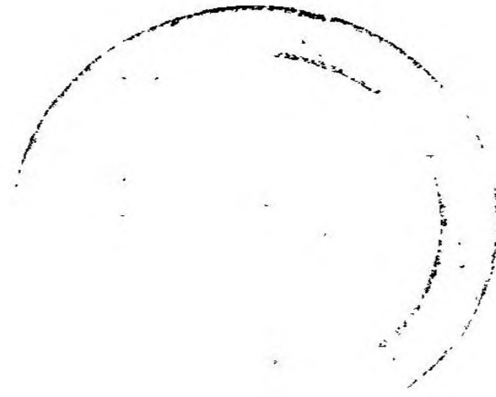

Novel mutations in the *rpoB* gene of rifampicin resistant *Mycobacterium tuberculosis*

A thesis submitted to the University of Colombo Sri Lanka for

the degree of

Doctor of Philosophy

by

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RG/ 20...

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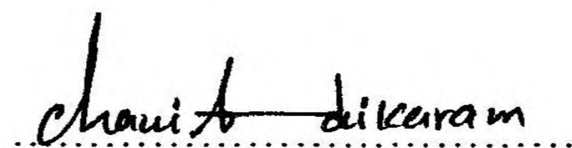
December 2012

To

My parents

Declaration by the candidate

This is to certify that the content of this PhD thesis titled "Molecular characterisation of rifampicin resistant *Mycobacterium tuberculosis* and development of PCR-ELISA for identification of rifampicin resistance" is my own work and whole or part was not presented for any other degree by me or anybody else. Except DNA sequencing all other research work presented in this thesis was carried out at the Department of Microbiology, Faculty of Medicine, University of Colombo, Sri Lanka by myself. DNA Sequencing was carried out by Macrogen DNA sequencing service- Korea.



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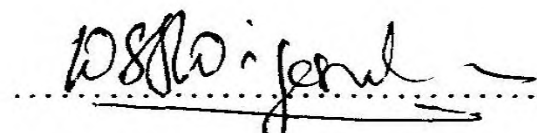

Prof. W.S.S. Wijesundera

Abstract

The thesis comprise studies on rifampicin resistance of *Mycobacterium tuberculosis* in Sri Lanka by sequentially addressing the isolation of *Mycobacterium tuberculosis*, drug susceptibility testing methods, characterisation of *rpoB* gene mutations and transmission pattern of rifampicin resistance.

Mycobacterium cultures (n=442) were isolated from acid fast bacilli (AFB) positive sputum specimens collected from clinically suspected tuberculosis patients in Sri Lanka. Four hundred and one (401) isolates were identified as belonging to *M. tuberculosis* complex while the remaining 41 were recognised as belonging to non-tuberculosis mycobacteria group. The prevalence of non-tuberculosis mycobacteria (9.7%) in an acid fast bacilli positive sputum cohort in Sri Lanka indicates the necessity for species identification of *Mycobacterium* isolates prior to treatment as non-tuberculosis mycobacteria are frequently resistant to conventionally used anti tuberculosis drugs.

In Sri Lanka, drug susceptibility testing of *M. tuberculosis* still depends on time consuming, conventional proportion method. Thus, the nitrate reductase assay (NRA) in broth medium and the manual mycobacteria growth indicator tube (MGIT) were evaluated as rapid culture based drug susceptibility testing methods for determination of rifampicin resistance. The nitrate reductase assay and the manual mycobacteria growth indicator tube demonstrated excellent agreement ($\kappa = 0.86$ and $\kappa = 0.94$ respectively) with the agar proportion method (APM). With the manual mycobacteria growth indicator tube and the nitrate reductase assay, it was possible to determine the rifampicin susceptibility within 8 and 10 days respectively from primary

M. tuberculosis isolates with high sensitivity (93% and 85% respectively) and specificity (100% and 99% respectively).

Rifampicin resistance has immerged due to point mutations in the *rpoB* gene and majority of world's prevailing mutations are restricted to the rifampicin resistance determining region (RRDR) of *rpoB* gene. However, the presence of mutations varies geographically and is not restricted to the rifampicin resistance determining region. Therefore, selected fragments (437bp, 872bp and 1395bp that cover RRDR and regions spanning the RRDR) of *rpoB* gene of the 31 rifampicin resistant *M. tuberculosis* strains isolated during the study were subjected to PCR amplification and DNA sequencing. The DNA sequences revealed 2 point mutations within the rifampicin resistance determining region at codon 526 (n=15, 48.4%) CAC (His) → TAC (Tyr) and codon 531 (n=3, 9.7%) TCG (Ser)→TTG (Leu). A significant proportion (n=15, 48.3%) showed two novel mutations in the region spanning the RRDR at codon 626 (n=13, 41.9%) GAC (Asp) →GAG (Glu) and codon 184 (n=2, 6.4%) GAC (Asp) → GAT (Asp), a silent mutation. Two isolates revealed double mutations (codons 626+526 and 626+184). The presence of new mutations with a high frequency and the different frequencies of the universally prevailing mutations, as reported here, emphasizes the need for expanding the geographical database of mutations for effective application of *rpoB* based diagnosis of drug resistant tuberculosis.

The commercialized molecular drug susceptibility testing methods are based on world's prevalent mutation in rifampicin resistance determining region of *rpoB* gene (codon, 531,526 and 516). Thus, available molecular drug susceptibility testing methods may not achieve the required sensitivity in Sri Lanka as they will only be able to identify 58% of drug resistant TB cases. Additionally, these methods are not accessible in developing countries due to the high cost per

test. Therefore, the polymerase chain reaction linked immunoabsorbent assay (PCR-ELISA) was developed using rifampicin resistant and susceptible *M. tuberculosis* isolates identified by agar proportion method. The dig labelled PCR amplified fragments of *rpoB* gene were hybridized with 5' biotinylated allele specific oligonucleotide probes corresponding to point mutations at codons 526, 531 & 626. The hybridization was determined by colour development. There was a good agreement between agar proportion method and PCR-ELISA with 86% sensitivity and 100% specificity for identification of rifampicin resistance of *M. tuberculosis.* The turnaround time of the assay was 2 days after isolation of primary cultures. Thus, PCR-ELISA is a rapid, sensitive and specific drug susceptibility testing method that can be customized as per user requirement.

DNA fingerprinting of IS6110 insertion element of rifampicin resistant and susceptible *M. tuberculosis* isolates revealed that none of the rifampicin resistant isolates have similar fingerprinting patterns to that of susceptible isolates. This observation indicated the absence of the acquisition of rifampicin resistance following infection of a rifampicin susceptible strain. Thus, the rifampicin resistance of *M. tuberculosis* in Sri Lanka is due to the transmission of rifampicin resistant strains (primary drug resistance) based on the IS6110 DNA fingerprinting.

Acknowledgements

To my supervisors, Professor Jennifer Perera and Prof. Sulochana Wijesundera, the brilliant teachers and great scientists, I have associated in my life. I am so lucky to have grown up underneath your vision of science and humanity in person. You always gave me a new life by encouraging me in all difficult situations. With deep respect, I express my sincere thanks to your invaluable support and proper guidance.

I gratefully acknowledge National Science Foundation (research grant: RG/ 2007/ BT/) for the financial assistance.

I gratefully remember Prof. S. Premawansa, Prof. Preethi Randeniya of the Faculty of Science, University of Colombo for directing me to the pathway of Postgraduate education.

I specially acknowledge the patients who participated in the study without any personal benefit. A special word of thanks to Consultant Chest Physicians, Dr. Vijitha Senarathna, Dr. Bandu Munasinghe and staff of the Ward No 5, 6, 7 & 8 of Chest Hospital Welisara for their assistance in collection of sputum specimen. Dr. Asha this warm word of gratitude is for your kind assistance in collection of sputum from patients.

The staff, Department of Microbiology, this especial word of thank to all of you. Mr. Sirithilak Gamage thank you very much for your kind support and technical assistance. Thank you very much Ms Shirani Hendalage, while extending your support and friendship, you always made my

life easy not only in research work but also my personal life. Mr. Priyantha, you warmly welcomed me on my arrival to the "TB lab", you always gave your support with a smile, thank you so much. Mr. Nimal and Mr. Sudath thank you, for the immense effort you took in transporting sputum specimen to the Department of Microbiology. This special thanks for Dr. Rohan Chinnaia, Dr. Ushani, Dr. Channa and Dr. Enoka, who always lent their hands to me in support of various activities. Thank you very much Mr. Aluthge, Mr. Gamini, and Mr. Nimal without you this work wouldn't have been a reality. Your support was immeasurable and I admire the kindness you showed throughout the years. Thank you very much Mr. Dinesh and Mr. Samarathunga, you always helped me when I really needed it and made my life easy. Ms Dilini, Mr. Suresh, Mr. Maga you made me smile and refreshed my mind with happiness. Thank you so much Ms. Gihani, you were the person who was always with me not only for research work; memories of quarrelling, laughing and struggling will never fade away in my life. Thank you very much Ms. Manjula, Ms.Jayanthi, Ms. Renuka, and Ms. Priayadarshanie you always understood my situation and made me smile. It was my pleasure to be with you during past few years. You were always with me, in happy, sad or bad movements and made my life wonderful at "Micro". Those happy memories will never die away.

I must gratefully acknowledge the support of the staff, Department of Parasitology. Thank you very much Dr. Sisira Pathirana you always supported and encouraged me. This warm words of thank to Mr. Kapilananda, Mr.Sudath and Mr. Jagath, you never ever hesitated to help me.
Mr. Mohan thank for your all the support, specially you kept the building C main door open early morning. Ms. Himali and Ms. Chandima and Ms. Chamika you were good neighbors and I could

share happy and sad moments with you. Thank you very much; your wonderful friendship will always remain with me.

Staff of Department of Biochemistry and Molecular Biology you were always ready to help me, thank you very much. I gratefully remember Mr. Premarathne, Department of Accounts for your kind assistance in handling accounts of the research grant.

My special thanks to Ms. Ravini, Ms. Praba University of Sri Jayewardenepura you were part of my new profession. Your support is immeasurable and made my research life easy. Ms. Pamoda and Iresha we shared our experience at J'pura. Thank you very much. My classmate Rangi and batch mate Dila, colleague Thilini, you are continued to be friends and never forgot me although distant physically. Thank you very much for your encouragement in my whole life.

My loving amma and thaththa you two are the best parents in the universe. Amma, you are wonderful, I am here today with your millennium encouragement; if you were not in my life I could have been just a woman who never had schooling. Thaththa, you always try to bring me to the top. My sister and your family, specially Laksha Duwa and Ama Putha you are the stars of my life and thank you very much for your love. My "Aiya" you always opened your mind to my words and made everything easy in my life. Thank you very much.

List of publications and communication

Published in peer reviewed journal

1. **Adikaram CP**, Perera J, Wijesundera S. Geographical profile of *rpoB* gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolated in Sri Lanka. Microbial Drug Resistance 2012; 18:525-30

2. **Adikaram CP**, Perera J, Wijesundera S. The Manual Mycobacteria Growth Indicator Tube and the Nitrate Reductase Assay are suitable alternatives for the rapid detection of rifampicin resistant *M. tuberculosis* in low resource settings. BMC infectious diseases 2012, 12:326. http://www.biomedcentral.com/1471-2334/12/326

Manuscript submitted

1. **Adikaram CP**, Perera J, Wijesundera S. Probe based PCR- Enzyme-Linked Immuno-absorbent Assay (PCR–ELISA) as a susceptibility testing method for *Mycobacterium tuberculosis*. Has been submitted to Journal of BMC infectious diseases

Manuscript in preparation

1. **Adikaram CP**, Perera J, Wijesundera S. Transmission of rifampicin resistance of *M. tuberculosis* in Sri Lanka

Research communication

1. **Adikaram CP**, Perera J, Wijesundera S. PCR-Enzyme Linked Immunoabsorbent Assay as a rapid molecular method for detection of rifampicin resistant *Mycobacterium tuberculosis* in low resource settings. The Ceylon Medical Journal 2012; 57: 31 (Presented at 125th anniversary international medical congress–Sri Lanka Medical Association)

2. **Adikaram CP**, Perera J, Wijesundera S, Perera GMM, Gamage S. Cord formation in a clinical isolate of *Mycobacterium abscessus*. Presented at 4th annual sessions and symposium, Association of the Pulmonologist-Sri Lanka 2012

3. **Adikaram CP**, Perera J, Wijesundera S. Genus *Mycobacterium* as an etiological agent of pulmonary infection in Sri Lanka. Presented at Research symposium-University of Colombo, August 2012. Page 223

4. **Adikaram CP**, Perera J, Wijesundera SW, Senaratne V. Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the *rpoB* Gene are Important Determinants of *M tuberculosis* (MTB) Rifampicin Resistance (RifR). 51st annual sessions of ICAAC, Chicago, USA 2011; C1: 627

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(This paper was awarded first place from oral presentation).

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7. **Adikaram CP**, Perera J, Wijesundera S. Comparison of Mycobacterium Growth Indicator Tube (MGIT) method and Nitrate Reductase Assay (NRA) with agar proportion method (APM) for detection of Rifampicin resistance. Proceedings of the 66th annual sessions of Sri Lanka Association for the Advancement of Science (SLAAS) 2010; 112A:12

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6. 6 Rooted Neighbour - joining dendrogram constructed from IS6110 RFLP fingerprinting patterns of RIF resistant *M. tuberculosis* isolates using Phylip 203

List of abbreviation

A	-	Adenine
AD	-	Anno Domini
AFB	-	Acid fast bacilli
APM	-	Agar proportion method
ADA	-	Adenosine deaminase
BC	-	Before Christ
BCG	-	Bacillus Calmette–Guérin
bp	-	Base pairs
C	-	Cytosine
CFU	-	Colony forming unit
CRI	-	Colorimetric redox indicator
CO_2	-	Carbon dioxide
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxy ribonucliec acid
dNTPs	-	Deoxynucleotide triphosphate
DST	-	Drug susceptibility testing
DOTS	-	Directly observed treatment short course
DR	-	Direct repeat
dUTP	-	Dexyuracil triphosphate
DW	-	Distilled water
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
EMB	-	Ethambutol

EPTB	-	Extra pulmonary tuberculosis
G	-	Guanine
h	-	Hours
HCl	-	Hydrochloric
HIV	-	Human immunodeficiency virus
INH	-	Isoniazid
IS	-	Insertion sequence
L-J	-	Lowenstein-Jensen
LPA	-	Line probe assay
M	-	Molar
MDR-TB	-	Multi drug resistant tuberculosis
$MgCl_2$	-	Magnisium chloride
MIC	-	Minimum inhibitory concentration
MGIT	-	Mycobacteria growth indicator tube
min	-	Minute
mRNA	-	messenger ribonucleic acid
MTC	-	*Mycobacterium tuberculosis* complex
NaCl	-	Sodium chloride
$NaNO_3$	-	Sodium nitrate
NaOH	-	Sodium hydroxide
NPTCCD	-	National Programme for Tuberculosis Control and Chest Diseases
NRA	-	Nitrate reductase assay
NTM	-	Non tuberculosis mycobacteria
PCR	-	Polymerase chain reaction

PZA	-	Pyrazinamide
RFLP	-	Restriction fragment length polymorphism
RIF	-	Rifampicin
RNA	-	Ribonucleic acid
RNAP	-	Ribonucleic acid polymerase
rpm	-	revolution per minute
RRDR	-	Rifampicin resistant determining region
SD	-	Standard deviation
SDS	-	Sodium dodecyl sulfate
SM	-	Streptomycin
T	-	Thymine
TB	-	Tuberculosis
TBE	-	Tris borate EDTA
TE	-	Tris EDTA
TNF-α	-	Tumor necrosis factor-alpha
UV	-	Ultra violet
VNTR	-	Variable numbers of tandem repeat sequences
WHO	-	World Health Organization
XDR-TB	-	Extensively drug resistant tuberculosis
ZN	-	Ziehl-Neelsen

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Chapter 1

General introduction

1.1 Genus *Mycobacterium*

The Genus *Mycobacterium* was first proposed in 1896 by Lehmann and Neumann. Currently, it contains about 151 species and 11 sub species (Skerman *et al.* 1980). Most species exist as free-living saprophytes and only minorities are successful as pathogens of higher vertebrates. The host-dependent mycobacteria are capable of reproducing *in vitro*. In contrast, *M. leprae* and *M. lepraemurium* are uncultivable and require the intracellular milieu for survival and propagation (Palomino *et al.* 2007).

1.1.1 Bacteriology of genus *Mycobacterium*

Mycobacterium is a non-motile, non sporulating, straight or slightly curved bacillus which is 1-10 µm in length and 0.2-0.6 µm in width. Unlike other actinomycetales, *M. tuberculosis* and most *Mycobacterium* species are rarely pleomorphic and do not elongate into filaments or branching chains in either clinical specimens or in culture. According to growth conditions and maturity of the culture, bacilli may vary in size and shape from short coccobacilli to long rods (Palomino *et al.* 2007). However, experiments have shown that *M. tuberculosis* cells grown in macrophages are filamentous (Chauhan *et al.* 2006).

