. To date, there is no standardized treatment for INH resistant TB and different countries have different treatment recommendations. The mechanisms of INH resistance are still not completely understood. Over 10% of INH resistant *M.tuberculosis* strains do not contain any

identified mutations in either *katG* or *inhA* genes; indicating there are other unknown drug resistance mechanism which remain to be identified. Despite these significant outstanding questions, research on drug resistant *M.tuberculosis* continues to focus on MDR TB due to the fact that it is more dangerous form of the disease. However, INH resistance is the initial step which fuels the development to MDR TB or further XDR TB. Trying to understand and to control the initial development and reduce transmission of INH resistance will 'turn off the tap' on MDR TB and reduce further drug resistance amplification to MDR and XDR TB. To achieve this, further studies should include:

- RCTs to optimize treatment of INH resistant *M.tuberculosis* which remains susceptible to RIF.
- Fully characterize all mechanisms of INH resistance in *M.tuberculosis* through whole genome sequencing of larger globally representative collections and in vitro functional studies.
- Establish the long-term impacts of large-scale IPT on the development of INH resistance.
- Conduct RCTs to determine if targeted therapy dependent on NAT2 acetylator status is effective.

References

Abebe, F. and G. Bjune (2006). "The emergence of Beijing family genotypes of *Mycobacterium tuberculosis* and low-level protection by bacille Calmette-Guerin (BCG) vaccines: is there a link?" Clin Exp Immunol **145**(3): 389-397.

Albert, H., F. Bwanga, et al. (2010). "Rapid screening of MDR-TB using molecular Line Probe Assay is feasible in Uganda." BMC Infect Dis **10**: 41.

Ambrose, P. G., S. M. Bhavnani, et al. (2007). "Pharmacokinetics-pharmacodynamics of antimicrobial therapy: it's not just for mice anymore." Clin Infect Dis **44**(1): 79-86.

Amuha, M. G., P. Kutyabami, et al. (2009). "Non-adherence to anti-TB drugs among TB/HIV co-infected patients in Mbarara Hospital Uganda: prevalence and associated factors." Afr Health Sci **9 Suppl 1**: S8-15.

Andersen, P. and T. M. Doherty (2005). "The success and failure of BCG - implications for a novel tuberculosis vaccine." Nat Rev Microbiol **3**(8): 656-662.

Argyrou, A., M. W. Vetting, et al. (2006). "*Mycobacterium tuberculosis* dihydrofolate reductase is a target for isoniazid." Nat Struct Mol Biol **13**(5): 408-413.

Baker, L. V., T. J. Brown, et al. (2005). "Molecular analysis of isoniazid-resistant *Mycobacterium tuberculosis* isolates from England and Wales reveals the phylogenetic significance of the ahpC -46A polymorphism." Antimicrob Agents Chemother **49**(4): 1455-1464.

Balganesh, M., N. Dinesh, et al. (2012). "Efflux Pumps of *Mycobacterium tuberculosis* play a significant role in anti-tuberculosis activity of potential drug candidates." Antimicrob Agents Chemother. **56**(5): 2643-2651

Baltussen, R., K. Floyd, et al. (2005). "Cost effectiveness analysis of strategies for tuberculosis control in developing countries." BMJ **331**(7529): 1364.

Banerjee, A., E. Dubnau, et al. (1994). "*inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*." Science **263**(5144): 227-230.

Banerjee, A., M. Sugantino, et al. (1998). "The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance." Microbiology **144 (Pt 10)**: 2697-2704.

Baronti, A. and N. Lukinovich (1968). "A pilot trial of rifampicin in tuberculosis." Tubercle **49**(2): 180-186.

Berenguer, J., S. Moreno, et al. (1992). "Tuberculous meningitis in patients infected with the human immunodeficiency virus." N Engl J Med **326**(10): 668-672.

Bergval, I. L., A. R. Schuitema, et al. (2009). "Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance." J Antimicrob Chemother **64**(3): 515-523.

Bhigjee, A. I., R. Padayachee, et al. (2007). "Diagnosis of tuberculous meningitis: clinical and laboratory parameters." Int J Infect Dis **11**(4): 348-354.

Blower, S. M. and T. Chou (2004). "Modeling the emergence of the 'hot zones': tuberculosis and the amplification dynamics of drug resistance." Nat Med **10**(10): 1111-1116.

Bobadilla-del-Valle, M., A. Ponce-de-Leon, et al. (2001). "*rpoB* Gene Mutations in Rifampin-Resistant *Mycobacterium tuberculosis* Identified by Polymerase Chain Reaction Single-Stranded Conformational Polymorphism." Emerging Infectious Diseases **7**(6): 1010-1013.

Bobrowitz, I. D. (1971). "Ethambutol compared to streptomycin in original treatment of advanced pulmonary tuberculosis." Chest **60**(1): 14-21.

Boehme, C. C., P. Nabeta, et al. (2010). "Rapid molecular detection of tuberculosis and rifampin resistance." N Engl J Med **363**(11): 1005-1015.

Burugina Nagaraja, S., S. Satyanarayana, et al. (2011). "How do patients who fail first-line TB treatment but who are not placed on an MDR-TB regimen fare in South India?" PLoS One **6**(10): e25698.

Buu, T. N., M. N. Huyen, et al. (2009). "*Mycobacterium tuberculosis* genotype and case notification rates, rural Vietnam, 2003-2006." Emerg Infect Dis **15**(10): 1570-1577.

Buu, T. N., M. N. Huyen, et al. (2009). "The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam." Int J Tuberc Lung Dis **13**(7): 900-906.

Byrd, T. F. and L. E. Davis (2007). "Multidrug-resistant tuberculous meningitis." Curr Neurol Neurosci Rep **7**(6): 470-475.

Cain, K. P., K. D. McCarthy, et al. (2010). "An algorithm for tuberculosis screening and diagnosis in people with HIV." N Engl J Med **362**(8): 707-716.

Caminero, J. A. (2006). "Treatment of multidrug-resistant tuberculosis: evidence and controversies." Int J Tuberc Lung Dis **10**(8): 829-837.

Canetti, G., W. Fox, et al. (1969). "Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes." Bull World Health Organ **41**(1): 21-43.

Canetti, G., M. Le Lirzin, et al. (1968). "Some comparative aspects of rifampicin and isoniazid." Tubercle **49**(4): 367-376.

Carr, R. E. and P. Henkind (1962). "Ocular manifestations of ethambutol, Toxic amblyopia after administration of an experimental antituberculous drug." Arch Ophthalmol **67**: 566-571.

Cattamanchi, A., R. B. Dantes, et al. (2009). "Clinical characteristics and treatment outcomes of patients with isoniazid-monoresistant tuberculosis." Clin Infect Dis **48**(2): 179-185.

Cattamanchi, A., J. L. Davis, et al. (2010). "Does bleach processing increase the accuracy of sputum smear microscopy for diagnosing pulmonary tuberculosis?" J Clin Microbiol **48**(7): 2433-2439.

Cavaco, I., S. Asimus, et al. (2007). "The Vietnamese Kinh population harbors particular N-acetyltransferase 2 allele frequencies." Clin Chem **53**(11): 1977-1979.

Cavusoglu, C., S. Hilmioglu, et al. (2002). "Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay." J Clin Microbiol **40**(12): 4435-4438.

Cavusoglu, C., A. Turhan, et al. (2006). "Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates." J Clin Microbiol **44**(7): 2338-2342.

Caws, M., P. M. Duy, et al. (2006). "Mutations prevalent among rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam." J Clin Microbiol **44**(7): 2333-2337.

Caws, M., G. Thwaites, et al. (2008). "The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*." PLoS Pathog **4**(3): e1000034.

Caws, M., G. Thwaites, et al. (2006). "Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with human immunodeficiency virus infection and multidrug resistance in cases of tuberculous meningitis." J Clin Microbiol **44**(11): 3934-3939.

CDC (2003). "Treatment of tuberculosis, American Thoracic Society, CDC and Infectious Diseases Society of America." MMWR **52** (RR-11): 1-77.

CDC (2006). "Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs--worldwide, 2000-2004." MMWR **55**(11): 301-305.

Chan, J. and S. H. E. Kaufmann (1994). "Immune Mechanisms of Protection. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 389-417.

Chauhan, L. S. (2007). "RNTCP 2007: looking ahead to future challenges." J Indian Med Assoc **105**(4): 192, 194, 196.

Chiang, C. Y., J. J. Lee, et al. (2009). "Tuberculosis-related deaths without treatment." Int J Tuberc Lung Dis **13**(12): 1563-1565.

Cho, H. J., W. J. Koh, et al. (2007). "Genetic polymorphisms of NAT2 and CYP2E1 associated with antituberculosis drug-induced hepatotoxicity in Korean patients with pulmonary tuberculosis." Tuberculosis (Edinb) **87**(6): 551-556.

Choi, J. C., S. Y. Lim, et al. (2007). "Drug resistance rates of *Mycobacterium tuberculosis* at a private referral center in Korea." J Korean Med Sci **22**(4): 677-681.

Churchyard, G., K. Fielding, et al. (2012). Community-wide Isoniazid Preventive Therapy Does Not Improve TB Control among Gold Miners: The Thibela TB Study, South Africa. 19th Conference on Retroviruses and Opportunistic Infections (CROI 2012). Seattle, March 5-8, 2012. Abstract 150aLB.

Coban, A. Y., A. Birinci, et al. (2004). "Drug Susceptibility Testing of *Mycobacterium tuberculosis* by the Broth Microdilution Method with 7H9 Broth." Mem Inst Oswaldo Cruz **99**(1): 111-113.

Cohen, T. and M. Murray (2004). "Modeling epidemics of multidrug-resistant M. tuberculosis of heterogeneous fitness." Nat Med **10**(10): 1117-1121.

Comas, I., S. Borrell, et al. (2011). "Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes." Nat Genet **44**(1): 106-110.

Comas, I., S. Homolka, et al. (2009). "Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies." PLoS One **4**(11): e7815.

Conte, J. E., Jr., J. A. Golden, et al. (2002). "Effects of gender, AIDS, and acetylator status on intrapulmonary concentrations of isoniazid." Antimicrob Agents Chemother **46**(8): 2358-2364.

Corbett, E. L., G. J. Churchyard, et al. (1999). "The impact of HIV infection on *Mycobacterium kansasii* disease in South African gold miners." Am J Respir Crit Care Med **160**(1): 10-14.

Cordice, J. W., Jr., L. M. Hill, et al. (1953). "Use of pyrazinamide (aldinamide) in the treatment of tuberculous lymphadenopathy and draining sinuses; a preliminary report." J Natl Med Assoc **45**(2): 87-98.

Cox, H. S., T. Kubica, et al. (2005). "The Beijing genotype and drug resistant tuberculosis in the Aral Sea region of Central Asia." Respir Res **6**: 134.

Crubezy, E., B. Ludes, et al. (1998). "Identification of Mycobacterium DNA in an Egyptian Pott's disease of 5,400 years old." C R Acad Sci III **321**(11): 941-951.

Cruciani, M., C. Scarparo, et al. (2004). "Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria." J Clin Microbiol **42**(5): 2321-2325.

D'Arcy Hart, P. (1999). "A change in scientific approach: from alternation to randomised allocation in clinical trials in the 1940s." Bmj **319**(7209): 572-573.

Da Silva, P. E., A. Von Groll, et al. (2011). "Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*." FEMS Immunol Med Microbiol **63**(1): 1-9.

Dai, Y., S. Leng, et al. (2009). "Effects of genetic polymorphisms of N-Acetyltransferase on trichloroethylene-induced hypersensitivity dermatitis among exposed workers." Ind Health **47**(5): 479-486.

Dalla Costa, E. R., M. O. Ribeiro, et al. (2009). "Correlations of mutations in *katG*, *oxyR-ahpC* and *inhA* genes and *in vitro* susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America." BMC Microbiol **9**: 39.

Daniel, T. M., J. H. Bates, et al. (1994). "History of Tuberculosis. Tuberculosis: pathogenesis, protection, and control." B. R. Bloom, American Society for Microbiology: 47-84

Daniel, V. S. and T. M. Daniel (1999). "Old Testament biblical references to tuberculosis." Clin Infect Dis **29**(6): 1557-1558.

Dannenberg, A., Jr., et al. (1994). "Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses - dual mechanisms that control bacillary multiplication. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 459-484.

De Beenhouwer, H., Z. Lhiang, et al. (1995). "Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay." Tuber Lung Dis **76**(5): 425-430.

De Jong, B. C., M. Antonio, et al. (2009). "Use of spoligotyping and large sequence polymorphisms to study the population structure of the *Mycobacterium*

tuberculosis complex in a cohort study of consecutive smear-positive tuberculosis cases in The Gambia." J Clin Microbiol **47**(4): 994-1001.

De Jong, B. C., P. C. Hill, et al. (2008). "Progression to active tuberculosis, but not transmission, varies by *Mycobacterium tuberculosis* lineage in The Gambia." J Infect Dis **198**(7): 1037-1043.

Deretic, V., J. Song, et al. (1997). "Loss of *oxyR* in *Mycobacterium tuberculosis*." Trends Microbiol **5**(9): 367-372.

Deshpande, P. S., R. S. Kashyap, et al. (2007). "Evaluation of the *IS6110* PCR assay for the rapid diagnosis of tuberculous meningitis." Cerebrospinal Fluid Res **4**: 10.

Dias, M. V., I. B. Vasconcelos, et al. (2007). "Crystallographic studies on the binding of isonicotinyl-NAD adduct to wild-type and isoniazid resistant 2-trans-enoyl-ACP (CoA) reductase from *Mycobacterium tuberculosis*." J Struct Biol **159**(3): 369-380.

Donald, P. R. (2010). "Cerebrospinal fluid concentrations of antituberculosis agents in adults and children." Tuberculosis (Edinb) **90**(5): 279-292.