The mycobacterial cell wall is unique and consists of peptidoglycolipids, mycolic acids, free lipids and interspersed proteins (Jarlier and Nikaido, 1994). Inner peptidoglycan layer contains a number of cross links and the outer end of the

arabinogalactan has esterified by mycolic acids. The arrangement of mycolic acids are species specific. The outer layer of the cell wall presents an array of free lipids such as phthiocerol dimycoserosates, phenolic glycolipid, trehalose-containing glycolipids and sulfolipids (Figure 1.1). The lipid composition of the cell wall depends on the availability of nutrients and may vary during the life cycle. However, lipids constitute more than half of the dry weight of the mycobacterial cell wall (Brennan, 2003).

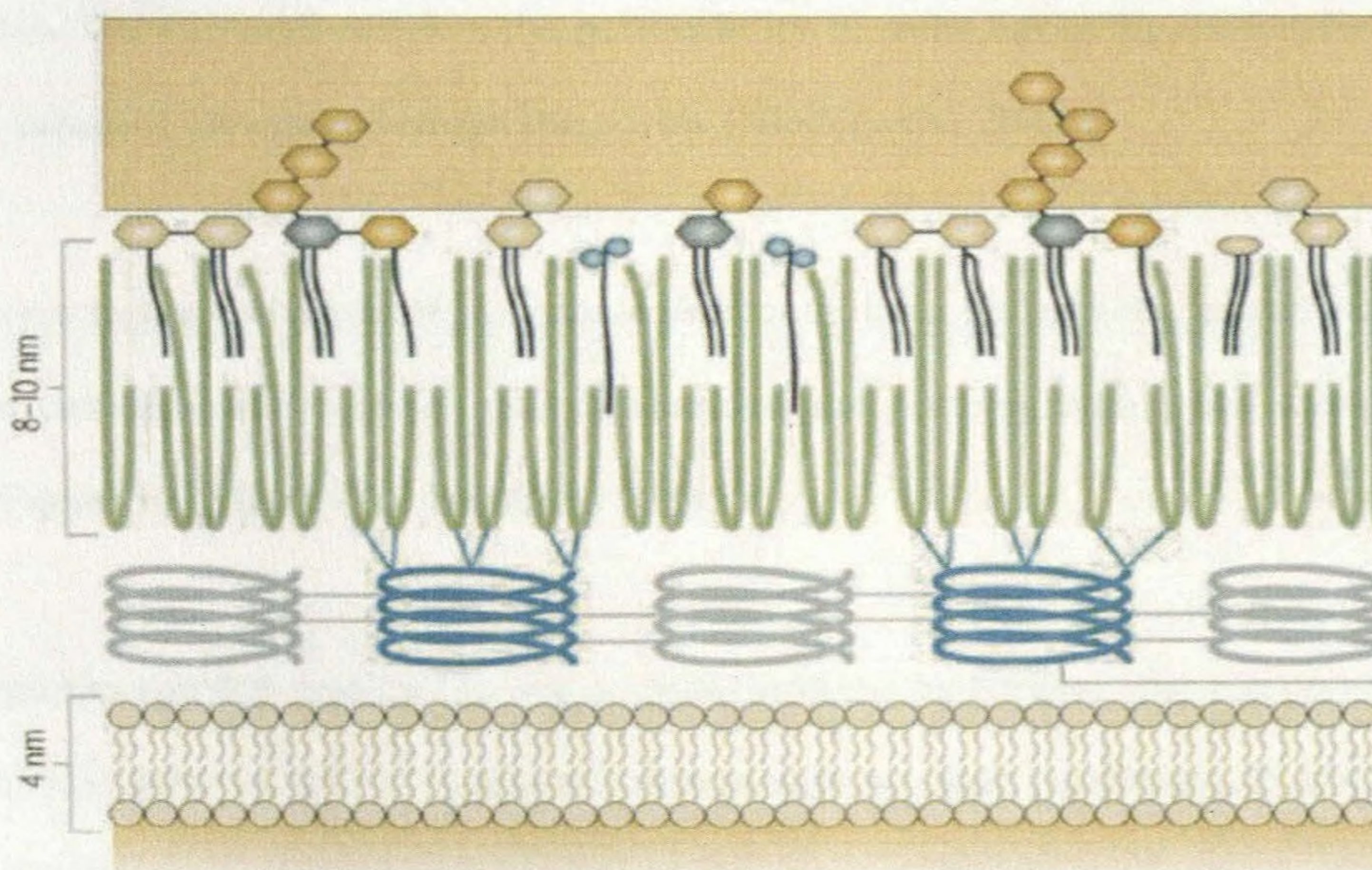


Figure 1.1 Schematic representation of the cell envelope of *M. tuberculosis*. The cell wall is mainly composed of peptidoglycan (grey), arabinogalactan (blue) and mycolic acids (green). The outer layer (brown), capsule mainly contains polysaccharides (Abdallah *et al.* 2007).

The waxy coat confers the characteristics of the genus comprising the distinctive immunological properties, resistance to antibiotics (Jarlier and Nikaido, 1994), slow growth rate and acid fastness (resistance to decolonization with acid-alcohol after staining with arylmethane dyes) (Harada, 1976). Trehalose 6, 6'-dimycolate (TDM),

the cord factor is an external constituent comprising the waxy coat of MTC (Actor, 2012). It is responsible for the virulence of the bacilli and (Rajni *et al.* 2010) protection from the macrophages (Hunter *et al.* 2006). However, cord factor is not characteristic for all species of the genus.

Some of the interspersed proteins are involved in construction of the cell wall and others known as porins form hydrophilic channels that are responsible for permeability of solutes. The cell wall restricts the permeability to most lipophilic molecules while other substances may pass through the porins (Niederweis, 2003).

In addition to the cell wall, *M. tuberculosis* accumulates an unbound pseudo-capsule in static culture and it contains polysaccharides and proteins with small amounts of lipids (Figure 1.1) (Daffe and Etienne, 1999).

Although the tubercle bacillus is not a spore-forming bacterium, the *Mycobacterium tuberculosis* complex (MTC) is able to tolerate unfavourable conditions (Pardini *et al.* 2005). Under such conditions, mycobacteria enter a latent or dormant state and multiplication can be suspended from days to many years (Caminero, 2004). The tubercle bacilli can survive at acid or alkaline microenvironment *in vivo* as well as *in vitro* (Vandal *et al.* 2009). This feature is made use of in processing clinical specimens as they can be treated with 4% NaOH prior to culture for reducing contamination (De Kantor *et al.* 1998). *M. tuberculosis* is highly resistant to freezing (from 2-4 °C to -70 °C), as viability, pathology and physiological properties are not altered by low temperature (Kubicaa and Kim, 1979). Additionally, tubercle bacilli can survive for several months on surfaces or in soil even at low oxygen level (Wayne, 1982).

However, *M. tuberculosis* is very sensitive to heat, sunlight and UV radiation. The ultraviolet killing effect ranges from 50% for *M. kansasii* to more than 99% for *M. tuberculosis* (Collins, 1971; Huber *et al.* 1970; Caminero, 2004).

Mycobacteria require a mesophilic well-nourished environment with high oxygen content for their optimum growth and carbon dioxide tension is important for successful multiplication (Gruft and Loder, 1971). Thus, the culture positivity rate, growth rate and yield from the acid fast bacilli (AFB) smear negative specimens could be enhanced with incubation of L-J medium at 10% CO_2 (Chauhan *et al.* 1991). *M. tuberculosis* can switch off from being aerobic to microaerophilic and utilize lipids (Wayne, 1982). Therefore, it can survive and grow as a successful pathogen in tissues, using fatty acids as the major carbon source (Neyrolles *et al.* 2006). *M. tuberculosis* is able to metabolize glycerol into pyruvate, whereas *M. bovis* does not show this ability due to the presence of inactive pyruvate kinase and utilizes pyruvate as carbon source (Keating *et al.* 2005). Recently it has been shown that *M. tuberculosis* can simultaneously co-catabolize multiple carbon sources to achieve enhanced monophasic growth (Carvalho *et al.* 2010).

1.1.2 The Genome of Mycobacteria

The genome of prokaryote is in a DNA/protein complex in the cytosol called the nucleoid, which lacks a nuclear envelope. The bacterial chromosome is a circular molecule of DNA that functions as a self-replicating genetic element. In addition, many important genes of prokaryotes are stored in extra chromosomal genetic elements such as plasmids and bacteriophages (Holmes and Jobling, 1996). However,

certain bacteria have multiple circular chromosomes, linear chromosomes and linear plasmids (Volff and Altenbuchner, 2000).

Bacterial genomes are generally small (<14,000 kbp) and less variant among species (Ribeiro *et al.* 2012). Unlike eukaryotes, bacteria show a strong correlation between genome size and number of functional genes in a genome as the bacteria have relatively small amounts of junk or non-coding DNA (Kuo *et al.* 2009). However, Cole *et al.* 1998 has shown that virulent *M. leprae* has a significantly higher percentage of pseudogenes to functional genes (Singh and Cole, 2011).

1.1.2.1 Genome of *Mycobacterium tuberculosis*

Integrated map of the circular chromosome of the virulent reference strain, *M. tuberculosis* H37Rv was the starting point for genome sequencing of the genus *Mycobacterium*. The complete genome sequence of *M. tuberculosis* H37Rv comprises 4,411,532 bp (Cole *et al.* 1998), second only to *Escherichia coli* (Blattner *et al.* 1997) with a 65.6 % of G+C content. The *Mycobacterium* genome is rich in repetitive DNA and duplicated housekeeping genes (Cole *et al.* 1998). The G + C content is relatively constant throughout the genome indicating the absence of probable horizontally transferred pathogenicity. The genome contains 3,924 open reading frames, accounting for > 91% of the potential coding capacity (Cole *et al.* 1998).

1.1.2.2 Comparative genomics of *M. tuberculosis* complex

Several approaches have been employed to compare the genomes of members of the *M. tuberculosis* complex by various DNA array technologies (Gordon *et al.* 1999; Behr *et al.* 1999; Kato-Maeda *et al.* 2001). Many of these studies have compared virulent and avirulent strains for discovering the links to understand changes in pathogenesis, develop novel vaccines and to investigate the effective diagnostic markers of tuberculosis.

The regions of difference (RD) in tandem repeat loci mainly RD1, RD2, RD4, RD7, RD8, RD9, RD10 and RD12 are candidates for the development of powerful tools for the rapid and unambiguous identification of members of the *M. tuberculosis* complex (Brosch *et al.* 2002). The RD7, RD8, RD9 and RD10 regions are absent in *M. microti, M. bovis* and BCG (Bacillus Calmette–Guerin) while present in all *M. tuberculosis* strains. Figure 1.2 demonstrates the differentiation of members of *M. tuberculosis* complex based on the regions of difference (Cole, 2002).

Tubercle bacilli

RD9

\+ | -

M. canettii
M. tuberculosis

TbD1

\+ | -

M. canettii
M. tuberculosis
(Aancestral)

M. tuberculosis
(Modern)

M. africanum, M. bovis
M. microti

Mmpl6^{551}

\+ | -

M. africanum

M. bovis
M. microti

RD4

\+ | -

M. bovis (Caprine, Seal)
M. microti

M. bovis (Classical)

RD1

\+ | -

M. bovis
(Classical)

BCG

Figure 1.2 Identification of Mycobacteria at the species level using the regions of difference (RD) in tandem repeat loci (Cole, 2002)

1.1.2.3 Insertion sequences (IS)

Insertion sequences are small (< 2-5 kb) segments of DNA that can be inserted at multiple sites in a genome (Mahillon and Chandler, 1998). Insertion sequences and

transposons are mobile genetic elements encoding genes which are essential for transposition. Insertion of these elements into structural genes leads to gene inactivation (Campbell *et al.* 1979). Thus, most of the insertion sequences are inserted in non-coding regions and clustered to exist as insertion hot-spots (Cole *et al.* 1998). More than 32 different insertion sequences have been detected in *M. tuberculosis* genome and most of them belong to the IS3 and IS256 families (Cole *et al.* 1998).

These elements are present in multiple copies in the genome of *M. tuberculosis*. In H37Rv, there are 56 copies of IS elements belonging to the well-known IS3, IS5, IS21, IS30, IS110, IS256, ISL3 and IS1535 families (Gordon *et al.* 1999).

IS6110, a member of the IS3 family, is the most abundant insertion element and play an important role in genome plasticity (Cole, 2000). The variable copy number of IS6110 between different strains of the tubercle bacillus is used as a genetic marker for molecular epidemiology of tuberculosis (see section 6.1 for further details) (Poulet and Cole, 1995) and *M. tuberculosis* strains may contain 0-25 IS6110 copies (McEvoy *et al.* 2007; Agasino *et al.* 1998).

1.1.3 Clinical relevance of the genus *Mycobacterium*

The obligatory causative agents of the genus *Mycobacterium*, responsible for tuberculosis (TB) are classified into *Mycobacterium tuberculosis* complex (MTC). It comprises *M. tuberculosis*, *M.bovis*, *M. africanum M. microti* (Wieten *et al.* 1983), *M. canettii* (Pfyffer *et al.* 1998) *M. caprae* (Aranaz *et al.* 1999) and *M. pinnipedii* (Cousins *et al.* 2003) species. *M. tuberculosis*, *M. africanum*, and *M. canettii* cause TB

primarily in human (Palomino *et al.* 2007) whereas, *M. bovis* (Moda *et al.* 1996), *M. microti* (Cavanagh *et al.* 2002), *M. caprae* (Aranaz *et al.* 1999) and *M. pinnipedii* (Cousins *et al.* 2003) infect cattle, domestic animals, goats and seals respectively and animal tuberculosis can also be zoonotic (Cvetnic *et al.* 2007; Kiers *et al.* 2008; Moda *et al.* 1996). Medically important other mycobacteria such as *M. avium, M. intracellulare* complex, *M. kansasii, M. marinum, M. fortuitum, M. chelonae* complex, *M. abscessus* and *M. scrofulaceum* are known as non-tuberculosis mycobacteria (NTM) species. They are responsible for diseases including lymphadenitis in children, chronic pulmonary diseases, skin and soft-tissue diseases and infections of the skeletal system (Wolinsky *et al.* 1992).

1.2 Tuberculosis in human

1.2.1 Annotation from the ancient history

Tuberculosis is an ancient infectious disease present before the beginning of recorded history and it has claimed millions of human lives throughout several centuries. Tuberculosis (*phthisis* in Greek terminology) was first recorded in Egypt, 5000 years ago. Around 460 BC, Hippocrates identified *phthisis* as the most widespread fatal disease in the age between 18 and 35 years (Palomino *et al.* 2007). Clarissimus Galen (131-201 AD), the most eminent Greek physician after Hippocrates, described *phthisis* as a disease of malnutrition causing ulceration of the lungs, chest or throat, accompanied by coughs, low fever, and wasting of the body because of pus (Palomino *et al.* 2007). In 1882, with the microscopic observation of tubercle bacilli in animal tissue by Robert Koch (1843-1910), the relationship between a causative microbe and TB was established (Kaufmann and Schaible, 2005).

According to the molecular evidence, it seems that the modern members of *M. tuberculosis* complex originated about three million years ago in Africa. It was then spread all over the world as a successful clone possibly coinciding with the waves of human migration out of Africa (Gutierrez *et al.* 2005). TB was also known as Great White Plague and 400 years ago, it was epidemic in Europe (Bates and Stead, 1993). Gradually, TB became a major public health concern in the world and currently it is the second infectious killer of adult, first being HIV/AIDS (WHO, 2010 a).

1.2.2 Mechanism of transmission

Tuberculosis is an air born contagious disease. Inhalation of micro droplets in the air, produced by patients during speaking, singing, laughing, sneezing, and especially coughing would infect healthy persons. Micro droplets are highly infectious as they can enter alveolar spaces in the subpleural zone and a person needs only to inhale a small number of bacilli to be infected. Each untreated person with transmissible TB disease will infect 10 to 15 healthy people every year (WHO, 2010 b). In addition to the respiratory tract, *M. tuberculosis* can use less conventional routes of infection such as digestive, mucocutaneous, placental and inoculation to enter the human body. Only 10% of infected persons will develop the active disease at some point of their life and half of them (5%) will present symptoms during the first two years after initial infection. The risk of developing active disease is increased by 10% annually for persons co-infected with HIV (Caminero, 2004; TBVI Annual Report, 2009).

1.2.3 Sites of infection

Human is the main reservoir or host for *M. tuberculosis* which is a facultative intracellular parasite usually in macrophages (Daffe and Etienne, 1999; Russell, 2001). The lungs especially in either the upper part of the lower lobe or the lower part of the upper lobe are primary sites of infection and this is called pulmonary tuberculosis (Russell, 2001). Extra pulmonary tuberculosis refers to disease outside the lungs such as lymph glands and abscesses, bones and joints, genitourinary tract, abdomen, spine, pericardium, skin and pleura (Sharma and Mohan, 2003).

1.2.4 Pathogenesis of *M. tuberculosis*

Infection of *M. tuberculosis* follows a well characterized sequence of events in human body. Once infectious bacilli reach the lower respiratory tract, the bacilli are phagocytosed by alveolar macrophages and replicate within the endosomes. They induce a localized pro inflammatory response with the recruitment of blood mononuclear cells such as monocytes and lymphocytes (Bhatt and Salgame, 2007). Before a cellular immune response is elicited, the bacilli may grow and enter the blood stream and spread to the apical regions of the lungs as well as other organs of the body. Further, TB bacilli that spread through the body set up many foci of infections by forming tubercles in the tissues and it causes a severe form of the disease called as Miliary TB (Kim *et al.* 2003).

The recruited inflammatory cells are the building blocks for the granuloma, or tubercle. The granuloma consists of a kernel of infected macrophages and foamy giant

cells surrounded by lymphocytes delineating the periphery of the structure. Bacilli are not always eliminated within the granuloma and it may become dormant resulting in a latent infection. Once an infected person becomes immunocompromised (in old age, malnutrition and HIV), the dormant bacilli can get activated and produce active tuberculosis (Kim *et al*. 2003).

1.2.5 Immune responses against *M. tuberculosis*

Most individuals exposed to *M. tuberculosis* never develop active disease, suggesting that adaptive immunity against tuberculosis is able to control the infection. Following inhalation of bacilli, dendritic cells present mycobacterial antigens to native T and B cells. The activated T cells traffic back to the lung, where waves of infiltrating cells, wall off infected macrophages in granuloma. This results in a dynamic interaction between *M. tuberculosis,* macrophages and T cells (Figure 1.3). Thus, priming of T cells and rapid migration into the infected lung are of critical importance in protection from TB (Young *et al*. 2008). After BCG vaccination, memory T cells that are specific for mycobacterial antigens will be generated. The priming of specific T cells requires efficient presentation of mycobacterial antigens, and both *M. tuberculosis* and BCG are able to interfere with these processes to persist in the host (Marino and Kirschner, 2004).

M. tuberculosis can survive within macrophages as it is able to interfere with membrane trafficking and arrest the maturation of the phagosome by inhibition of phagosome-lyzosome fusion. This protects the pathogen from degradation and maintains the delivery of nutrients to the *M. tuberculosis*-containing vacuole. This is

critical for propagation and persistence of *M. tuberculosis* in the human body (Vergne *et al.* 2004).

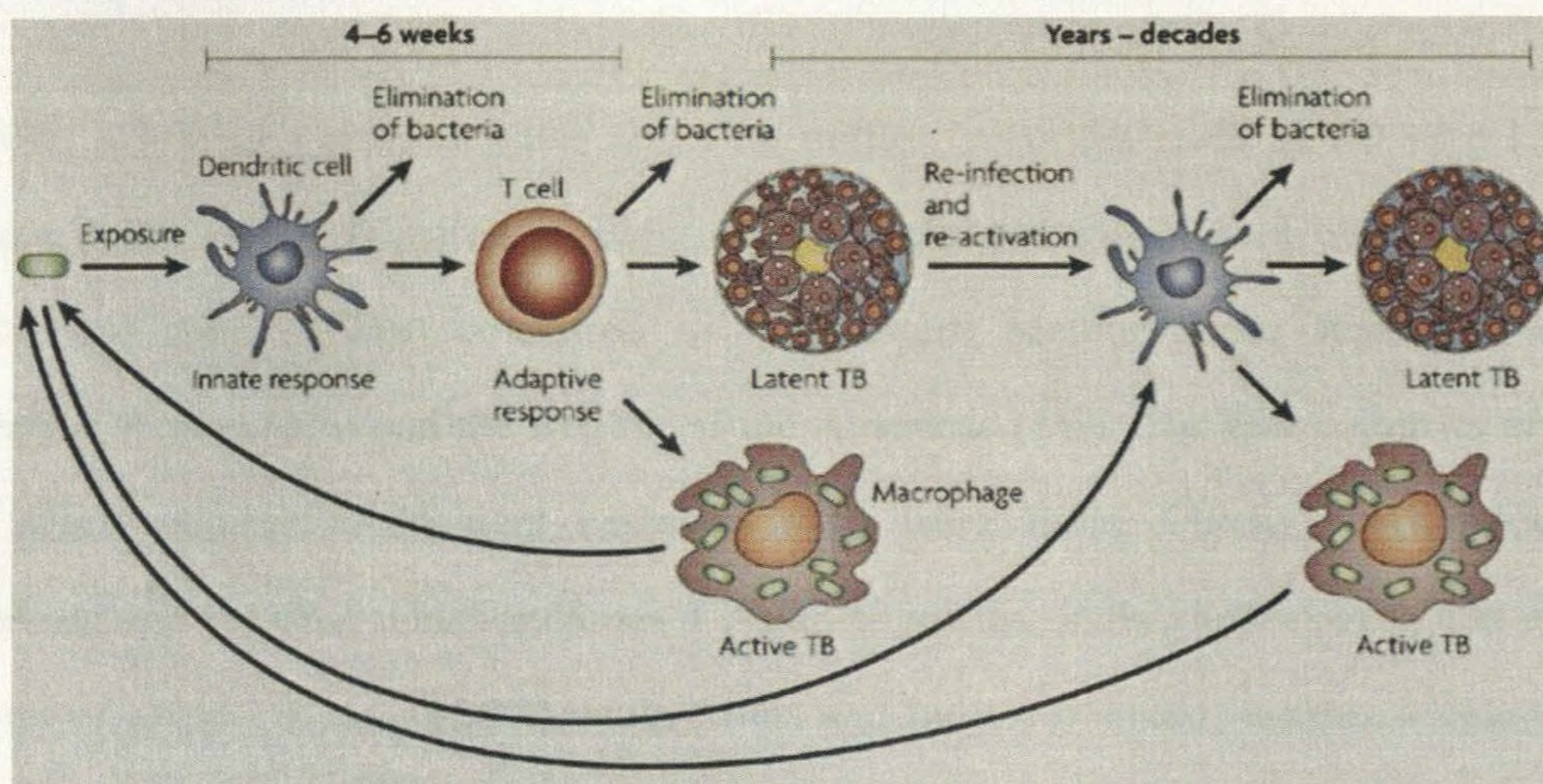


Figure1.3 Activation of immune responses after exposure to *M. tuberculosis.* Macrophages are key effector cells in mycobacterial killing, but can also provide a niche for bacterial multiplication. Dendritic cells engulf bacteria and control the infection by activating T cells. Bacteria can persist within granulomas and cause latent TB. If the T-cell response is insufficient to control the initial infection, clinical symptoms will develop within ~1 year (Young *et al.* 2008).

1.3 Burden of tuberculosis

1.3.1 Global burden

The burden of tuberculosis is being measured in terms of incidence, prevention and mortality. It can be expressed as the account of illness as well as mortality and the disability-adjusted life years lost (WHO, 2011). A one third of the world's population is infected with TB and someone dies from TB every 15 seconds. The vast majority of TB deaths are in the developing countries with the contribution of more than half of all

deaths from the Asian region. The greatest TB burden per population (over 350 cases per 100,000 population) and highest number of deaths per population was in Africa by 2009 (WHO Fact sheet, 2010).

In 2010, 8.8 million incident cases and 12.0 million prevalent cases of TB with 1.45 million deaths were estimated globally. Most of the estimated cases occur in Asia (59%) and Africa (26%) compared to the Eastern Mediterranean Region (7%), European Region (5%) and the Region of the Americas (3%). The five countries with the largest number of incident cases in 2010 were India, China, South Africa, Indonesia and Pakistan which includes 4 Asian countries. India alone contributed one quarter of world TB cases (26%) while China and India combined together accounted for 38% (Figure 1.4) (WHO, 2011).

Globally, in 2010, 6.2 million people were diagnosed as having TB including 5.4 million new cases and 0.3 million recurrent incidence after being cured of TB in the past. Besides 0.4 million were retreatment cases due to treatment failure or interruption. In remaining (0.1 million), the history of treatment was not recorded. Among new TB cases, 4.6 million were pulmonary TB cases (2.6 million of sputum smear-positive and 2.0 million sputum smear-negative) and 0.8 million were extra pulmonary TB cases. Case notification has been increased with involvement of public-private and public-public mix in controlling of TB. Globally, the number of TB cases diagnosed and notified per 100,000 population has stabilized since 2008 and clear gap exists between the numbers of notified and the estimated cases (WHO, 2011).

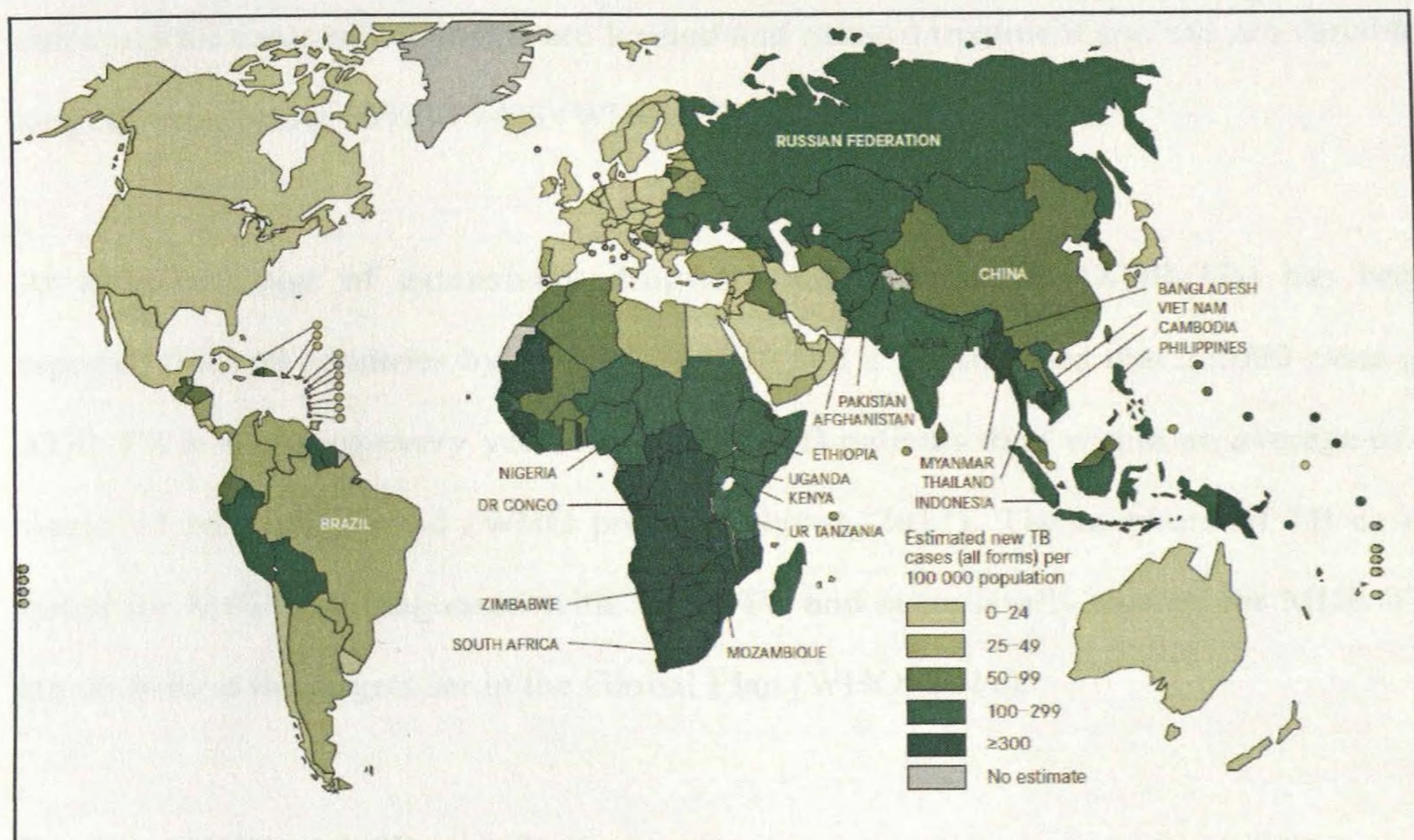


Figure 1.4 Estimated TB incidence rate, 2010 (WHO, 2011). Highest incident rate is estimated in Asia and most of African countries have ≥ 300 estimated new TB cases per 100,000 population.

1.3.2 Burden of drug resistant tuberculosis

About 440,000 multi drug resistant tuberculosis (MDR-TB) cases are estimated to emerge each year and 150,000 persons are estimated to die from MDR-TB. The four countries that had the largest number of estimated cases of MDR-TB were China, India, the Russian Federation and South Africa (WHO, 2010). In 2010, there were an estimated 650,000 cases of MDR-TB including 290,000 cases of pulmonary TB. However, the actual diagnosis of MDR-TB is less than 10% in most of the countries that have high MDR-TB estimation (WHO, 2011).

In 2010, only 16% of estimated MDR-TB cases (45,553 patients) were enrolled on treatment for recommended second-line drug regimens. National data on treatment

outcomes for cases of MDR-TB are limited and rates of treatment success are variable, ranging from below 50% to 74% (WHO, 2011).

At least one case of extensively drug-resistant tuberculosis (XDR-TB) has been reported from 69 countries by the end of 2010 and it is estimated that 25,000 cases of XDR-TB is emerging every year. Most XDR-TB patients died within an average of 3 weeks of being diagnosed (WHO progress report, 2011). The numbers of TB cases tested for MDR-TB, diagnosed with MDR-TB and successfully treated for MDR-TB lag far behind the targets set in the Global Plan (WHO, 2011).

1.3.3 Burden of HIV and TB co-infection

TB and HIV are frequently referred to as co-or dual-epidemics due to their high rate of co-infection. Since the 1980s HIV has been largely responsible for the resurgence of the TB epidemic becoming a massive challenge for global TB control. As TB is harder to diagnose and progresses more rapidly within immunocompromised patients, TB is a leading cause of death among people with HIV, especially in developing countries (WHO Fact sheet, 2010). People co-infected with TB and HIV are 21-34 times more likely to develop active TB disease than People without HIV. The highest rates of HIV co-infection in TB patients are in the African region, (44%) followed by the regions of the America (17%) (WHO, 2011). In 2010, 1.1 million new TB cases were among people living with HIV and approximately 82% of them were in the African region. Globally, there was an estimated 0.35 million deaths from TB among people who were HIV-positive and 34% of notified TB cases were aware of their HIV status (WHO, 2011).

1.3.4 TB in women and children

In 2010, there were 3.2 million estimated TB cases (36%) among women (WHO, 2011). Globally, 700,000 women died from TB including 200,000 of HIV-TB co infected cases in 2008. In many countries, even though men carry more of the TB burden, higher TB incidences are detected among women in some settings such as Afghanistan, and in parts of Pakistan. Women of reproductive age are more susceptible to develop TB than men of the same age and TB can cause infertility. Globally, TB is the third leading cause of death among women in reproductive age. The feminization of the HIV epidemic has led to a greater burden of TB among women (Tuberculosis Women and TB, 2009).

The percentage of prevalence of childhood TB cases varies from 3% to more than 25% of estimated cases worldwide. Of these childhood cases, 75% occur annually in high TB burden countries (WHO, 2006). TB is the cause of death for around 100,000 children worldwide in each year and the majority of these cases occur in low income countries where the prevalence of HIV is high (WHO, 2011).

1.3.5 TB in Sri Lanka

The population of Sri Lanka is about 21 million and is considered a low TB prevalence country in the Asian region. In Sri Lanka, the estimated incidence rate of all forms of tuberculosis in 2010 was 66 per 100,000 population and the case notification was 95% of the total. In 2010, 9328 new TB cases (6780 pulmonary cases and 2548 extra pulmonary cases) had been notified and 4635 among them were

sputum smear positive TB cases (WHO country profile, 2012). Total of 380 retreatment cases were notified including 219 relapse cases, 62 treatment after failure and 99 treatment after default by 2010. HIV co-infection rates among TB patients are currently estimated to be less than 0.1% (WHO regional report, 2011).

In Badulla, Batticaloa, Monaragala, Jaffna and Ampara districts, the numbers of smear negative cases were higher than the smear positives cases in 2009. The highest numbers of TB cases as well as new smear positive cases were reported from Colombo district while the lowest was reported from Mannar in 2009 (NPTCCD Annual Report, 2009).

The drug resistant rate in Sri Lanka is low and drug resistance among new TB patients is 0.2% and 18%–21% among re-treatment cases (WHO regional report, 2011). The estimated MDR-TB cases among notified new and retreatment pulmonary TB cases were 14 and 6.1 respectively in 2010. Further, 11 cases (5 new and 6 retreatment cases) confirmed as MDR-TB after testing of suspected 1221 cases (839 new cases and 378 retreatment cases) (WHO country profile, 2012). In year 2009, the overall treatment success rate was 86% among registered new smear positive cases, 88% in smear negative and extra pulmonary and 73% among re-treatment patients (WHO regional report, 2011).

The majority of new TB cases were among 45-54 years age group while the lowest numbers were seen in 0-14 years age group. In 2009, the proportion of infected males was higher than the females among all age groups. During the last five years, the

number of infected females was more than the males in the age group of 15-24 (NPTCCD annual report, 2009).

1.4 Diagnosis of tuberculosis

Pulmonary TB is suggested by chest radiography and clinical symptoms such as persistent productive cough for three weeks or longer, blood stained sputum, weight loss, night sweats and chest pain. Reliable diagnosis can be made by demonstrating the presence of tubercle bacilli in the sputum or other specimen from an infected site of the body by means of microscopy and culture in the laboratory (De Kantor *et al.* 1998). Several molecular based automated methods have also been developed recently for the rapid and accurate laboratory diagnosis of TB (Palomino *et al.* 2007).