Donald, P. R., D. P. Parkin, et al. (2007). "The influence of dose and N-acetyltransferase-2 (NAT2) genotype and phenotype on the pharmacokinetics and pharmacodynamics of isoniazid." Eur J Clin Pharmacol **63**(7): 633-639.

Donald, P. R. and J. F. Schoeman (2004). "Tuberculous meningitis." N Engl J Med **351**(17): 1719-1720.

Drobniewski, F. A., S. Hoffner, et al. (2006). "Recommended standards for modern tuberculosis laboratory services in Europe." Eur Respir J **28**(5): 903-909.

Dunner, E., W. B. Brown, et al. (1949). "The effect of streptomycin with para-amino salicylic acid on the emergence of resistant strains of tubercle bacilli." Dis Chest **16**(6): 661-666.

Duong, T. H., P. H. Nguyen, et al. (2009). "Risk factors for hepatitis B infection in rural Vietnam." Asian Pac J Cancer Prev **10**(1): 97-102.

Ebrahimi-Rad, M., P. Bifani, et al. (2003). "Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family." Emerg Infect Dis **9**(7): 838-845.

Ellard, G. A., M. J. Humphries, et al. (1993). "Cerebrospinal fluid drug concentrations and the treatment of tuberculous meningitis." Am Rev Respir Dis **148**(3): 650-655.

Ewer, K., J. Deeks, et al. (2003). "Comparison of T-cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak." Lancet **361**(9364): 1168-1173.

Farhat, M., C. Greenaway, et al. (2006). "False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria?" Int J Tuberc Lung Dis **10**(11): 1192-1204.

Faustini, A., A. J. Hall, et al. (2005). "Tuberculosis treatment outcomes in Europe: a systematic review." Eur Respir J **26**(3): 503-510.

Fielding, K. L., A. D. Grant, et al. (2011). "Thibela TB: design and methods of a cluster randomised trial of the effect of community-wide isoniazid preventive therapy on tuberculosis amongst gold miners in South Africa." Contemp Clin Trials **32**(3): 382-392.

Find Diagnostics. accessed 04/18, 2010, www.finddiagnositics.org
Flores, L., T. Van, et al. (2007). "Large sequence polymorphisms classify *Mycobacterium tuberculosis* strains with ancestral spoligotyping patterns." J Clin Microbiol **45**(10): 3393-3395.
Gagneux, S., K. DeRiemer, et al. (2006). "Variable host-pathogen compatibility in *Mycobacterium tuberculosis*." Proc Natl Acad Sci U S A **103**(8): 2869-2873.
Gagneux, S. and P. M. Small (2007). "Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development." Lancet Infect Dis **7**(5): 328-337.
Gandhi, N. R., A. Moll, et al. (2006). "Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa." Lancet **368**(9547): 1575-1580.
Garcia-Martin, E. (2008). "Interethnic and intraethnic variability of NAT2 single nucleotide polymorphisms." Curr Drug Metab **9**(6): 487-497.
Garcia de Viedma, D., M. del Sol Diaz Infantes, et al. (2002). "New Real-Time PCR Able To Detect in a Single Tube Multiple Rifampin Resistance Mutations and High-Level Isoniazid Resistance Mutations in *Mycobacterium tuberculosis*." J. Clin. Microbiol. **40**(3): 988-995.
Garg, R. K. (1999). "Tuberculosis of the central nervous system." Postgrad Med J **75**(881): 133-140.
Garnier, T., K. Eiglmeier, et al. (2003). "The complete genome sequence of *Mycobacterium bovis*." Proc Natl Acad Sci U S A **100**(13): 7877-7882.
Gillespie, S. H. (2002). "Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective." Antimicrob Agents Chemother **46**(2): 267-274.
Ginsburg, A. S., S. C. Woolwine, et al. (2003). "The Rapid Development of Fluoroquinolone Resistance in *M. tuberculosis*." The New England Journal of Medicine **349**: 1977-1978.
Girgis, N. I., Z. Farid, et al. (1990). "The use of the bromine partition test in the diagnosis and prognosis of tuberculous meningitis." East Afr Med J **67**(6): 404-406.
Glynn, J. R., M. D. Yates, et al. (2004). "DNA fingerprint changes in tuberculosis: reinfection, evolution, or laboratory error?" J Infect Dis **190**(6): 1158-1166.
Greco, S., E. Girardi, et al. (2006). "Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis." Thorax **61**(9): 783-790.
Greco, S., M. Rulli, et al. (2009). "Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression." J Clin Microbiol **47**(3): 569-576.
Gross National Product per capital accessed 10/10, 2010, http://www.studentsoftheworld.info/rank/PNBH2.html
Guo, H., Q. Seet, et al. (2006). "Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA." J Med Microbiol **55**(Pt 11): 1527-1531.
Gupta, R., A. Irwin, et al. (2004). "Scaling-up treatment for HIV/AIDS: lessons learned from multidrug-resistant tuberculosis." Lancet **363**(9405): 320-324.

Ha, D. T., N. T. Lan, et al. (2009). "Microscopic observation drug susceptibility assay (MODS) for early diagnosis of tuberculosis in children." PLoS One **4**(12): e8341.

Hazbon, M. H., M. Brimacombe, et al. (2006). "Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*." Antimicrob Agents Chemother **50**(8): 2640-2649.

Heemskerk, D., J. Day, et al. (2011). "Intensified treatment with high dose rifampicin and levofloxacin compared to standard treatment for adult patients with tuberculous meningitis (TBM-IT): protocol for a randomized controlled trial." Trials **12**: 25.

Heep, M., B. Brandstatter, et al. (2001). "Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates." J Clin Microbiol **39**(1): 107-110.

Heifets, L. B. and R. C. Good (1994). "Current Laboratory Method for the Diagnosis of Tuberculosis. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 85-110.

Helb, D., M. Jones, et al. (2010). "Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology." J Clin Microbiol **48**(1): 229-237.

Herrera-Leon, L., T. Molina, et al. (2005). "New multiplex PCR for rapid detection of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates." Antimicrob Agents Chemother **49**(1): 144-147.

Higuchi, N., N. Tahara, et al. (2007). "NAT2 6A, a haplotype of the N-acetyltransferase 2 gene, is an important biomarker for risk of anti-tuberculosis drug-induced hepatotoxicity in Japanese patients with tuberculosis." World J Gastroenterol **13**(45): 6003-6008.

Hillemann, D., S. Rusch-Gerdes, et al. (2007). "Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens." J Clin Microbiol **45**(8): 2635-2640.

Hippocrates' Aphorisms. accessed 06/28, 2010, http://classics.mit.edu/Hippocrates/aphorisms.html

Hobby, G. L., A. P. Holman, et al. (1973). "Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis." Antimicrob Agents Chemother **4**(2): 94-104.

Honore-Bouakline, S., J. P. Vincensini, et al. (2003). "Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction." J Clin Microbiol **41**(6): 2323-2329.

Hopewell, P. C. (1994). "Overview of clinical tuberculosis. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 25-46.

Hosoglu, S., M. F. Geyik, et al. (2002). "Predictors of outcome in patients with tuberculous meningitis." Int J Tuberc Lung Dis **6**(1): 64-70.