1.4.1 Direct microscopic observation

Mycobacteria appear as red colour rods under light microscopy after Zeihl-Neelsen (ZN) staining. Microscopy for AFB is the gold standard for the diagnosis of active TB especially in resource poor settings (De Kantor *et al.* 1998). AFB smear microscopy is a rapid, inexpensive method of laboratory diagnosis and useful to identify highly infectious smear positive TB patients. However, the specificity and sensitivity of the method is low (Somoskovi *et al.* 2001) especially in the diagnosis of extra pulmonary TB cases (De Kantor *et al.* 1998). This technique is based on the acid fast nature and high lipid content of the wall of bacteria. It resists decolourization by 3% acid-alcohol (HCl and ethanol) after primary staining. Auramine O staining is more sensitive than

Zeihl- Neelsen but requires a fluorescent microscope for observation (Murray *et al.* 2003).

The sensitivity of AFB microscopy is influenced by numerous factors (Lipsky *et al.* 1984) such as prevalence and severity of disease, type of specimen, quality of specimen (McCarter *et al.* 1996), number of mycobacteria present in the specimen, quality of the examination (De Kantor *et al.*1998), method of processing (direct or concentrated) and method of centrifugation (Rickman, *et al.* 1980; Perera *et al.* 1999). The minimum number of bacilli that need to be present for detection by stained smears has been estimated to be 5,000-10,000/ ml of sputum. However, AFB microscopy is unable to differentiate mycobacteria into species level based on microscopic morphology (De Kantor *et al.* 1998).

1.4.2 Cultures based identification

Different egg-based media, agar-based media and liquid media are used for cultivating tubercle bacilli. Most conventional solid media such as Lowenstein-Jensen (L-J) and Ogawa medium are egg based and contain high concentrations of malachite green to prevent contamination from other bacteria (De Kantor *et al.* 1998). Middlebrook 7H10, 7H11 (agar based solid media) and Middlebrook 7H9 (broth media) are available in powder form. Addition of growth supplement (OADC- mixture of oleic acid, albumin, dextrose and catalase) and antibiotics (PANTA- mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) will enhance the growth of pure *Mycobacterium* cultures on Middlebrook media (De Kantor *et al.* 1998). Slightly higher isolation rate has been detected on Middlebrook medium than

egg-based media (Flournoy *et al.* 2001). However, Middlebrook media require incubation in a 5% to 10% CO_2 atmosphere for achieving better sensitivity and are more expensive than L-J medium (De Kantor *et al.* 1998). Additionally, blood agar media is a good alternative for L-J medium for rapid isolation of *M. tuberculosis* from sputum in low resource settings. *M. tuberculosis* grows within one to two weeks on blood agar plates with significantly higher number of colonies than L-J medium (Mathur *et al.* 2009; Drancourt *et al.* 2003). Culture is more sensitive than microscopy as it detects low numbers of viable organisms and generally 10-100 viable bacilli per ml of sputum are adequate to produce colony forming units (CFU) on a culture medium (Monoca *et al.* 1999).

In the last two decades, semi-automated and fully automated liquid culture methods such as BACTEC TB-460 system, Mycobacteria Growth Indicator Tube (MGIT), Bact/Alert, ESP Mice, MB Redox and KRD Niche B, biphasic Septic-Check AFB and Mice-Acid and BACTEC MGIT960 have been introduced for cultivation of mycobacteria. A satisfactory culture isolation rate can be achieved within a short time by using these automated liquid culture systems (Palomino *et al.* 2007).

In the automated BACTEC TB-460, a modified Middlebrook 7H9 medium containing ^{14}C radio labeled palmitic acid is used. The vials containing the medium remain sealed through the entire culture process. The specimen is inoculated by puncturing the rubber septum with a sterile needle and the growth of bacteria is detected radiolabeled CO_2 (Middlebrook, 1977).

Manual or automated MGIT have designed for the rapid detection of mycobacteria in all types of clinical specimens except blood and urine. MGIT contains modified Middlebrook 7H9 with a silicon film embedded ruthenium salt at the bottom of the tube as a fluorescence indicator. This fluorescence compound is sensitive to the presence of dissolved oxygen in the broth medium. The oxygen normally present in the medium quenches the natural fluorescence of the ruthenium salt. In the presence of bacterial growth, oxygen is consumed due to their metabolism. The quenching effect lowers accordingly, and the bottom of the tube fluoresces when exposed to ultraviolet light. Growth supplement OADC and PANTA antibiotic mixture are added to the tube before inoculation of the decontaminated sample. The tubes are incubated at 37°C and tested daily with a UV lamp from the second day of inoculation (Somoskovi *et al.* 2000). However, the high contamination rate in MGIT cultures has been recorded as a major drawback (Lee *et al.* 2003).

VersaTREK is a good alternative to automated MGIT 960 in isolation of *M. tuberculosis* with low rate of contamination (Yuksel *et al.* 2011). The technology of the VersaTREK/ESP culture system is based on the detection of headspace pressure changes within a sealed bottle. It indicates presence of viable mycobacteria by monitoring change of internal pressure either due to gas production or gas consumption and special algorithm has been developed for detection of very slow growing Mycobacteria (Gravet *et al.* 2010).

However, the ideal medium for isolation of tubercle bacilli should be economical, simple to prepare from readily available ingredients, inhibit the growth of contaminants, support luxuriant growth of a small number of bacilli and permit

preliminary differentiation of isolates on the basis of colony morphology (De Kantor *et al.* 1998).

1.4. 3 Nucleic acid based diagnostic methods

DNA amplification by PCR is a more reliable rapid method for detection of *M. tuberculosis* in clinical samples (Kox *et al.* 1994) and variety of *in house* PCR methods have been developed for diagnosis of pulmonary and extra pulmonary TB. Insertion elements IS6110 (Githui *et al.* 1999) and IS1081 (Bahador *et al.* 2005) that are present in multiple copies and some signal copy genes such as the gene encoding 65 kDa heat-shock protein (Baba *et al.* 2008), the gene encoding 126 kDa fusion protein (Palomino *et al.* 2007), and the gene encoding the β-subunit of RNA polymerase (Kim *et al.* 2004) are common amplification targets for detection of MTC. As PCR based methods more sensitive and rapid compared to culture and it is more reliable especially for final diagnosis of EPTB (Magana-Arachchi, 2001). However, relatively low sensitivity has been observed in the diagnosis of smear negative pulmonary TB cases (Parvez *et al.* 2003).

Further, the nested PCR and the Real Time-PCR have been introduced as powerful tools for rapid and accurate diagnosis of tuberculosis meningitis (Takahashi and Nakayama, 2006). However, the occurrence of false-positive results is known to be a major challenge in the interpretation of results of PCR based methods (Honore-Bouakline *et al.* 2003).

Recently, several PCR based commercial methods such as Amplified Mycobacterium Tuberculosis Direct test (AMTD) (Bradley *et al.*1996), the AccuProbe system (Ichiyama *et al.* 1997), INNO LiPA Mycobacteria (Perandin *et al.* 2006), GenoType Mycobacterium (Kiraz *et al.* 2010) and GenoType MTC (Richter *et al.* 2003) test have been made available. In all these methods, specific gene target that allows species differentiation is amplified and hybridized with the specific probes. The hybridization detection method is specific to each test. For example, in AMTD test, the amplified fragment of 16S rRNA gene of *M. tuberculosis* is detected by hybridization with chemiluminescent acridinium ester-labeled probes (Ichiyama *et al.* 1996; Barrett *et al.* 2002).

DNA sequencing of an amplified specific gene fragment is the reference standard molecular identification method of *M. tuberculosis* in clinical specimens (Kim *et al.* 1999). Additionally, PCR- restriction fragment length polymorphism (PCR-RFLP) using gene markers such as *hsp65* (Varma-Basil *et al.* 2010), *rpoB* (Lee *et al.* 2000), *gyrB* (Goh *et al.* 2006) & *pncA* (Bannalikar and Verma, 2006) can be used in the identification of mycobacterial species. In this method, a selected gene fragment is PCR amplified and digest with restriction enzymes such as *BstEII* and *HaeIII*. The products of the digestion reaction are separated and visualized by agarose gel electrophoresis (Lee *et al.* 2000).

1.5 Chemotherapy for tuberculosis and drug resistance

1.5.1 Anti TB drugs and treatment regimens

Treatment for TB commenced in 1944 with the discovery of Streptomycin (SM) and later development of new antibiotics improved the efficacy of the treatment and reduced the duration of the treatment. The present 6 month short course therapy was started with introduction of rifampicin (RIF) and pyrazinamide (PZA) since 1980 (palomino *et al.* 2007). The rationale of treatment of TB is two-fold, killing of bacteria for prevention of the transmission and elimination of bacteria from the body for preventing relapses (Centers for Disease Control and Prevention, 2003). Thus, the anti tuberculosis drugs have 3 main properties; bactericidal activity, sterilizing activity and ability to prevent resistance development which are essential properties of anti tuberculosis drugs. These properties are manifested to different extents by different drugs (WHO, 2003).

The newly identified TB patients (A patient who has never been treated for TB or who has taken anti tuberculosis drugs for less than four weeks) are treated with combination of first line anti tuberculosis drugs including isoniazid (INH), ethambutol (EMB), RIF, PZA and SM. RIF and INH are most powerful bactericidal drugs against all populations of bacteria and RIF has powerful sterilizing ability too. PZA and SM are bactericidal drugs active against certain population of TB bacilli. PZA is active in an acid environment and SM acts only on rapidly multiplying TB bacilli. EBM are used to prevent the emergence of resistant bacilli (WHO, 2003). However, EMB is not used in children and RIF is not recommended for the paediatric cases. The recommended doses for first line drugs are given in Table 1.1.

Second-line drugs include the aminoglycosides (kanamycin and amikacin), the polypeptide (capreomycin, PAS, cycloserine), the thioamides (ethionamide and prothionamide) and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin (WHO, 2003; WHO, 2010).

Table 1.1 Recommended doses of first line anti tuberculosis drugs for adults (WHO, 2010)

Drug	**Recommended doses**			
	Daily		**3 times per week**	
	Dose and range (mg/kg body)	**Maximum (mg)**	**Dose and range (mg/kg body)**	**Daily maximum weight (mg)**
Isoniazid	5(4-5)	300	10 (8-12)	900
Rifampicin	10 (8-12)	600	10 (8-12)	600
Pyrazinamide	25(15-20)	-	35 (30-40)	-
Ethambutol	15(15-20)	-	30 (25-35)	-
Streptomycin*	15(12-18)		15 (12-18)	1000

* For patient over 60 years and patient weighting < 50kg modified doses are Administered

The current, 6 month TB treatment course is divided into 2 phases. The initial phase that last 2 months for rapid killing of bacilli is followed by a continuation phase for killing of any remaining or dormant bacilli. The drug regimen and duration of the treatment may vary with the response of patients to drugs. As an example, the treatment of new TB cases consists of a two-month intensive phase and four-month continuation phase while retreatment cases are treated with a 3 month intensive phase followed by a five months continuation phase (Table 1.2) (WHO, 2003; WHO, 2010). Relapse cases, treatment failure cases and treatment after interruption (default) cases should be treated with at least 4 anti TB drugs, including second line injectable drugs.

An initial phase of at least 6 months should be followed by a continuation phase of 12-18 months for these patients.

Directly observed treatment short course (DOTS) is a WHO recommended strategy for TB treatment. In DOTS, the patients should be treated under the observation of recognized health care person and it prevents the interruption of treatment. Thus, it has been recognized as a highly efficient and cost-effective plan. DOTS has been promoted as a global strategy since the mid-1990s. Countries applying DOTS on a wide scale have witnessed remarkable results including reduction of TB transmission, TB deaths and drug resistance (WHO, 2003).

Table 1.2 Recommended drugs and duration of chemotherapy for different categories of TB patients (WHO, 2010; WHO, 2003)

Type of Patient	**Intensive phase**		**Continuation Phase**	
	Drug	**Duration**	**Drug**	**Duration**
New smear –positive PTB New smear- negative PTB New severe extra pulmonary TB	H,R,Z,E	2 months daily	H,E,R	4 Months daily
Retreatment cases-smear positive	S,H,R,Z, E	3 months daily with S given only for 1st two months	H,R,E	5 months daily
Chronic(still sputum-positive after re-treatment) or suspected MDR TB cases	Specially designed standard or individual regimen			

* H= isoniazid, R= rifampicin, Z = pyrazinamide, E= ethambutol, S = streptomycin
PTB= pulmonary tuberculosis

1.5.2 Drug resistance of *M. tuberculosis*

Drug resistance may be defined in the light of the probable response of the patient to chemotherapy with the concerned drug (Aziz *et al.* 2003). The emergence of drug resistance in TB undermines the efficacy of treatment in individuals and control programs in populations. As multiple drug resistance of TB is often attributed to sequential monotherapy, the standard initial treatment regimens that contain 4 different antibiotics have been designed. Drug resistant bacilli have emerged in many settings even though combination therapy seemed to be sufficient for controlling the disease (Colijn *et al.* 2011). Previously, drug resistant TB has been described as a man-made amplification of a natural phenomenon (Pablos-Mendez *et al.* 1997). However, it may not be an entirely man-made marvel and drug resistant forms of TB have independently emerged in many settings. As per current findings, probability of drug resistant at the time of diagnosis (new patients) may be 1000–10,000 times higher than previously suggested (Colijn *et al.* 2011). Treatment with a single drug, irregular drug supply, inappropriate prescription and poor adherence to treatment permit further multiplication of drug-resistant strains (Pablos-Mendez *et al.* 1997).

Drug resistant cases require second line drugs that are more costly, more toxic, less effective and have more side effects. Recently, XDR-TB has emerged and poses an important challenge to the control of TB (WHO, 2011).

The important definitions with reference to drug-resistance are listed in Table 1.3 (WHO, 2011).

Table 1.3 Definitions with reference to drug-resistance (WHO, 2011).

Category of resistance	**Definition**
Primary resistance	Theoretical concept that refers to transmission of a drug-resistant strain to TB patients who have not been previously treated
Resistance among new cases	Drug-resistant TB cases with less than one month of treatment. This is a proxy for primary or initial resistance
Acquired resistance	Theoretical concept that refers to the development of resistance among patients diagnosed with TB who have received anti-TB treatment for more than one month. In the past, resistance among previously treated cases was used as a proxy of acquired resistance. Currently, this category also includes patients re-infected with a resistant strain, those with treatment failure and relapse. Therefore, the only way to truly define acquired resistance cases is to determine the DST pattern before and after the start of treatment.
Multidrug-resistant TB	Resistance to at least INH and RIF, the most effective first-line drugs
Extensively drug-resistant TB	Resistance to at least INH and RIF with resistance to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin

1.5.3 Mechanisms of drug resistance

M. tuberculosis characteristically develops natural genetic resistance to anti-TB drugs without exposure to any antibiotics. However, with the presence of cytotoxic drugs, resistance can arise in many ways including alteration of the drug target protein, decreasing membrane permeability and reducing drug metabolism.

1.5.3. Natural drug resistance

The natural resistance develops due to random, spontaneous mutations during bacterial multiplication with a defined frequency. Unlike many other bacteria, *M. tuberculosis* does not show evidence of horizontal gene transfer that leads to acquisition of resistant plasmids or transposons (Pablos-Mendez *et al.* 1997).

Genetic mutations that donate RIF resistance of *M. tuberculosis* occur at a rate of 10^{-10} per cell division and lead to an estimated prevalence of 1 in 10^8 bacilli in drug-free environments. The resistant mutation rate for INH is approximately 10^{-7} to 10^{-9} per cell division resulting in a prevalence of 1 out of 10^6 bacilli in drug-free environments (David, 1970). Thus, spontaneous occurrence of genetic resistance is diluted by the majority of drug-susceptible micro-organisms in a drug free environment. This suggests the presence of a large bacterial load such as in lung cavities is required for emergence of natural MDR strain. The existence of antimicrobials delivers the selective pressure for resistant organisms to become predominant and lead the rising numbers of MDR strains (Dots-plus for standardized management of multidrug-resistant tuberculosis in South Africa - policy guidelines, 2004). Figure 1.5

demonstrates the development of primary and acquired drug resistance due to the spontaneous mutations and selective pressure of the treatment.

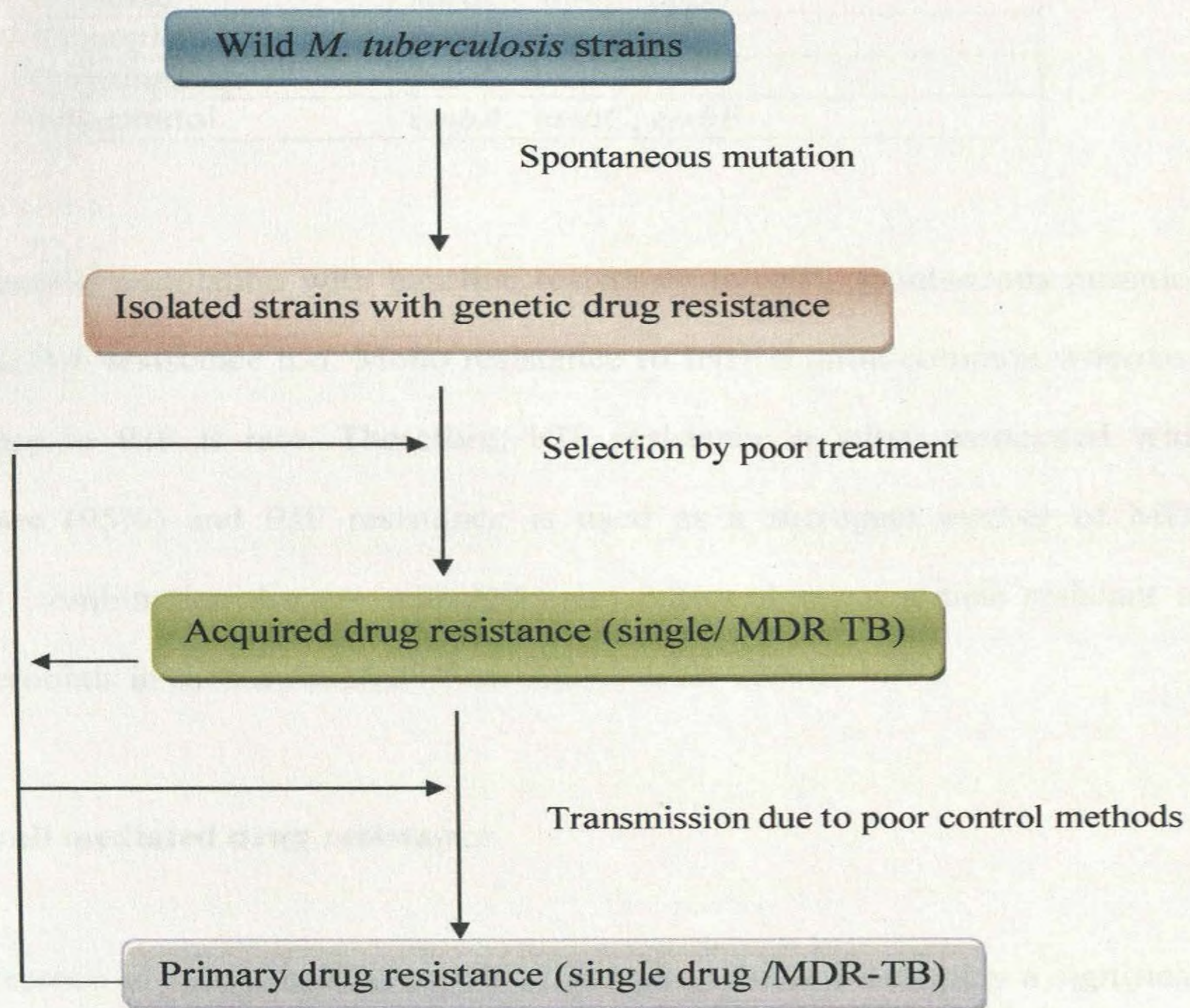


Figure 1.5 The schematic diagram displaying the development and spread of drug resistant tuberculosis (Pablos-Mendez *et al.* 1997)

The multi-drug resistant phenotypes are mounted by sequential accumulation of point mutations in different genes that interfere with each anti tuberculosis drug activity (Table 1.3) (Zhang and Telenti, 2000; Silva *et al.* 2003; Tessema *et al.* 2012; Jureen *et al.* 2008). So far, there is no evidence for pleiotropic mutation that is responsible for MDR-TB.

Table 1.4 Genetic markers responsible for resistant of first line anti TB drugs

Drug	Genetic marker of drug resistant
Isoniazid	*katG, inhA, ahpC*
Rifampicin	*rpoB*
Pyrazinamide	*pncA*
Ethambutol	*embA, embC, embB*

In a bacterial population with baseline resistance to INH, spontaneous mutation may result in RIF resistance too. Mono resistance to INH is quite common whereas mono resistance to RIF is rare. Therefore, RIF resistance is often associated with INH resistance (95%) and RIF resistance is used as a surrogate marker of MDR-TB. Further, combination therapy with INH and RIF will select strains resistant to both antimicrobials in such a situation (Somoskovi *et al.* 2001).

1.5.3.2 Cell wall mediated drug resistance

The presence of active multi-drug efflux pumps in the cell wall play a significant role in development of natural and induced drug resistance in mycobacteria (Gupta *et al.*2006). Efflux-mediated drug resistance in *M. tuberculosis* could be due to one or more efflux pumps working alone or in coordination. Several ATP-binding cassettes (ABC) major facilitator family (MFS), resistance-nodulation-division (RND) and the small multidrug resistance (SMR) efflux pumps have been characterized as antibiotic transporters in mycobacterial cell wall (Meenakshi *et al.* 2012; Pasca *et al.* 2004; Pasca *et al.* 2005; De Rossi *et al.*1998). At least 14 members of MFS and 26 ABC transporters are encoded by *M. tuberculosis* genome while ABC transporters account for 2.5% of the whole genome (Cole *et al.* 1998; Braibant *et al.* 2000). Differences in

efflux pump expression between mycobacterial species are important because they offer insights into the acquisition of drug resistance (Sarathy *et al.* 2012).

The exposure to various anti-tuberculous drugs can trigger expression of selected efflux pumps and it leads to drug-mediated phenotypic resistance. According to De Steenwinkel *et al.* 2010 isoniazid resistant bacterial population that emerged as the subsequent re-growth of susceptible population represented efflux pump-mediated drug tolerance. Further, isoniazid susceptible and rifampicin mono-resistant *M. tuberculosis* strains can develop resistance to isoniazid within three weeks of exposure and this can be effectively prevented by efflux pump inhibitors (Machado *et al.* 2012). The higher expression of pump-encoding genes such as *mmpl7, p55, efpA, mmr, Rv1258 and Rv2459* (Machado *et al.*2012) can be due to the transitory induction by the substrate of these pumps or mutations in the promoter and regulatory region (De Rossi *et al.*2006; Nguyen and Thompson, 2006). The simultaneous expression of *Rv2459, Rv3728* and Rv3065 gens is associated with resistance to the combination of isoniazid and ethambutol (Gupta *et al.* 2010).

1.5.2.3 Specific molecular mechanisms for anti TB drug resistance

There is a clear correlation between the genetic mechanism and the resistant phenotype. Resistance conferring chromosomal alterations are highly restricted to the genes that are targeted by the drug. Mutations in *rpsL* (streptomycin), *rpoB* (RIF) or *16S* ribosomal RNA (kanamycin, amikacin; 2-deoxystreptamine aminoglycosides) are related with high- level drug resistance while mutations in *gldB* (streptomycin), *eis*

(kanamycin), and *inhA* (INH) confer a low-level resistant phenotype (Zhang and Yew 2009).

Isoniazid

Isoniazid (INH) is highly specific antimycobacterial agent against the *M. tuberculosis* complex. It is a prodrug which needs to be converted into active form by the bacterial enzyme catalase peroxidase (*KatG*) (Zhang *et al.* 1992). Activated INH interferes with the synthesis of essential mycolic acids by inhibiting enoyl- acyl carrier protein reductase that is encoded by *InhA* (Rawat *et al.* 2003). Thus, mainly INH resistance is genetically heterogeneous as it may be raised with mutations in *katG* and mutations in *inhA*, or more frequently in its promoter region (Silva *et al.* 2003). Additionally, mutations in *ahpC, kasA* and *ndh* genes have been associated with isoniazid resistance (Zhang *et al.* 1992). However, in clinical isolates, the mutations in *InhA* are associated with low level resistance to INH while mutations in *katG* confer moderate to high level resistance.

More than hundred mutations including missense and nonsense mutations, insertions and deletions in *katG* have been reported with MICs ranging from 0.2 to 256 mg/L. Further, full gene deletion has been detected more rarely. The most prevalent mutation is at amino acid position 315 of *KatG* (serine → threonine) and it accounts for more than 60-80% of INH resistance in clinical isolates (Leung *et al.* 2006) with MIC values ranging from 2 to >10 mg/l INH. Also, this mutation occurs more frequently in MDR than in isoniazid mono resistant strains (Hazbon *et al.* 2006). The most common *inhA* mutation occurs in its promoter region (215C→T) and it has been found more frequently to be associated with mono-resistant strains (Leung *et al.*

2006). However, the mutation profile for INH resistance may vary geographically and diagnostic tests should be adapted accordingly.

Rifampicin

Rifampicin is the key element of short course anti-tuberculosis therapy. It binds with the RNA polymerase β subunit that is encoded by *rpoB* gene and inhibits the elongation of messenger RNA. Majority of *M. tuberculosis* clinical isolates resistant to RIF show mutations in *rpoB* gene and it leads conformational changes in the RNA polymerase that determine a low affinity for the drug (Campbell *et al.* 2001). More than 90% of rifampicin-resistant *M. tuberculosis* isolates confer mutations within the hot-spot region of 81bp of *rpoB* gene and mutations at codon 531 and 526 are the most frequently reported point mutations responsible for RIF resistance (Mani *et al.* 2001; Zhang and Telenti, 2000; Ramaswamy and Musser, 1998). Also, studies have reported mutations outside of the hot-spot region of *rpoB gene* in RIF resistant *M. tuberculosis* isolates (for further details refer chapter 5). Cross resistance between RIF and other rifamycins may occur. As an example, mutations in codons 516, 518, 522, 529 and 533 are associated with low-level resistance to rifampicin while susceptible to rifabutin and rifalazil (Yang *et al.* 1998).

Pyrazinamide

Pyrazinamide inhibits semi dormant bacilli residing in acidic environments by disrupting bacterial membrane energetics and inhibiting membrane transport (Mitchison, 1985). It is a pro-drug and activate into pyrazinoic acid (activated from) by the enzyme pyrazinamidase/nicotinamidase (PZase) (Konno *et al.* 1967). PZase in *M. tuberculosis* is encoded by the *pncA* gene (Scorpio and Zhang. 1996) and mutations

in *pncA* are the main mechanisms for pyrazinamide resistance in *M. tuberculosis*. Most alterations occur in a 561 bp region of the open reading frame or in an 82 bp region of its putative promoter (Jureen *et al.* 2008). High degree of diversity of mutations in *pncA* gene had been observed among pyrazinamide resistant strains. However, some clinical pyrazinamide resistant strains do not show mutations in *pncA* or its promoter region but may occur due to mutations in an unknown *pncA* regulatory gene (Cheng *et al.*2000)

Streptomycin

In prokaryotes, streptomycin (SM) interferes with the protein synthesis during translation by binding to the 30S subunit of bacterial ribosome (Moazed and Noller 1987). In *M. tuberculosis*, the genetic basis of resistance to streptomycin is mostly due to mutations in *rrs* or *rpsL* gene, which produce alterations in the streptomycin binding site (Gillespie, 2002). The site of action of SM is the ribosomal protein S12 and the *16S* rRNA of the 30S subunit of the ribosome. Thus, resistance is caused by mutations in the S12 protein encoded by *rpsL* gene and *16S* rRNA encoded by *rrs* gene (Honore and Cole, 1994; Finken *et al.* 1993). Mutations of the *rrs* gene occur in the loops of the *16S* rRNA and are clustered in two regions around nucleotides 530 and 915. The most common mutation in *rpsL* is K43R79 (Nair *et al.*1993). However, such mutations are identified in slightly more than 50% of the clinical strains studied to date (Gillespie, 2002) and clinical isolates showing low-level resistance have no mutation in *rpsL* or *rrs*. Further, mutations in *gidB*, which encodes a conserved 7-methylguanosine methyltransferase specific for the *16S* rRNA confer a low level of streptomycin resistance (Okamoto *et al.*2007).

Ethambutol

Ethambutol interferes in the biosynthesis of cell wall arabinogalactan (Takayama *et al.* 1979) and arabinosyl transferase, an enzyme involved in the synthesis of arabinogalactan that is encoded by *embB* gene. Nearly, 50% of ethambutol resistant clinical isolates are associated with mutations at codon 306 of *embB* gene (Telenti *et al.* 1997) and it is responsible for low-level of resistance. Also, mutations at *embB*406 and *embB*497 play an important role in presenting ethambutol resistance. However, certain amino acid substitutions in *embB* gene have little or no effect on ethambutol resistance (Safi *et al.* 2002). It is reported that a significant percentage of ethambutol-resistant isolates are not associated with mutations in *embB* and there must be other mechanisms for ethambutol resistance that has not yet been described (Shi *et al.* 2011).

Fluoroquinolones

In *M. tuberculosis*, targets of the quinolones are the DNA gyrase (type-II DNA topoisomerase) that is composed of two A and two B subunits encoded by genes *gyrA* and *gyrB* respectively (Takiff, 1994). High-level resistance to fluoroquinolones in clinical strains of *M. tuberculosis* is due to the amino acid substitutions in the putative fluoroquinolone binding region of the *M. tuberculosis* (Aubry, 2004; Cambau 1994). The most prevailing mutation is Asp-94 (Zhu *et al.* 2012; Cheng *et al.* 2004) in the conserved region of *gyrA* while Ala-75, Gly-88, Ala-90 and Ser-91 have also associated with fluoroquinolones resistance (Pitaksajjaku *et al.* 2011). The polymorphism occurring at Codon 95 of *gyrA* gene is not related to fluoroquinolone resistance and the presence of mutations at position 80 of *gyrA* gene lead to

hypersusceptibility, especially if present together with other resistance mutations (Aubry *et al.*2006).

1.5.3.4 Fitness of bacteria and drug resistance

As mutations conferring drug resistance usually affect replication of resistant bacteria, they have to compete with sensitive bacteria in the absence of antibiotics. Antibiotic resistance therefore confers a reduction in fitness as the efficiency of multiplication of the mutant cell is less compared to that of the wild-type strain. The reduction of fitness is expressed as reduced growth, virulence or transmission of bacteria (Andersson, 2006). Thus, microorganisms require a physiological cost for the acquisition of drug resistance and the outcome of the competition process depends on its relative fitness. This is a disadvantage for propagation of drug resistant bacteria. Therefore, the relation between drug resistance and fitness cost may contribute to the elimination of resistant bacteria from a bacterial population (Andersson and Levin, 1999). As an example RIF resistant isolates habouring *rpoB* gene mutations have less fitness cost *in vitro* (O'Sullivan *et al.* 2005) suggesting there is a possibility for reversion to susceptibility (Mariam *et al.* 2004). However, there are limited data available on the fitness cost in *M. tuberculosis* (Gagneux, 2006; Mariam *et al.* 2004) and these studies have been based mainly on *in vitro* models or non-isogenic strains.

1.6 Scope of the thesis

The molecular drug susceptibility testing is based on the detection of mutations that are responsible for resistance of each drug. The available commercialised molecular DST methods for detection of RIF resistance have been customized to detect the world's prevailing mutations in the RIF resistance determining region (RRDR) of *rpoB* gene. The recent literature have recorded that mutations of *rpoB* gene responsible for RIF resistance are not restricted to the RRDR and vary geographically (Lingala *et al.* 2010; Tan *et al.* 2011). Thus, investigation of the *rpoB* gene mutations in a specific geographical area is vital prior to initiation of a proper molecular DST method for determination of RIF resistance. Based on the literature review, it was hypothesized that mutations of the *rpoB* gene of *M. tuberculosis* isolates in Sri Lanka differ from those identified in other countries.

Further, several important concepts such as the molecular characterisation of drug resistant *M. tuberculosis* and transmission pattern of drug resistance in Sri Lanka remain unknown. Additionally, molecular based identification methods of drug resistant *M. tuberculosis* are yet to be established. Thus, remaining parts of the thesis cover in depth studies on the molecular based identification of RIF resistant isolates in Sri Lanka and transmission of RIF resistance.

1.7 Objectives

General objective 1

To study the RIF resistant gene mutations in a selected group of *M. tuberculosis* strains in Sri Lanka.

Specific objectives

(a) To determine the proportionate case load of MTC or NTM strains among patients presenting with acid fast bacilli positive sputum.

(b) To determine the incidence of RIF resistance among *M. tuberculosis* isolates using conventional agar proportion method.

(c) To evaluate of manual Mycobacteria Growth Indicator Tube (MGIT) and nitrate reductase assay (NRA) for rapid detection of RIF resistance.

(d) To identify the RIF resistant mutations of the *rpoB* gene of *M. tuberculosis* among drug resistant isolates using DNA sequencing.

General objective 2

To develop a rapid molecular method for identification of rifampicin resistance

Specific objectives

(a) To develop a PCR-ELISA as a rapid molecular method for identification of RIF resistant mutations.

(b) To validate the PCR-ELISA as a diagnostic method for determination of RIF resistance of *M. tuberculosis* culture isolates (indirect method).

General objective 3

To describe the genetic diversity and the transmission pattern of RIF resistant *M. tuberculosis* in Sri Lanka, using molecular fingerprinting

Specific objectives:

(a) To determine the Restriction Fragment Length Polymorphism (RFLP) patterns of 31 RIF resistant and 46 RIF susceptible *M. tuberculosis* isolates.

(b) To compare evolutional relationships between RIF susceptible and resistant strains of *M. tuberculosis*.