Hu, Y., S. Hoffner, et al. (2010). "Extensive transmission of isoniazid resistant *M. tuberculosis* and its association with increased multidrug-resistant TB in two

rural counties of eastern China: a molecular epidemiological study." BMC Infect Dis **10**: 43.

Hu, Y., X. Ma, et al. (2011). "A major subgroup of Beijing family *Mycobacterium tuberculosis* is associated with multidrug resistance and increased transmissibility." Epidemiol Infect: **139**(1):130-8.

Jayaram, R., R. K. Shandil, et al. (2004). "Isoniazid pharmacokinetics-pharmacodynamics in an aerosol infection model of tuberculosis." Antimicrob Agents Chemother **48**(8): 2951-2957.

Jindani, A., A. J. Nunn, et al. (2004). "Two 8-month regimens of chemotherapy for treatment of newly diagnosed pulmonary tuberculosis: international multicentre randomised trial." Lancet **364**(9441): 1244-1251.

Johansen, I. S., B. Lundgren, et al. (2003). "Direct Detection of Multidrug-Resistant *Mycobacterium tuberculosis* in Clinical Specimens in Low- and High-Incidence Countries by Line Probe Assay." J. Clin. Microbiol. **41**(9): 4454-4456.

Johnson, R., R. Warren, et al. (2006). "An outbreak of drug-resistant tuberculosis caused by a Beijing strain in the western Cape, South Africa." Int J Tuberc Lung Dis **10**(12): 1412-1414.

Johnston, J. C., N. C. Shahidi, et al. (2009). "Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis." PLoS One **4**(9): e6914.

Kadhiravan, T. and S. Deepanjali (2010). "Role of corticosteroids in the treatment of tuberculosis: an evidence-based update." Indian J Chest Dis Allied Sci **52**(3): 153-158.

Kamerbeek, J., L. Schouls, et al. (1997). "Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology." J Clin Microbiol **35**(4): 907-914.

Kaplan, J. E., C. Benson, et al. (2009). "Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America." MMWR Recomm Rep **58**(RR-4): 1-207; quiz CE201-204.

Kapur, V., L. L. Li, et al. (1994). "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 **32**(4): 1095-1098.

Karagoz, T., O. Yazicioglu Mocin, et al. (2009). "The treatment results of patients with multidrug resistant tuberculosis and factors affecting treatment outcome." Tuberk Toraks **57**(4): 383-392.

Katiyar, S. K., S. Bihari, et al. (2008). "A randomised controlled trial of high-dose isoniazid adjuvant therapy for multidrug-resistant tuberculosis." Int J Tuberc Lung Dis **12**(2): 139-145.

Katrak, S. M., P. K. Shembalkar, et al. (2000). "The clinical, radiological and pathological profile of tuberculous meningitis in patients with and without human immunodeficiency virus infection." J Neurol Sci **181**(1-2): 118-126.

Kelley, C., D. Rouse, et al. (1997). "Analysis of *ahpC* gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*." Antimicrob. Agents Chemother. **41**(9): 2057-2058.

Keshavjee, S., I. Y. Gelmanova, et al. (2008). "Treatment of extensively drug-resistant tuberculosis in Tomsk, Russia: a retrospective cohort study." Lancet **372**(9647): 1403-1409.

Kinzig-Schippers, M., D. Tomalik-Scharte, et al. (2005). "Should we use N-acetyltransferase type 2 genotyping to personalize isoniazid doses?" Antimicrob Agents Chemother **49**(5): 1733-1738.

Kita, T., Y. Tanigawara, et al. (2001). "N-Acetyltransferase2 genotype correlated with isoniazid acetylation in Japanese tuberculous patients." Biol Pharm Bull **24**(5): 544-549.

Kong, Y., M. D. Cave, et al. (2007). "Association between *Mycobacterium tuberculosis* Beijing/W lineage strain infection and extrathoracic tuberculosis: Insights from epidemiologic and clinical characterization of the three principal genetic groups of *M. tuberculosis* clinical isolates." J Clin Microbiol **45**(2): 409-414.

Kremer, K., J. R. Glynn, et al. (2004). "Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers." J Clin Microbiol **42**(9): 4040-4049.

Krishnan, N., W. Malaga, et al. (2011). "*Mycobacterium tuberculosis* lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles." PLoS One **6**(9): e23870.

Kukongviriyapan, V., A. Prawan, et al. (2003). "Arylamine N-acetyltransferase-2 genotypes in the Thai population." Br J Clin Pharmacol **55**(3): 278-281.

Kuznetsov, I. B., M. McDuffie, et al. (2009). "A web server for inferring the human N-acetyltransferase-2 (NAT2) enzymatic phenotype from NAT2 genotype." Bioinformatics **25**(9): 1185-1186.

Lancet_report (1973). "Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Second report." Lancet **1**(7816): 1331-1338.

Larsen, M. H., C. Vilcheze, et al. (2002). "Overexpression of *inhA*, but not *kasA*, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis*." Mol Microbiol **46**(2): 453-466.

Lavender, C., M. Globan, et al. (2005). "Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* isolates collected in Australia." Antimicrob Agents Chemother **49**(10): 4068-4074.

Lee, A. S. G., I. H. K. Lim, et al. (1999). "Contribution of *kasA* Analysis to Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* in Singapore." Antimicrob. Agents Chemother. **43**(8): 2087-2089.

Lee, H., S. Cho, et al. (1998). "Molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* isolated from Korea by polymerase chain reaction-single strand conformation polymorphism sequence analysis." Int J Tuberc Lung Dis. **2**(7): 585-589.

Lei, B., C. J. Wei, et al. (2000). "Action mechanism of antitubercular isoniazid. Activation by *Mycobacterium tuberculosis* KatG, isolation, and characterization of inha inhibitor." J Biol Chem **275**(4): 2520-2526.

Lew, W., M. Pai, et al. (2008). "Initial drug resistance and tuberculosis treatment outcomes: systematic review and meta-analysis." Ann Intern Med **149**(2): 123-134.

Lienhardt, C., S. V. Cook, et al. (2011). "Efficacy and safety of a 4-drug fixed-dose combination regimen compared with separate drugs for treatment of pulmonary tuberculosis: the Study C randomized controlled trial." Jama **305**(14): 1415-1423.

Lipin, M. Y., V. N. Stepanshina, et al. (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**(6): 620-626.

Loeliger, A., A. B. Suthar, et al. (2012). "Protease inhibitor-containing antiretroviral treatment and tuberculosis: can rifabutin fill the breach?" Int J Tuberc Lung Dis **16**(1): 6-15.

Lonnroth, K., L. M. Thuong, et al. (2003). "Private tuberculosis care provision associated with poor treatment outcome: comparative study of a semi-private lung clinic and the NTP in two urban districts in Ho Chi Minh City, Vietnam. National Tuberculosis Programme." Int J Tuberc Lung Dis **7**(2): 165-171.

Ma, Z., C. Lienhardt, et al. (2010). "Global tuberculosis drug development pipeline: the need and the reality." Lancet **375**(9731): 2100-2109.