1.8 Presentation of the thesis

The specific aims under the first objective of the research have been achieved by studies described in chapters 2-4 of the thesis. Chapter 2 explains the isolation of the *Mycobacterium* cultures from sputum specimens of suspected TB patients in Sri Lanka and identification of MTC isolates using phenotypic characters of colonies, biochemical test and PCR amplification of specific gene fragment for MTC. The isolation of rifampicin resistant *M. tuberculosis* by conventional method and evaluation of two culture based methods for determination of rifampicin resistance is addressed in chapter 3. Chapter 4 describes the investigation of mutations in RIF resistant isolates and the DNA sequencing was used for confirming the results of conventional methods. Thus, the studies described in chapter 2-4 collectively investigated the rifampicin resistance in a selected group of *M. tuberculosis* strains in Sri Lanka.

The specific aims of the second objective of the research were achieved by the study described in the chapter 5 of the thesis and it describes the optimization and validation of PCR-ELISA for determination of RIF resistance of *M. tuberculosis*.

Chapter 6 explains the investigation of the polymorphism among *M. tuberculosis* strains by RFLP analysis and transmission pattern of RIF resistance. Thus, the chapter 6 covers the specific aims of the third objective of the research.

Chapter 7 discusses the relevance of the present findings to the advancement of knowledge in the field of science and benefits to control programmes and community that could be derived from the research.

Chapter 2

Genus *Mycobacterium* as an etiological agent in pulmonary diseases

2.1 Background

2.1.1 *Mycobacterium* species and pulmonary diseases

A considerable number of species in the genus *Mycobacterium* are non-pathogenic while a few are obligatory etiological agents of pulmonary diseases. *M. tuberculosis* complex (MTC), consisting of *M. tuberculosis, M. bovis, M. africanum M. microti* and *M. canettii* are causative agents for tuberculosis in human (Zumla and Grange, 2002). Among these, *M. tuberculosis* is responsible for human tuberculosis in the vast majority of cases worldwide. However, geographical variation of the MTC species distribution has been identified. As an example, *M. africanum* is a common cause of human pulmonary TB (39%) as much as *M. tuberculosis* (55%) in West Africa (De Jong *et al.* 2010). In Ghana, 3% of pulmonary TB cases are represented by *M. bovis*, while 20% are *M. africanum* and 73% are *M. tuberculosis* (Addo *et al.* 2007).

Non tuberculosis mycobacteria (NTM) are ubiquitous in nature and are widely distributed in water, soil and animals. Among prevailing NTM species, only a few species have a clinical impact on humans as opportunistic pathogens (Falkinham, 2009). *M. avium* complex (MAC), *M. abscessus, M. kansasii, M. fortuitum, M. chelonae, M. szulgai, M. triviale* and *M. scrofulaceum* are common NTM species that cause pulmonary diseases in humans (Simons *et al.* 2011). Additionally, *M. riyadhense* was recently proposed as a causative agent of pulmonary NTM disease (Godreuil *et al.* 2012). However, NTM are

increasingly recognized as a significant cause of chronic human pulmonary infections in both immunocompromised and immunocompetent patients. Further, it is an important cause of mortality in Western countries (Nightingale *et al.* 1992; Katoch, 2004).

In contrast to tuberculosis, diseases caused by NTM have varied clinical manifestations, triggering a wide spectrum of infections with generally low virulence than TB (Simons *et al.* 2011). Patients with underlying structural lung diseases such as chronic obstructive pulmonary diseases, cystic fibrosis, bronchiectasis, history of tuberculosis and chronic aspiration are more vulnerable to develop NTM lung disease (Griffith *et al.* 2007). Additionally, working in mining industry and advanced age are risk factors for NTM lung diseases. However, there is no evidence on animal-to-human (zoonosis) or human-to-human transmission of NTM and human diseases are generally acquired from environmental exposure (Meissner and Anz, 1977; Griffith *et al.* 2007).

Clinical relevance of pulmonary NTM species vary geographically and MAC is the main cause of pulmonary diseases in Asia (specially South Korea and Japan), North America and most parts of Europe (Table 2.1), (Simons *et al.* 2011; Marras and Daley, 2002). However, Simon *et al* (2011) has shown that the *M. fortuitum* is the main cause of NTM pulmonary disease in India. There is very little information on NTM infections in the South East Asian region including Sri Lanka. The systematic reporting on molecular epidemiology of NTM pulmonary infectious is also limited (Simons *et al.* 2011).

Table 2.1 Non tuberculosis mycobacteria species causing pulmonary infections in human (Simons *et al.* 2011)

Country (no. of infections tested)	NTM Species %								
	M. absce -ssus	*M. avium*	*M. chelonae*	*M. fortuitum*	*M. gordonae*	*M. kansasii*	*M. triviale*	*M. scrofula -aceum*	*M. szulgai*
India (15)	-	-	-	40	-	33	20		?
Hong Kong(28)	-	54	14	-	4	4	-		4
South Korea (131)	39	50	2	3	-	4	-		0
Japan (1064)	-	81	0.6	2	-	14	-		0.5
Thailand (132)	-	43	5	5	-	17	-	8	-
Singapore (15)	-	60	7	-	-	27	-	7	-
Taiwan (302)	19	43	10	10	-	9	-		-

2.1.2 Laboratory diagnosis of *Mycobacterium* pulmonary infections

The initial laboratory identification of the genus *Mycobacterium* can be made by microscopic observation for the presence of AFB. The definitive diagnosis demands the recovery of *Mycobacterium* species on a culture medium, followed by species identification tests. Although numerous novel, rapid and direct molecular methods have been developed, culture remains the gold standard for identification of *Mycobacterium* species from clinical specimens (Ogbaini-Emovon, 2009).

The general microbiological measures of growing clinical material on a selective or differential culture media and sub-culturing to obtain pure cultures cannot be applied to *Mycobacterium*. Genus *Mycobacterium* will not grow on simple, chemically defined media and it requires special, enriched, selective media. Slow replication rate is a characteristic feature in culturing of *Mycobacterium* (De Kantor *et al.* 1998). Generally, an AFB positive sputum will require 3 weeks for producing visible colonies of *Mycobacterium* on solid medium (Palomino *et al.* 2007). However, NTM species such as *M. fortuitum, M abscessus,* and *M. chelonae* are considered as rapid growers as they grow into visible colonies within 3–5 days of incubation (Griffith *et al.*2007; Falkinham, 2002).

2.1.2.1 Selective culture media for *Mycobacterium* species

Culture isolation is a primary requirement in conventional species identification and indirect drug susceptibility testing. Solid media (egg-based or agar-based) and liquid media have been optimized as special, selective media for cultivating bacilli of the genus *Mycobacterium* (De Kantor *et al.* 1998). Egg based L-J medium is the most commonly used solid medium for isolation of *Mycobacterium* species especially in low resource settings (De Kantor *et al.* 1998). Middlebrook media (7H10 and 7H11 agar medium and 7H9 broth medium) are modified media for better isolation of *Mycobacterium* species and Table 2.2 shows a comparison of different culture media. The selective nature of culture media is provided by addition of certain antibiotics that do not interfere with the growth of *Mycobacterium* species (Rothlau *et al.*1981). The activity of each antibiotic used for the Middlebrook media is summarized in table 2.3.

Table 2.2 Comparison of different culture media used in the isolation of *Mycobacterium* species

Culture media	Special components of the medium		Advantages	Disadvantages	References
	Component	Role			
Lowenstein – Jensen (L-J)	• Egg albumin • Asparagine • Ribonucleic acids • Malachite green	• Provide solid form & nutrients • Growth stimulant • Growth stimulant • Prevent contaminations	• Less expensive • Contaminations during preparation is less	• Longer incubation period (8 weeks)	• Fonseca *et al.* 2011 • De Kantor *et al.* 1998
Middlebrook 7H10 agar	• Oleic acid • Albumin • Dextrose • Catalase • Sodium chloride • Agar	• Metabolism of mycobacteria • Protects the organisms by binding free fatty acids • Energy source • Destroys toxic peroxides • Enhances the presence of essential electrolytes • Provide solid form	• Early detection of micro colonies • Slightly higher isolation rate than L-J • Lower contamination rate • Less turnaround time (10-15 days) than L-J	• Cost per test is high	• Fonseca *et al.* 2011 • Difco™ & BBL™ Manual • Cohn *et al.* 1968 • Schaefer and Lewis. 1965 • Lynn *et al.* 1979 • Ghatak *et al.* 2011 • Finlayson and Edson. 1949 • Manca *et al.* 1999
Middlebrook 7H11 agar	• Pancreatic digest of casein • Other components similar to 7H10 agar medium	• Essential source of nitrogen for the growth of tubercle bacilli	• Early detection of micro colonies • Higher isolation rate than other solid media • Lower contamination rate • Less turnaround time	• Cost per test is high	• Fonseca *et al.* 2011 • Lorian. 1967 • Cohn *et al.* 1968 • Mitchison *et al.* 1973
Middlebrook 7H9 broth	• Components similar to 7H10 agar medium • No agar		• More sensitive than solid media (10%) • Less turnaround time (10-15 days)	• Cost per test is high • Preliminary differentiation of isolates using colony morphology is impossible • Contamination rate is high	Fonseca *et al.* 2011

Table 2.3 Mode of action of antibiotics in Middlebrook culture media

Antibiotic	Activity	Reference
Polymyxin B	Inhibit growth of Gram (-) bacteria	Dixon and Chopra,1986
Amphotericin B	Inhibit growth of Gram (+) bacteria and Fungi	Reeves *et al.* 2004
Trimethoprim lactate	Inhibit growth of Gram-positive bacteria	Gleckman *et al.* 1981
Nalidixic acid	Active against both Gram (-) and (+) bacteria	Cook *et al.*1966
Azolicilin	Inhibit growth of Gram (+) and (-)	·White *et al.* 1980

2.1.2.2 Laboratory differentiation of *Mycobacterium* species

Microscopic observation of ZN stained smear prepared from the culture will provide evidence only for the presence of mycobacteria, purity of the culture and cord formation. These basic characters are not sufficient for definitive species level identification of the genus *Mycobacterium* (Leao *et al.* 2004). The conventional taxonomic differentiation of the genus *Mycobacterium* is based on phenotypic characters of the cultures and biochemical properties of bacteria. In the past decade, the species characterization of genus *Mycobacterium* was addressed by various molecular biological measures and it increased the species pool of the genus with refreshing knowledge on known species (Kasai *et al.* 2000; Kim *et al.* 1999).

The phenotypic characters of cultures such as slow growth rate, cord formation, rough and crumbly colony appearance, non pigmentation and inhibition of growth in the presence of

para nitrobenzoic acid (PNB) are characteristic features of MTC cultures. In contrast, the characters of rapid growth, pigmentation (schotochromogens, photochromogens or nonchromogens) ability to grow in PNB incorporated media and creamy like watery colonies indicate the presence of NTM (De Kantor *et al.* 1998; Garcla-Agudo and Garcla-Martos, 2011). Several biochemical properties of the genus *Mycobacterium* including nitrate reductase, niacin production, catalase activity, production of arylsulfatase and urease, tween 80 hydrolysis, growth in the presence of 5% NaCl and MacConkey agar without crystal violet and the use of mannitol, inositol and sorbitol are adequate to identify majority of clinically relevant Mycobacterial species (De Kantor *et al.* 1998; Garcla-Agudo and Garcla-Martos, 2011).

Biochemical analysis and phenotypic characters may occasionally fail to arrive at a definitive identification. Thus, analysis of the lipids of mycobacteria by thin layer chromatography (TLC) (Jenkins, 1981) and high performance liquid chromatography (HPLC) (Duffey *et al.* 1996) has been recommended as alternate approaches for identification of *Mycobacterium* cultures. Presently, molecular based methods such as PCR amplification, DNA sequencing (Chen *et al.*1996), probe hybridization (Tortoli *et al.* 2003; Richter *et al.* 2003) and Polymorphism analysis of restriction fragments (PCR-RFLP) are used for differentiation of MTC and NTM (Lee *et al.* 2000; Ong *et al.* 2010).

Further, differentiation of members in MTC may be important for epidemiological purposes and individual patient management. For example, patients who are infected with *M. bovis* may not respond to pyrazinamide, a first line anti TB drug. MTC members can

be identified by using several conventional phenotypic and biochemical characters (Table 2.4) and definitive confirmation can be made by molecular based methods.

Table 2.4 The common phenotypic characteristics of *Mycobacterium tuberculosis* complex members (Leao *et al.* 2004)

MTC member	**Specific phenotypic characters**
M. tuberculosis	Nitrate positivity, niacin positivity, cord formation, susceptibility to PZA and cycloserine, resistant to 2-thiophen- carboxylic acid hydrazide (TCH), sensitive to PNB
M. bovis	Growth on pyruvate media, susceptible to TCH and cycloserine, resistant to PZA , niacin negativity
M. africanum	Susceptible to TCH, PZA and cycloserine
M. canettii	Niacin negativity, nitrate positivity, resistant to TCH, PZA and streptomycin

2.2 Justification and objectives of the present chapter

The pulmonary mycobacterial diseases may be a result of an infection caused either by MTC or NTM. The individual patient management in pulmonary mycobacterial diseases depends on the causative agent. Thus, the species level identification of genus *Mycobacterium* enhances the quality of the treatment and control programmes.

The literature reviewed so far reveals that the AFB microscopy is not sufficient to differentiate MTC from NTM even the laboratory diagnosis of suspected pulmonary

mycobacterial diseases is mainly based on the AFB microscopy in Sri Lanka. Thus, the objective of the present chapter is,

1. To isolate of *Mycobacterium* cultures from AFB positive sputum samples
2. To identify the importance of differentiation of *Mycobacterium* cultures as MTC or NTM

2. 3 Research methodology

The suitability of culture for the identification of *Mycobacterium* species compared to the AFB microscopy was evaluated by calculating the overall culture positivity rate, culture positivity of primary and secondary suspected TB cases.

2.3.1 Study population

Patients attending chest hospital-Welisara, central chest clinic-Colombo and prison-hospital Colombo were enrolled for the study during the period from March 2008 to October 2011. Only suspected secondary TB patients (including retreatment, default, treatment failure and recurrent) were considered from the chest hospital. Sputum samples were collected from both new and secondary TB patients attending chest clinic Colombo, following confirmation of AFB smear positivity. Also symptomatic respiratory patients undergoing evaluation for pulmonary tuberculosis at the prison hospital Colombo were screened by smear examination and these included both new cases and retreatment cases. In addition, a few samples received from the routine diagnostic laboratory of the

Department of Microbiology were used for the study. H37Rv was used as the control strain.

2.3.2 Sample collection

Early morning sputum samples on 3 consequent days were collected into new, sterile, leak proof, wide mouthed specimen containers. Patient records including demographic information and clinical history on tuberculosis were collected through a specially prepared request form (Appendix 1). The specimens were refrigerated immediately after collection and transported to the laboratory.

2.3.3 Preparation of culture media

Tissue culture grade chemicals were purchased from Sigma Company, St. Louis, MO, USA (unless otherwise stated) and consumables were sterilized by autoclaving at 121 °C for 15 min (Hirayama, USA). Pipettes were disinfected by exposing to UV light and wiping with 70% ethanol.

L-J, and 7H9 Middlebrook media (BD, Difco^{TM}) were used for isolation of *Mycobacterium* species and prepared according to the manufacturers' guidelines (Appendix 2.1 and 2.3). Additionally, PNB incorporated L-J was prepared and used to differentiate MTC from NTM species.

The antibiotic mixture (PANTA- polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) (Appendix 2.4) and OAD (oleic acid, albumin and dextrose) growth supplement for Middlebrook medium were prepared manually. In preparation of OAD, 300 µl of oleic acid was warmed with 15 ml of 0.05 M NaOH at 56 °C in order to safonify oleic acid. Then it was added into 450 ml of 6% bovine albumin fraction V in fresh 0.85% NaCl. The pH was adjusted to 7.0 with 4% NaOH. The mixture was warmed at 56 °C for 1 h and 20 ml of 50% dextrose solution was added into the mixture. Finally, 20 ml of filter sterilized aliquots were stored at 4 °C for future use. Randomly selected aliquots were incubated at 37 °C for 48 h for determination of contamination.

2.3.4 Sample processing

Decontamination of sputum was carried out according to Petroff's method (De Kantor *et al.*1998). Briefly, 3 consequent sputum samples of a patient were pooled into a new sterile plastic container and two volumes of 4% NaOH (x ml of sputum + 2x ml of 4% NaOH) was added. Following vortexing (30 seconds), the specimens were left at room temperature (25°C) with occasional shaking. After 15 min, the mixture was transferred into a new 15 ml sterile plastic tube and, centrifuged at 3000 x *g* for 15 min at 4 °C. Supernatant was discarded. The pellet was washed twice with 10 ml of sterile distilled water by re-suspending and centrifuging under the same conditions. Finally, the washed pellet was re-suspended in 1 ml sterile distilled water and 200 µl each of the prepared suspension was inoculated into L-J, 7H9 Middlebrook broth and PNB incorporated L-J. The inoculated culture media were incubated at 37 °C in an atmosphere of 5% CO_2 partial

pressure. Cultures were observed twice a week for presence of any growth and media that had no growth were discarded after 2 months. For microscopic examination, 20 µl of the suspension was used to prepare the smear. The smear was stained with ZN stain (Appendix 2.5) and examined microscopically under oil immersion (x1000) for AFB. The remaining suspension was stored in sterile glycerol at -20 °C for further use.

2.3.5 Identification of *Mycobacterium* cultures

Isolated *Mycobacterium* cultures (n=442) were differentiated as MTB or NTM by using phenotypic methods, nitrate reductase activity and molecular markers. The ability of differentiation by each method was calculated as percentage.

2.3.5.1 Phenotypic identification

The phenotypic characters; colony appearance, growth rate, pigmentation, presence of growth on PNB and cord formation in broth medium were used for the differentiation of mycobacterial cultures into MTC and NTM.

2.3.5.2 Nitrate reductase activity

Mycobacterium cultures were further identified as MTC or NTM by nitrate reductase activity (De Kantor *et al.* 1998). Briefly, 1 loop (~ 10 µg) of colony or 100 µl of a broth culture was added into 200 µl of sterilized $NaNO_3$ (0.1%) in a micro-centrifuge tube (1.5

ml) and tubes were incubated at 37 °C for 3 h. Colour change (colourless to pink) was examined by sequentially adding 1 drop of 50% HCl, 2 drops of 0.2% sulfanilamide and 2 drops of 0.1% N-napthtylethylene-diamine into the incubated tube. The colour of the culture medium was interpreted using a standard colour series (Appendix 2.6) as shown in Figure 2.1. The optical density of colour intensity was determined using a spectrophotometer at 570 nm.

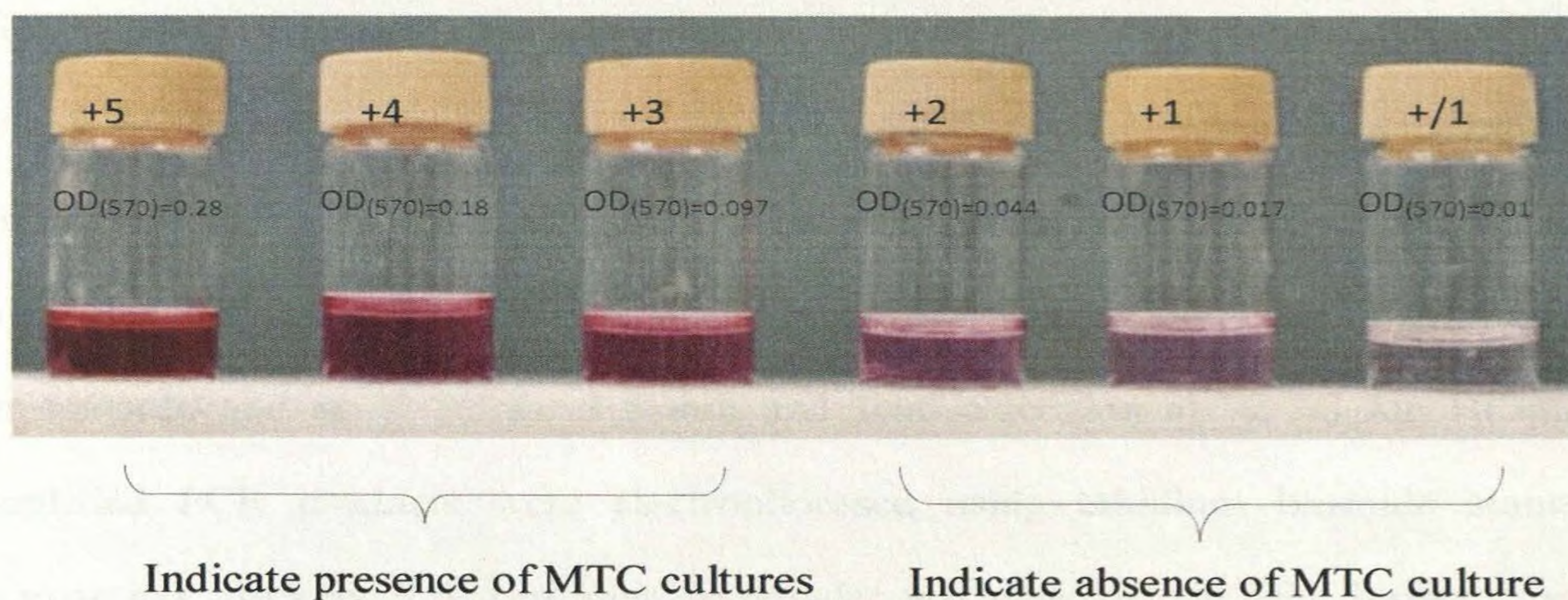


Figure 2.1 Standard colour series for nitrate reductase test interpretation (De Kantor *et al.*1998). Colour range from +5 to +3 (from OD $_{(570)}$ 0.28 to 0.097) indicates significant nitrate reductase activity (presence of MTC) and + 2 to +/-1(from OD $_{(570)}$ 0.044 to 0.01) indicates insignificant nitrate reductase activity (not supportive of presence of MTC)

In order to detect nitrite that may have been further reduced to nitric oxide (which cannot be detected by the reagents used above), a small amount of powdered zinc was added to each culture media that did not show colour change.

2.3.5.3 Identification of isolates by molecular markers

The conventionally classified culture isolates were further confirmed by DNA amplification. A 240 bp fragment of the insertion element IS6110 was amplified with specific primers PT18 (5'-GAACCGTGAGGGCATCGAGG-3') and INS2 (5'-GC GTA GGCGTCGGTGACAAA-3') (Mulcahy *et al.*1996). The 25 µl PCR mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 200 µM of deoxynucleotides triphosphates (Promega, USA), 1 U *Taq* polymerase (GenScript, USA), 20 pmol of each primer (1st base, Singapore), and 2.5 µl of crude genomic DNA from heat-killed bacterial culture was used for each PCR reaction. The thermo-cycling parameters were as follows. Initial denaturation at 94 °C for 10 min, 40 cycles following at 94 °C for 1.5 min, 65 °C for 1.5 min and at 72 °C for 1.5 min and final extension at 72 °C for 10 min. The amplified PCR products were electrophoresed using ethidium bromide stained 1% agarose gel. Briefly, 2.5 g of agarose powder was dissolved in TBE buffer (Appendix 3.1) by heating in a microwave oven. After cooling to around 56 °C, ethidium bromide (0.5 µg/ml) was added to gel solution and poured into a gel casting tray. The PCR products (5 µl) were mixed with gel loading buffer (3 µl) (Appendix 3.2) and loaded on to the gel and electrophoresed at 50 V for 2.5 h. The amplified DNA products on electrophoresed gel were visualized using UV illuminator.

2.4 Results

2.4.1 AFB microscopy

Five hundred and thirty four (534) AFB positive sputum specimens were collected during the study period. In the analysis of clinical data retrieved from patient's records, 87 out of 534 AFB positive sputum specimens were denoted as secondary TB patients. The remaining samples (n= 447) were from primary pulmonary infections. Table 2.5 shows the number of AFB positive sputum specimens received from each sampling centre.

Table 2.5 AFB positive sputum specimens received from each sampling centres during study period (March 2008 to October 2011)

Collection center	**No. of specimen**	**Suspected treatment category of patient**
Chest hospital-Welisara	80	Only secondary TB patients (default, retreatment, treatment failure)
Central chest clinic –Colombo	418	Primary and secondary TB patients
Prison hospital –Colombo	31	Primary and secondary TB patients
Dept. of Microbiology	5	Primary and secondary TB patients
Total	534	Primary and secondary TB patients

2.4.2 *Mycobacterium* culture isolates

As shown in Figure 2.2, 442 *Mycobacterium* cultures were yielded from 534 AFB positive sputum specimens in solid and liquid culture media collectively. Among the remaining 92 AFB positive sputum specimens (17%), 64 did not yield a positive culture in any of the media used, while 28 sputum specimens yielded contaminants. Among the 92 sputum specimens that did not show *Mycobacterium* growth, 25 (28.7%) were collected from suspected secondary TB patients while reaming 67 (15 %) were from suspected new TB patients. The culture isolation rate was 82.7% among the selected AFB positive pulmonary infectious cohort in Sri Lanka. The culture recovery rate in suspected secondary TB patients was 71.2 % (62 out of 87) while it was slightly higher among new TB patients 85% (380 out of 447).

Three hundred twenty seven (327) AFB positive sputum samples showed a growth on both solid and liquid medium. Among 442 *Mycobacterium* cultures, 68 cultures were recovered only from broth medium while 47 grew only on solid medium on initial culture. Thus, the higher isolation rate was observed in broth medium (89.3%) compared to the L-J medium (84.6%).

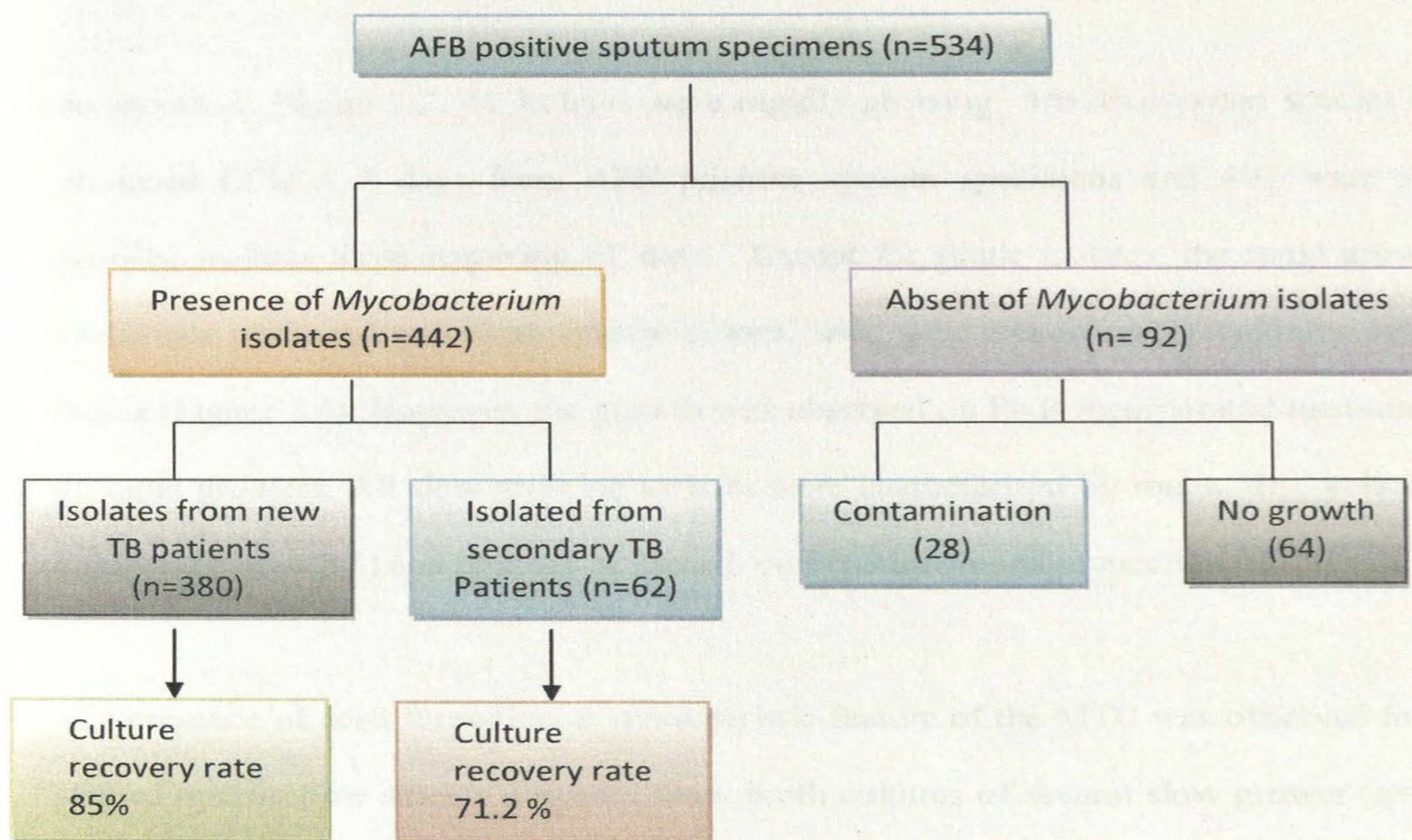


Figure 2.2 Flow diagram for the summary of the isolation of *Mycobacterium spp* from AFB positive sputum specimens in the presence study

2.4.3 Differentiation of *Mycobacterium* cultures

Four hundred forty two (442) *Mycobacterium* isolates that were recovered from AFB positive sputum specimens were identified as MTC or NTM by their phenotypic, biochemical and molecular characteristics. Out of 442 cultures, 401(90.72%) were observed as MTC and remaining 41 (9.27%) were classified as NTM species. The NTM culture population was isolated from both suspected primary TB (n=30) and secondary TB (n=11) patients.

2.4.3.1 Classification by phenotypic characters

As shown in Figure 2.3, 41 isolates were rapidly growing *Mycobacterium* species that produced CFU < 7 days from AFB positive sputum specimens and 401 were slow growing isolates those requiring >7 days. Except for single isolates, the rapid growing specimens produced yellowish orange colour, soft, wet, creamy moist colonies on L-J media (Figure 2.4). However, the growth was observed on PNB incorporated medium for all rapid growers. All slow growing isolates were characterized by rough, dry, yellowish colonies (Figure 2.5) and absence of growth on PNB incorporated medium.

The presence of cord formation, a characteristic feature of the MTC was observed in ZN stained microscopic smears prepared from broth cultures of several slow grower (n=26). However, one rapidly growing culture obtained from a suspected secondary TB patient demonstrated the cord formation in broth medium (Figure 2.6).

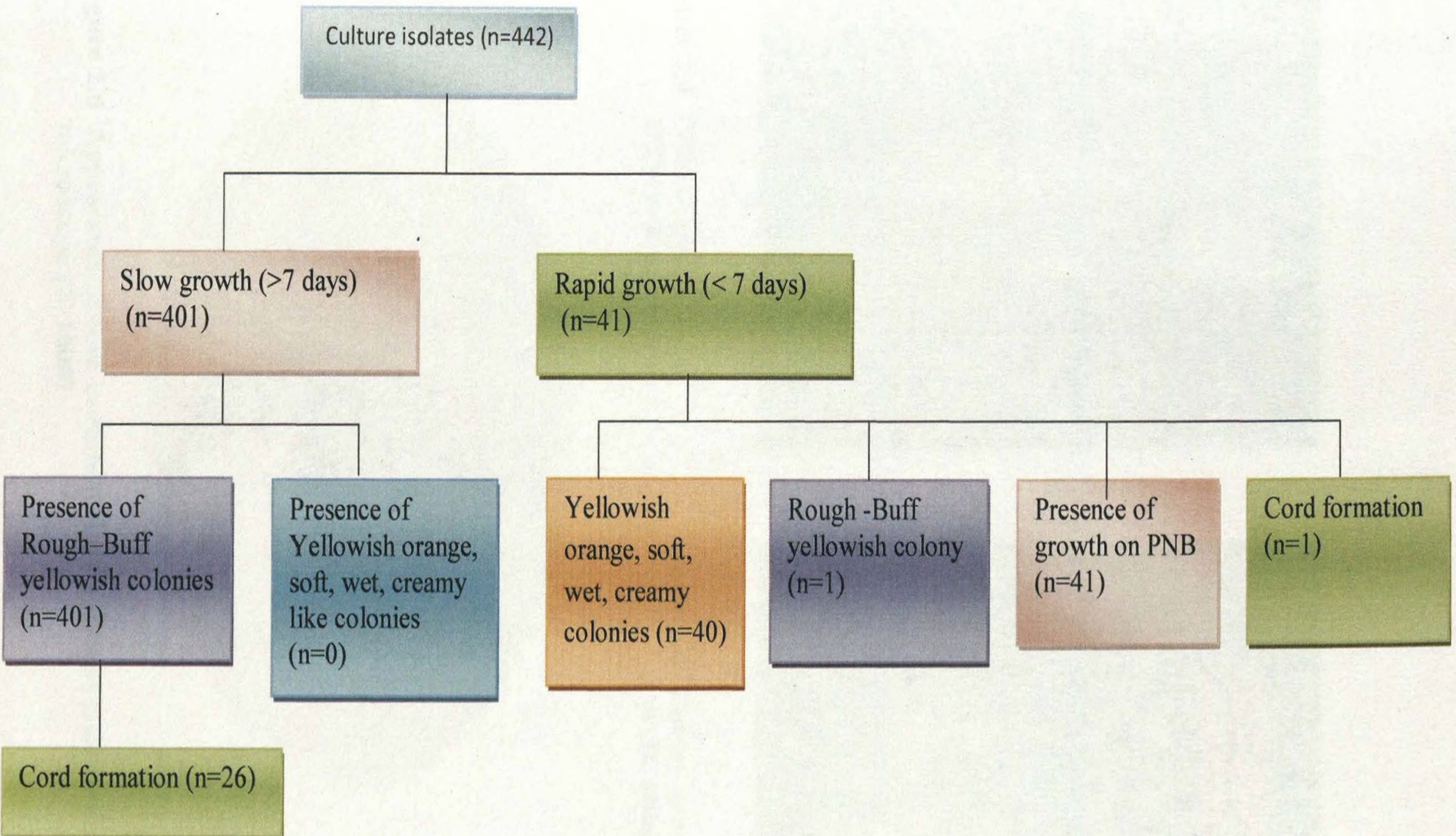


Figure 2.3 Phenotypic observations of isolated *Mycobacterium* cultures (n=442) during the study.