Makinen, J., H. J. Marttila, et al. (2006). "Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*." J Clin Microbiol **44**(2): 350-352.

Malessa, R., H. C. Diener, et al. (1994). "Successful treatment of meningoencephalitis caused by *Mycobacterium avium intracellulare* in AIDS." Clin Investig **72**(11): 850-852.

Marais, S., G. Thwaites, et al. (2010). "Tuberculous meningitis: a uniform case definition for use in clinical research." Lancet Infect Dis **10**(11): 803-812.

Marttila, H., H. Soini, et al. (1996). "*katG* mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates recovered from Finnish patients." Antimicrob. Agents Chemother. **40**(9): 2187-2189.

Matthys, F., L. Rigouts, et al. (2009). "Outcomes after chemotherapy with WHO category II regimen in a population with high prevalence of drug resistant tuberculosis." PLoS One **4**(11): e7954.

McDermott, W. (1948). "The Significance of the Finding of Tubercle Bacilli Resistant to Streptomycin *in vitro* in the Anti-Microbial Therapy of Tuberculosis." Bull N Y Acad Med **24**(2): 131.

McIlleron, H., M. Willemse, et al. (2009). "Isoniazid plasma concentrations in a cohort of South African children with tuberculosis: implications for international pediatric dosing guidelines." Clin Infect Dis **48**(11): 1547-1553.

Menon, P. R., R. Lodha, et al. (2011). "Intermittent or daily short course chemotherapy for tuberculosis in children: meta-analysis of randomized controlled trials." Indian Pediatr **47**(1): 67-73.

Menzies, D., A. Benedetti, et al. (2009). "Effect of duration and intermittency of rifampin on tuberculosis treatment outcomes: a systematic review and meta-analysis." PLoS Med **6**(9): e1000146.

Merid, Y., M. A. Yassin, et al. (2009). "Validation of bleach-treated smears for the diagnosis of pulmonary tuberculosis." Int J Tuberc Lung Dis **13**(1): 136-141.

Mitchell, R. S. (1955). "Fatal toxic encephalitis occurring during iproniazid therapy in pulmonary tuberculosis." Ann Intern Med **42**(2): 417-424.

Mitchison, D. A. (2005). "The diagnosis and therapy of tuberculosis during the past 100 years." Am J Respir Crit Care Med **171**(7): 699-706.

Mitnick, C. D., S. S. Shin, et al. (2008). "Comprehensive treatment of extensively drug-resistant tuberculosis." N Engl J Med **359**(6): 563-574.

Mokrousov, I., T. Otten, et al. (2002). "Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* Strains by a Multiplex Allele-Specific PCR Assay Targeting *katG* Codon 315 Variation." J. Clin. Microbiol. **40**(7): 2509-2512.

Monedero, I. and J. A. Caminero (2011). "Evidence for promoting fixed-dose combination drugs in tuberculosis treatment and control: a review." Int J Tuberc Lung Dis **15**(4): 433-439.

Moore, D. A., C. A. Evans, et al. (2006). "Microscopic-observation drug-susceptibility assay for the diagnosis of TB." N Engl J Med **355**(15): 1539-1550.

Moore, D. A., D. Mendoza, et al. (2004). "Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings." J Clin Microbiol **42**(10): 4432-4437.

Morgan, M., S. Kalantri, et al. (2005). "A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis." BMC Infect Dis **5**: 62.

Murthy, J. M. (2010). "Tuberculous meningitis: The challenges." Neurol India **58**(5): 716-722.

Musser, J. M. (1995). "Antimicrobial Agent Resistance in Mycobacteria: Molecular Genietic Insights." Clinical Microbiology Reviews **8**(4): 496-514.

Nahid, P., E. E. Bliven, et al. (2010). "Influence of *M. tuberculosis* lineage variability within a clinical trial for pulmonary tuberculosis." PLoS One **5**(5): e10753.

Nau, R., F. Sorgel, et al. (2010). "Penetration of drugs through the blood-cerebrospinal fluid/blood-brain barrier for treatment of central nervous system infections." Clin Microbiol Rev **23**(4): 858-883.

Nguyen, V. T., M. L. McLaws, et al. (2007). "Highly endemic hepatitis B infection in rural Vietnam." J Gastroenterol Hepatol **22**(12): 2093-2100.

NICE Guidelines. Tuberculosis: Clinical diagnosis and management of tuberculosis, and measures for its prevention and control. The National Institute for Health and Clinical Excellence, Issued: March 2011.

Niemann, S., R. Diel, et al. (2010). "*Mycobacterium tuberculosis* Beijing Lineage favours the spread of multidrug resistant tuberculosis in the Republic of Georgia." J Clin Microbiol. **48**(10):3544-50

Nikolayevsky, V., T. Brown, et al. (2004). "Detection of Mutations Associated with Isoniazid and Rifampin Resistance in *Mycobacterium tuberculosis* Isolates from Samara Region, Russian Federation." J. Clin. Microbiol. **42**(10): 4498-4502.

Nitti, V. (1972). "Antituberculosis activity of rifampin. Report of studies performed and in progress (1966-1971)." Chest **61**(6): 589-598.

NTP of Vietnam (2008). National Tuberculosis Programme of Viet Nam: Guidelines for tuberculosis diagnosis, treatment and prevention: 23-84

Nuermberger, E. L., T. Yoshimatsu, et al. (2004). "Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis." Am J Respir Crit Care Med **169**(3): 421-426.

Nyeki, A., T. Buclin, et al. (2003). "NAT2 and CYP1A2 phenotyping with caffeine: head-to-head comparison of AFMU vs. AAMU in the urine metabolite ratios." Br J Clin Pharmacol **55**(1): 62-67.

Ohkado, A., L. Aguiman, et al. (2006). "Tuberculosis drug resistance and treatment outcomes under DOTS settings in large cities in the Philippines." Int J Tuberc Lung Dis **10**(3): 283-289.

Ohno, M., I. Yamaguchi, et al. (2000). "Slow N-acetyltransferase 2 genotype affects the incidence of isoniazid and rifampicin-induced hepatotoxicity." Int J Tuberc Lung Dis **4**(3): 256-261.

Pablos-Mendez, A., D. K. Gowda, et al. (2002). "Controlling multidrug-resistant tuberculosis and access to expensive drugs: a rational framework." Bull World Health Organ **80**(6): 489-495; discussion 495-500.

Pai, M., L. L. Flores, et al. (2003). "Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis." Lancet Infect Dis **3**(10): 633-643.

Pai, M., A. Ramsay, et al. (2008). "Evidence-based tuberculosis diagnosis." PLoS Med **5**(7): e156.

Parwati, I., R. van Crevel, et al. (2010). "Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains." Lancet Infect Dis **10**(2): 103-111.

Patin, E., L. B. Barreiro, et al. (2006). "Deciphering the ancient and complex evolutionary history of human arylamine N-acetyltransferase genes." Am J Hum Genet **78**(3): 423-436.

Patterson, R. J. and G. P. Youmans (1970). "Multiplication of *Mycobacterium tuberculosis* Within Normal and "Immune" Mouse Macrophages Cultivated With and Without Streptomycin." Infect Immun **1**(1): 30-40.

Portevin, D., S. Gagneux, et al. (2011). "Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages." PLoS Pathog **7**(3): e1001307.

Prasad, K. and M. B. Singh (2008). "Corticosteroids for managing tuberculous meningitis." Cochrane Database Syst Rev(1): CD002244.

Pym, A. S., B. Saint-Joanis, et al. (2002). "Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans." Infect Immun **70**(9): 4955-4960.

Quy, H. T., T. N. Buu, et al. (2006). "Drug resistance among smear-positive tuberculosis patients in Ho Chi Minh City, Vietnam." Int J Tuberc Lung Dis **10**(2): 160-166.

Quy, H. T., F. G. Cobelens, et al. (2006). "Treatment outcomes by drug resistance and HIV status among tuberculosis patients in Ho Chi Minh City, Vietnam." Int J Tuberc Lung Dis **10**(1): 45-51.

Quy, H. T., N. T. Lan, et al. (2003). "Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate?" Int J Tuberc Lung Dis **7**(7): 631-636.

Quy, H. T., K. Lonnroth, et al. (2003). "Treatment results among tuberculosis patients treated by private lung specialists involved in a public-private mix project in Vietnam." Int J Tuberc Lung Dis **7**(12): 1139-1146.

R Development Core Team (2010) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0. URL http://www.R-project.org [accessed July 21, 2011].

Radhakrishnan, V. V. and A. Mathai (1993). "Correlation between the isolation of *Mycobacterium tuberculosis* and estimation of mycobacterial antigen in cisternal, ventricular and lumbar cerebrospinal fluids of patients with tuberculous meningitis." Indian J Pathol Microbiol **36**(4): 341-347.

Raviglione, M. C., D. E. Snider, Jr., et al. (1995). "Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic." JAMA **273**(3): 220-226.

Resch, S. C., J. A. Salomon, et al. (2006). "Cost-effectiveness of treating multidrug-resistant tuberculosis." PLoS Med **3**(7): e241.

Rihs, H. P., A. John, et al. (2007). "Concordance between the deduced acetylation status generated by high-speed: real-time PCR based NAT2 genotyping of seven single nucleotide polymorphisms and human NAT2 phenotypes determined by a caffeine assay." Clin Chim Acta **376**(1-2): 240-243.

Rodrigues, L., J. A. Ainsa, et al. (2011). "Inhibition of drug efflux in mycobacteria with phenothiazines and other putative efflux inhibitors." Recent Pat Antiinfect Drug Discov **6**(2): 118-127.

Rouse, D. A., J. A. DeVito, et al. (1996). "Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance." Mol Microbiol **22**(3): 583-592.

Ruxrungtham, K., T. Brown, et al. (2004). "HIV/AIDS in Asia." Lancet **364**(9428): 69-82.

Rybak, M. J. (2006). "The pharmacokinetic and pharmacodynamic properties of vancomycin." Clin Infect Dis **42 Suppl 1**: S35-39.

Sabbagh, A., A. Langaney, et al. (2008). "Worldwide distribution of NAT2 diversity: implications for NAT2 evolutionary history." BMC Genet **9**: 21.

Sajduda, A., A. Brzostek, et al. (2004). "Molecular Characterization of Rifampin- and Isoniazid-Resistant *Mycobacterium tuberculosis* Strains Isolated in Poland." J. Clin. Microbiol. **42**(6): 2425-2431.

Salfinger, M., A. J. Crowle, et al. (1990). "Pyrazinamide and pyrazinoic acid activity against tubercle bacilli in cultured human macrophages and in the BACTEC system." J Infect Dis **162**(1): 201-207.

Samb, B., P. S. Sow, et al. (1999). "Risk factors for negative sputum acid-fast bacilli smears in pulmonary tuberculosis: results from Dakar, Senegal, a city with low HIV seroprevalence." Int J Tuberc Lung Dis **3**(4): 330-336.

Sandman, L., N. W. Schluger, et al. (1999). "Risk factors for rifampin-monoresistant tuberculosis: A case-control study." Am J Respir Crit Care Med **159**(2): 468-472.

Sarkar, S. and M. R. Suresh (2011). "An overview of tuberculosis chemotherapy - a literature review." J Pharm Pharm Sci **14**(2): 148-161.

Schaaf, H. S., D. P. Parkin, et al. (2005). "Isoniazid pharmacokinetics in children treated for respiratory tuberculosis." Arch Dis Child **90**(6): 614-618.

Schluger, N. W. (2005). "The pathogenesis of tuberculosis: the first one hundred (and twenty-three) years." Am J Respir Cell Mol Biol **32**(4): 251-256.

Schutte, C. M., C. H. Van der Meyden, et al. (2000). "The impact of HIV on meningitis as seen at a South African Academic Hospital (1994 to 1998)." Infection **28**(1): 3-7.

Selvakumar, N., B. N. Murthy, et al. (2005). "Lot quality assurance sampling of sputum acid-fast bacillus smears for assessing sputum smear microscopy centers." J Clin Microbiol **43**(2): 913-915.

Shafer, R. W., D. S. Kim, et al. (1991). "Extrapulmonary tuberculosis in patients with human immunodeficiency virus infection." Medicine (Baltimore) **70**(6): 384-397.

Siddiqi, N., R. Das, et al. (2004). "*Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump." Infection **32**(2): 109-111.

Silva, M. S. N., S. G. Senna, et al. (2003). "Mutations in *katG*, *inhA*, and *ahpC* Genes of Brazilian Isoniazid-Resistant Isolates of *Mycobacterium tuberculosis*." J. Clin. Microbiol. **41**(9): 4471-4474.

Siqueira, H. R., F. A. Freitas, et al. (2009). "Isoniazid-resistant *Mycobacterium tuberculosis* strains arising from mutations in two different regions of the *katG* gene." J Bras Pneumol **35**(8): 773-779.

Sirgel, F. A., P. B. Fourie, et al. (2005). "The early bactericidal activities of rifampin and rifapentine in pulmonary tuberculosis." Am J Respir Crit Care Med **172**(1): 128-135.

Slayden, R. A. and C. E. Barry, 3rd (2002). "The role of KasA and KasB in the biosynthesis of meromycolic acids and isoniazid resistance in *Mycobacterium tuberculosis*." Tuberculosis (Edinb) **82**(4-5): 149-160.

Smith, P. G. and A. R. Moss (1994). "Epidemiology of Tuberculosis. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 47-60.

Somner, A. R. and A. A. Brace (1962). "Ethionamide, pyrazinamide and cycloserine used successfully in the treatment of chronic pulmonary tuberculosis." Tubercle **43**: 345-360.

Somoskovi, A., J. Dormandy, et al. (2006). "Use of smear-positive samples to assess the PCR-based genotype MTBDR assay for rapid, direct detection of the *Mycobacterium tuberculosis* complex as well as its resistance to isoniazid and rifampin." J Clin Microbiol **44**(12): 4459-4463.

Srivastava, S. and T. Gumbo (2011). "In vitro and in vivo modeling of tuberculosis drugs and its impact on optimization of doses and regimens." Curr Pharm Des **17**(27): 2881-2888.

Steele, M. A. and R. M. Des Prez (1988). "The role of pyrazinamide in tuberculosis chemotherapy." Chest **94**(4): 845-850.

Sugamori, K. S., S. Wong, et al. (2003). "Generation and functional characterization of arylamine N-acetyltransferase *Nat1/Nat2* double-knockout mice." Mol Pharmacol **64**(1): 170-179.

Sun, Y. J., A. S. Lee, et al. (2007). "Genotype and phenotype relationships and transmission analysis of drug-resistant tuberculosis in Singapore." Int J Tuberc Lung Dis **11**(4): 436-442.

Swaminathan, S., C. N. Paramasivan, et al. (2004). "Unrecognised tuberculosis in HIV-infected patients: sputum culture is a useful tool." Int J Tuberc Lung Dis **8**(7): 896-898.

Takayama, K. and J. O. Kilburn (1989). "Inhibition of synthesis of arabinogalactan by ethambutol in Mycobacterium smegmatis." Antimicrob Agents Chemother **33**(9): 1493-1499.

Tanira, M. O. M., M. Simsek, et al. (2003). "Distribution of Arylamine N-acetyltransferase 2 (NAT2) Genotypes among Omanis." SQU J. Sci. Res.: Med. Sci. **5**: 9-14.

Telenti, A., N. Honore, et al. (1997). "Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level." J. Clin. Microbiol. **35**(3): 719-723.

The consensus human NAT2 alleles nomenclature. accessed 10/10, 2010, http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/nat_pdf_files/Human_NAT2_alleles.pdf

Tho, D. Q., D. T. Ha, et al. (2008). "Comparison of MAS-PCR and GenoType MTBDR assay for the detection of rifampicin-resistant *Mycobacterium tuberculosis*." Int J Tuberc Lung Dis **12**(11): 1306-1312.

Tho, D. Q., N. T. Lan, et al. (2011). "Multiplex allele-specific polymerase chain reaction for detection of isoniazid resistance in *Mycobacterium tuberculosis*." Int J Tuberc Lung Dis **15**(6): 799-803.

Thomas, J. P., C. O. Baughn, et al. (1961). "A new synthetic compound with antituberculous activity in mice: ethambutol (dextro-2,2'-(ethylenediimino)-di-l-butanol)." Am Rev Respir Dis **83**: 891-893.

Thuong, N. T., S. J. Dunstan, et al. (2008). "Identification of tuberculosis susceptibility genes with human macrophage gene expression profiles." PLoS Pathog **4**(12): e1000229.

Thuy, T. T., N. S. Shah, et al. (2007). "HIV-associated TB in An Giang Province, Vietnam, 2001-2004: epidemiology and TB treatment outcomes." PLoS One **2**(6): e507.

Thwaites, G., M. Caws, et al. (2008). "Relationship between *Mycobacterium tuberculosis* genotype and the clinical phenotype of pulmonary and meningeal tuberculosis." J Clin Microbiol **46**(4): 1363-1368.

Thwaites, G., M. Fisher, et al. (2009). "British Infection Society guidelines for the diagnosis and treatment of tuberculosis of the central nervous system in adults and children." J Infect **59**(3): 167-187.

Thwaites, G. E., T. T. Chau, et al. (2002). "Isoniazid resistance, mycobacterial genotype and outcome in Vietnamese adults with tuberculous meningitis." Int J Tuberc Lung Dis **6**(10): 865-871.

Thwaites, G. E., N. Duc Bang, et al. (2005). "The influence of HIV infection on clinical presentation, response to treatment, and outcome in adults with Tuberculous meningitis." J Infect Dis **192**(12): 2134-2141.

Thwaites, G. E., N. T. Lan, et al. (2005). "Effect of antituberculosis drug resistance on response to treatment and outcome in adults with tuberculous meningitis." J Infect Dis **192**(1): 79-88.

Thwaites, G. E., D. B. Nguyen, et al. (2004). "Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults." N Engl J Med **351**(17): 1741-1751.

Tisdall, F. F. and A. Brown (1926). "The Relative Value of Different Tuberculin Skin Tests." Can Med Assoc J **16**(8): 939-943.

Tonjum, T., D. B. Welty, et al. (1998). "Differentiation of Mycobacterium ulcerans, M. marinum, and M. haemophilum: mapping of their relationships to M. tuberculosis by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis." J Clin Microbiol **36**(4): 918-925.

Torok, M. E., T. T. Chau, et al. (2008). "Clinical and microbiological features of HIV-associated tuberculous meningitis in Vietnamese adults." PLoS One **3**(3): e1772.

Torok, M. E., H. D. Nghia, et al. (2007). "Validation of a diagnostic algorithm for adult tuberculous meningitis." Am J Trop Med Hyg **77**(3): 555-559.

Torok, M. E., N. T. Yen, et al. (2011). "Timing of initiation of antiretroviral therapy in human immunodeficiency virus (HIV)-associated tuberculous meningitis." Clin Infect Dis **52**(11): 1374-1383.

Truffot-Pernot, C., B. Ji, et al. (1991). "Activities of pefloxacin and ofloxacin against mycobacteria: in vitro and mouse experiments." Tubercle **72**(1): 57-64.

Tsenova, L., E. Ellison, et al. (2005). "Virulence of selected *Mycobacterium tuberculosis* clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli." J Infect Dis **192**(1): 98-106.

Tsenova, L., R. Harbacheuski, et al. (2007). "BCG vaccination confers poor protection against *M. tuberculosis* HN878-induced central nervous system disease." Vaccine **25**(28): 5126-5132.

Tsukamura, M., E. Nakamura, et al. (1985). "Therapeutic effect of a new antibacterial substance ofloxacin (DL8280) on pulmonary tuberculosis." Am Rev Respir Dis **131**(3): 352-356.

Tuon, F. F., H. R. Higashino, et al. (2010). "Adenosine deaminase and tuberculous meningitis--a systematic review with meta-analysis." Scand J Infect Dis **42**(3): 198-207.

Van Deun, A., A. K. Maug, et al. (2010). "Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis." Am J Respir Crit Care Med **182**(5): 684-692.

Van Doorn, H. R., P. E. de Haas, et al. (2006). "Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands." Clin Microbiol Infect **12**(8): 769-775.

Van Rie, A., R. Warren, et al. (2001). "Analysis for a limited number of gene codons can predict drug resistance of *Mycobacterium tuberculosis* in a high-incidence community." J Clin Microbiol **39**(2): 636-641.

Van Soolingen, D., P. E. de Haas, et al. (2000). "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." J Infect Dis **182**(6): 1788-1790.

Velayati, A. A., M. R. Masjedi, et al. (2009). "Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran." Chest **136**(2): 420-425.

Verdon, R., S. Chevret, et al. (1996). "Tuberculous meningitis in adults: review of 48 cases." Clin Infect Dis **22**(6): 982-988.

Vijdea, R., M. Stegger, et al. (2008). "Multidrug-resistant tuberculosis: rapid detection of resistance to rifampin and high or low levels of isoniazid in clinical specimens and isolates." Eur J Clin Microbiol Infect Dis **27**(11): 1079-1086.

Vinnard, C., C. A. Winston, et al. (2010). "Isoniazid resistance and death in patients with tuberculous meningitis: retrospective cohort study." Bmj **341**: c4451.

Volmink, J. and P. Garner (2007). "Directly observed therapy for treating tuberculosis." Cochrane Database Syst Rev (4): CD003343.

Wada, T., S. Maeda, et al. (2004). "Dual-Probe Assay for Rapid Detection of Drug-Resistant *Mycobacterium tuberculosis* by Real-Time PCR." J. Clin. Microbiol. **42**(11): 5277-5285.

Wagner, B. G., J. S. Kahn, et al. (2010). "Should we try to eliminate HIV epidemics by using a 'Test and Treat' strategy?" AIDS **24**(5): 775-776.

Walker, K., G. Ginsberg, et al. (2009). "Genetic polymorphism in N-Acetyltransferase (NAT): Population distribution of NAT1 and NAT2 activity." J Toxicol Environ Health B Crit Rev **12**(5-6): 440-472.

Wang, C., P. Peyron, et al. (2010). "Innate immune response to *Mycobacterium tuberculosis* Beijing and other genotypes." PLoS One **5**(10): e13594.

Wang, F., P. Jain, et al. (2010). "*Mycobacterium tuberculosis* Dihydrofolate Reductase is Not a Target Relevant to the Anti-tubercular Activity of Isoniazid." Antimicrob Agents Chemother **54**(9): 3776-3782.

Ward, H. A., D. D. Marciniuk, et al. (2005). "Treatment outcome of multidrug-resistant tuberculosis among Vietnamese immigrants." Int J Tuberc Lung Dis **9**(2): 164-169.

Wayne, L. G. (1994). "Cultivation of *Mycobacterium tuberculosis* for research purposes. Tuberculosis: pathogenesis, protection and control." B. R. Bloom, American Society for Microbiology: 73-84.

White, I. R., P. Royston, et al. (2011). "Multiple imputation using chained equations: Issues and guidance for practice." Statistics in Medicine **30**(4): 377-399.

WHO (1993). WHO declares tuberculosis a global emergency. Soz Praventivmed. **38:** 251-252.

WHO (1997). Anti-tuberculosis drug resistance in the world: The WHO/IUATLD global project on drug resistance surveillance, 1994-1997. WHO, Geneva (WHO/TB/97.229)

WHO (1999). What is DOTS? A guide to understanding the WHO-recommended TB control strategy known as DOTS. WHO, Geneva. (WHO/CDS/CPC/TB/99.270)

WHO (2001). DOTS for all country reports. WHO, Geneva. (WHO/CDS/STB/ 2001.13)

WHO (2004). Anti-tuberculosis drug resistance in the world. report no. 3. WHO, Geneva. (WHO/ HTM/TB/2004.343)

WHO (2004). TB/HIV: a clinical manual. Second edition. WHO, Geneva. (WHO/HTM/TB/2004.329)

WHO (2006). Global TB control: surveillance, planning, financing. WHO, Geneva. (WHO/ HTM/TB/2006.362)

WHO (2006). The stop TB strategy. WHO, Geneva. (WHO/ HTM/TB/2006.368)

WHO (2007). Report of the meeting of the WHO Global Task Force on XDR-TB, 9-10 October 2006. WHO, Geneva. (WHO/ HTM/TB/2007.375)

WHO (2008). Anti-tuberculosis drug resistance in the world. Report no. 4. WHO, Geneva. (WHO/ HTM/TB/2008.394)

WHO (2008). Guidelines for the programmatic management of drug resistant tuberculosis: Emergency update 2008. WHO, Geneva. (WHO/HTM/TB/2008.402)

WHO (2008). Laboratory-based evaluation of 19 commercially available rapid diagnostic tests for tuberculosis. 2nd. WHO/TDR, Geneva. http://www.who.int/tdr/publications/documents/diagnostic-evaluation-2.pdf

WHO (2008). WHO policy statement: molecular line probe assays for rapid screening of patients at risk of MDR TB. WHO, Geneva. http://www.who.int/tb/features_archive/policy_statement.pdf

WHO (2009). Global Tuberculosis control: epidemiology, strategy, financing. WHO, Geneva. (WHO/HTM/TB/2009.411)

WHO (2009). "Guidelines for surveillance of drug resistance in tuberculosis. fourth edition. WHO, Geneva. (WHO/HTM/TB/2009.422)

WHO (2009). Treatment of tuberculosis Guidelines, Fourth edition. WHO, Geneva. (WHO/HTM/TB/2009.420)

WHO (2010). Global tuberculosis control. WHO,Geneva. (WHO/HTM/TB/2010.7)

WHO (2010). Multidrug and Extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. WHO, Geneva. (WHO/HTM/TB/2010.3)

WHO (2010). Rapid advice: Treatment of tuberculosis in children. WHO, Geneva. (WHO/HTM/TB/2010.13)

WHO (2011). Commercial serodiagnostic tests for diagnosis of tuberculosis: policy statement. WHO, Geneva. (WHO/HTM/TB/2011.5)

WHO (2011). Global tuberculosis control 2011. WHO, Geneva. (WHO/HTM/TB/ 2011.16)

WHO (2011). Guidelines for intensified tuberculosis case-finding and isoniazid preventive therapy for people living with HIV in resource-constrained settings. WHO, Geneva. (WHO/HTM/TB/2011.11)

WHO and UNAIDS (2009). AIDS epidemic update. WHO/UNAIDS, Geneva. (UNAIDS/09.36E/ JC1700E)

Williams, B. G., V. Lima, et al. (2011). "Modelling the impact of antiretroviral therapy on the epidemic of HIV." Curr HIV Res **9**(6): 367-382.

Youmans, G. P., G. W. Raleigh, et al. (1947). "The Tuberculostatic Action of para-Aminosalicylic Acid." J Bacteriol **54**(4): 409-416.

Zhang, Y. and A. Telenti (2000). "Genetics of Drug Resistance in *Mycobacterium tuberculosis*". Book: 235-254

Zhao, B., A. Seow, et al. (2000). "Correlation between acetylation phenotype and genotype in Chinese women." Eur J Clin Pharmacol **56**(9-10): 689-692.

Zheng, H., L. Lu, et al. (2008). "Genetic basis of virulence attenuation revealed by comparative genomic analysis of *Mycobacterium tuberculosis* strain H37Ra versus H37Rv." PLoS One **3**(6): e2375.

Zignol, M., M. S. Hosseini, et al. (2006). "Global incidence of multidrug-resistant tuberculosis." J Infect Dis **194**(4): 479-485.

Zink, A. R., C. Sola, et al. (2003). "Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping." J Clin Microbiol **41**(1): 359-367.