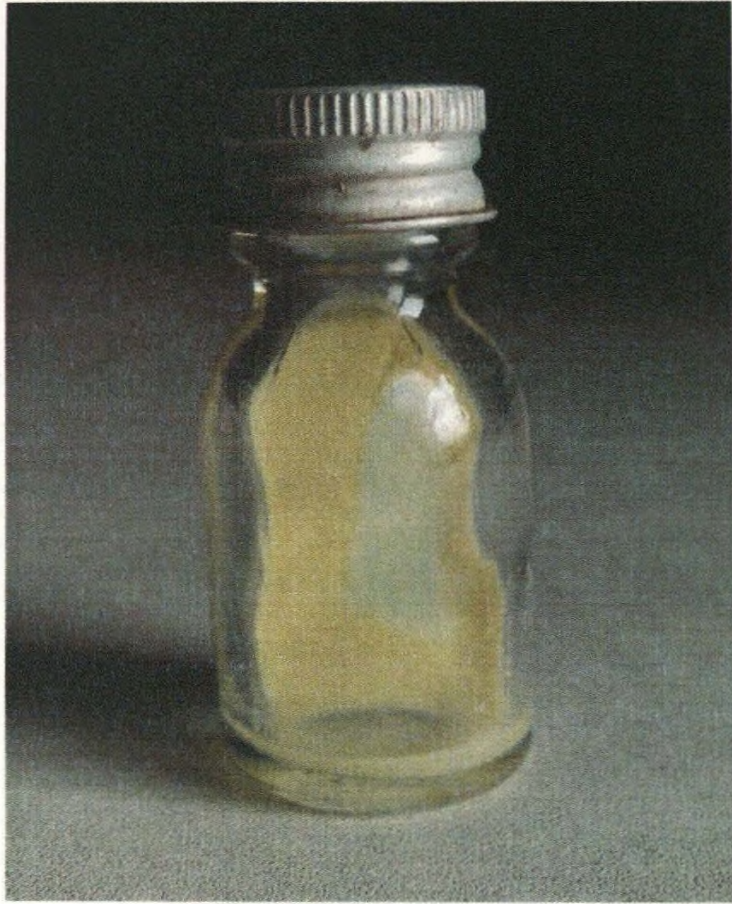


Figure 2.4 Colony appearance of NTM culture on L-J

Figure 2.5 Colony appearance of MTC culture on L-J

Figure 2.6 Appearance of cord in Ziehl–Neelsen stained slide under the light microscope (X 1000)

2.4.3.2 Application of nitrate reductase test for culture differentiation

In biochemical identification of *Mycobacterium* isolates by nitrate reductase test, the isolates that demonstrated the colour change within the +5 to +3 of standard colour series (Figure 2.1) were considered as MTC. Slow growing culture isolates showed nitrate reductase activity except in 3 cultures 99.2%) . No colour change within +5 to +3 was observed for any rapid growing isolates. Figure 2.7 represents an example of colour change observed for the nitrate reductase test of *Mycobacterial* isolates.

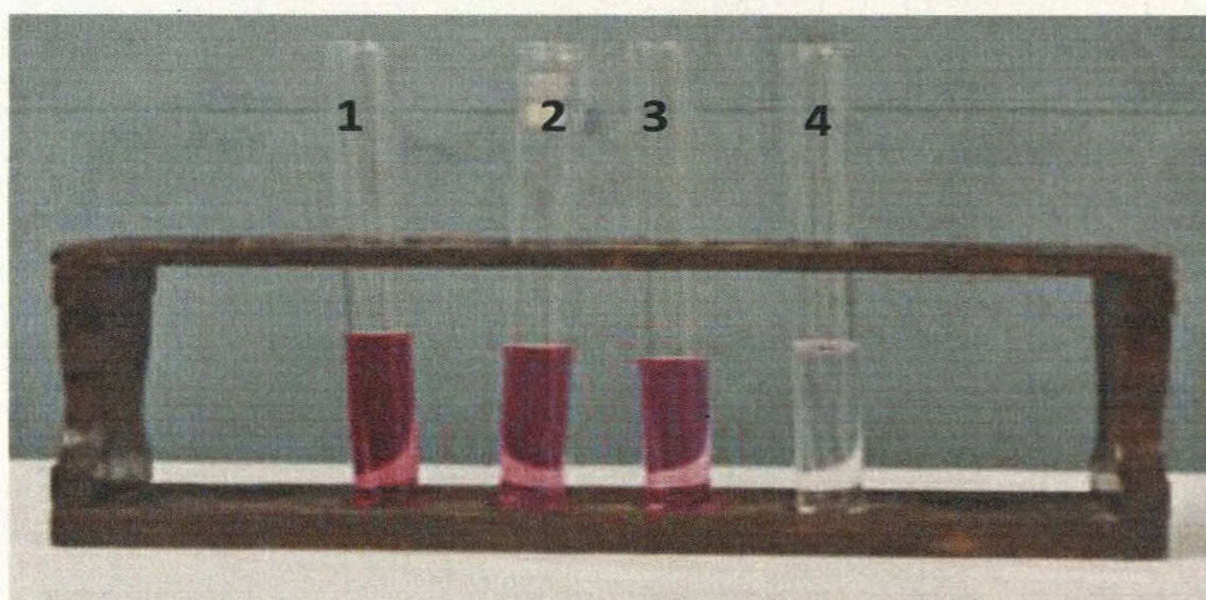


Figure 2.7 Four representative samples showing the results of nitrate reductase activity. Sample No. 1-3 shows a colour change compatible with positive range in the standard colour series indicating nitrate reductase activity (MTC). Sample No.4 has no significant colour change (NTM)

2.4.3.3 Molecular identification of *Mycobacterium* cultures

The amplification of 240 bp fragment of IS6110 insertion element was observed for DNA from all slow growing cultures (n=401, 100%) confirming these isolates to be MTC. Cultures that failed to amplify this fragment were confirmed as non MTC. The single rapidly growing isolate that mimicked MTC due to cord formation was confirmed as NTM due to the absence of 240 bp fragment. Figure 2.8 shows the photograph of electrophoresed agarose gel for several isolates under UV illumination.

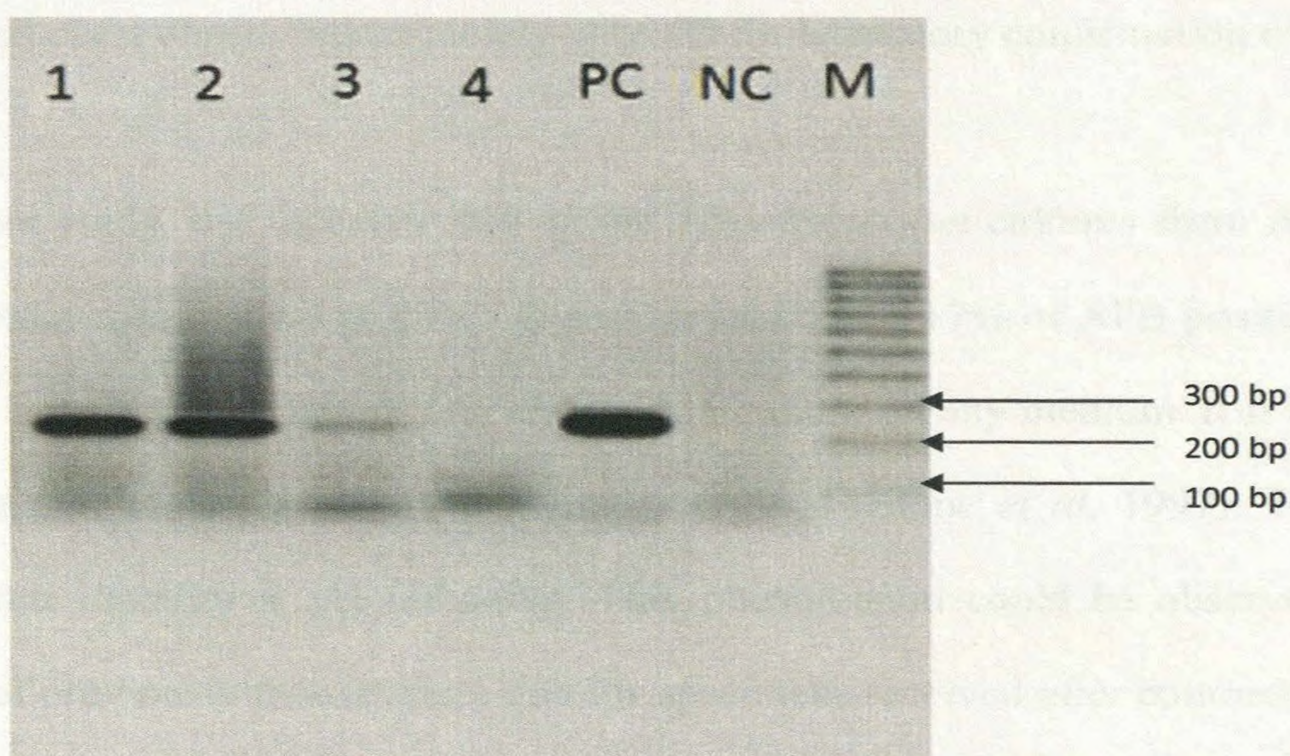


Figure 2.8 Gel photograph showing agarose gel electrophoresis of PCR amplified IS6110 fragment (240 bp) for selected isolates. Lane 1-3 *M. tuberculosis* isolates, lane 4- NTM isolate, PC- positive control, NC negative control, M- 100 bp molecular marker.

2.5 Discussion

2.5.1 Recovery of *Mycobacterium* cultures from AFB positive sputum

Although definitive identification is limited by AFB microscopy, it is the key diagnostic tool for clinically suspected TB cases in low resource settings. In Sri Lanka, the availability of *Mycobacterium* culture facility is restricted to the reference laboratory and a few private laboratories. Molecular identification is yet to be established in the government health care settings. The definitive diagnosis of pulmonary TB is limited for suspected drug resistant cases (as cultures are done to provide DST results to clinicians). All peripheral chest clinics depend solely on AFB for laboratory confirmation of TB.

In the present study, the recovery rate of the *Mycobacterium* cultures from AFB smear positive sputum specimens was 83%. It was observed that 17% of AFB positive sputum samples did not show subsequent *Mycobacterium* culture on any medium. It is a common phenomenon that could be observed in many settings (Stone *et al.* 1997). The precise reason for this inability is yet unknown. This phenomenon could be observed in high percentage of previously treated cases and for specimens received after commencement of the anti TB treatment. With commencing of treatment the viability of the bacilli will be lost and dead bacilli may remain in the sputum, making AFB microscopy is positive.

Frequently, the culture isolation rate of the broth medium is slightly higher than L-J medium as observed by the present study. The broth medium ensures optimum nutrition for growth of mycobacteria than the egg based L-J medium (Ang *et al.* 2001; Sula and

Sundaresan, 1963). However, the possibility of contamination is higher in broth medium compared to L-J medium. As an example, Irfan *et al.* (2008) has revealed an 8% contamination rate in broth medium compared to 3% in L-J medium. Further, the study described in this chapter had 4% and 1% contamination rate for broth and L-J medium respectively.

2.5.2 Prerequisite of species differentiation of pathogenic NTM

The prevalence of MTC and NTM was 90.7% and 9.3% respectively among a selected AFB sputum positive pulmonary disease cohort in Sri Lanka. Although the percentage of NTM is small, it is of significant importance as NTM are resistant to conventional anti tuberculosis drugs. Of the 41 NTM isolates retrieved from the present study, 11 isolates were from suspected secondary patients. These patients are resistant to conventional first line anti-TB drugs and AFB positivity remain after 6 months of treatments. Thus, clinicians may misunderstand as MDR-TB cases.

The drug susceptibility profile of NTM is quite different to that of MTC and thus chemotherapy of patients should be different. Further, rapidly growing *Mycobacterium* species are frequently resistant to RIF and INH, the most important among the first line anti -TB drugs. They are susceptible to inexpensive broad spectrum antibiotics (Katoch *et al.* 2004). As examples, RIF and INH have no role in the treatment of infections due to *M. chelonei* complex and combination of cefoxitin and ciprofloxacin will be successive against it (Singh and victory, 1992). Similarly, the combination of ciprofloxacin,

clarithromycin and amikacin is sufficient for eradication of infections caused by *M. fortuitum*. Further, members of the *M. fortuitum* group are usually susceptible to ciprofloxacin and ofloxacin, while *M. chelonae* and *M. abscessus* are resistant to these drugs. Also, *M. chelonae* is resistant to cefoxitin and susceptibility to tobramycin, whereas *M. abscessus* is susceptible to cefoxitin and resistant to tobramycin (Garcia-Agudo and Garcia-Martos, 2011). Thus, the correct laboratory identification of *Mycobacterium* species is of primary importance prior to commencing treatment (Arora *et al.* 2012).

2.5.3 Limitations of current differentiation methods of *Mycobacterium* species

The use of nitrate reductase activity for differentiation of *Mycobacterium* species may not be fully reliable as several species of the MTC complex show mutable behaviours. As an example *M. bovis* a member of MTC has weak nitrate reductase activity in aerobic growth and Bacille Calmette-Guerin (BCG), vaccine strain of *M. bovis* entirely lacks this activity (Sohaskey and Modesti, 2009). Practical limitations such as yielding of few, small CFU may be less informative in phenotypic characters and is not sufficient for nitrate reductase test. To make an exact conclusion a subculture will be required that increase the turnaround time for diagnosis.

The molecular markers are reliable in identification of *Mycobacterium* species, especially, when inconclusive results are observed for phenotypic and biochemical characters. As an example, *M. marinum* and *M. abscessus*, common pathogens responsible for NTM lung

disease have the ability to mimic the presence of *M. tuberculosis* by forming microscopic cords in broth medium (Sanchez-Chardi *et al.* 2011; Staropoli and Branda, 2008). Further, a rough colony morphotype is also present among virulent forms of *M. abscessus* (Howard *et al.* 2006). In the present study, one of the isolates retrieved from a young woman with pulmonary disease, showed rough cream coloured colonies after 6 days of incubation on L-J and PNB incorporated L-J. The cord formation was observed in the microscopic smear that was prepared from broth culture. However, nitrate reductase activity and DNA amplification of IS6110 fragment were negative. Rapid growth, positive growth in the presence of PNB, absence of nitrate reductase activity and failure to PCR amplify the IS6110 fragment confirmed that the isolate is a NTM species, although rough colony appearance and cord formation are characteristic features of MTC. The analysis of DNA sequence of fragment of the *rpoB* gene that is commonly used for the speciation of *Mycobacterium* species (Kim *et al.* 1999; Kim *et al.* 2004), confirmed the isolate as *Mycobacterium abscessus* which is one of the most pathogenic and chemotherapy resistant NTM species. This is the first report of isolating *M. abscessus* from a clinical specimen in Sri Lanka and it clearly demonstrates that cord formation is not specific to MTC and detection of cording in broth culture should be further investigated before arriving at a conclusion.

Chapter 3

Evaluation of rapid culture based drug susceptibility testing of *M. tuberculosis*

3.1 Background

3.1.1 Drug susceptibility testing

The drug susceptibility testing (DST) is an *in-vitro* microbiological assay performed for determination of susceptibility of an organism to a drug while making a distinction between susceptible and resistant strains. DST decides which antibiotic will be most successful in treating a bacterial infection (Forbes *et al.* 2004) and ensures correct treatment. Drug susceptibility profile of a bacterial species is a very important phenomenon in treating of relapse or re-treatment cases, changing the drug regimen with suspected drug resistance and drug resistance surveillance in a particular region (WHO, 2010).

Drug susceptibility testing of *M. tuberculosis* is crucial due to the increasing rates of MDR-TB and the emergence of XDR-TB (WHO policy statement, 2010). WHO has recommended to carryout DST for at least RIF and INH in resource limited settings especially for previously treated patients and HIV co-infected patients to face obstacles of management and controlling of MDR-TB. Drug susceptibility tests of TB are basically two different types; phenotypic and genotypic. Phenotypic, methods assess the growth of *M. tuberculosis* in the presence of antibiotics while genotype methods are based on the characterization of mutations in genes that are responsible for the activity of the drug.

Phenotypic DST methods are widely used all over the world especially in resource-constrained settings (WHO policy statement, 2010).

3.1.2 Phenotypic DST methods

3.1.2.1 Conventional methods

The absolute concentration method (AC), the resistance ratio method (RR) and the proportion method (PM) are common conventional DST methods performed on solid media (Aziz *et al.* 2003). The proportion method (on 7H10 agar medium) is the gold standard conventional phenotypic method and therefore, new DST methods are validated against the PM (Curry, 2008). The critical concentration of the drug used in the PM may vary with the medium, either L-J medium or agar medium (Table 3.1). The proportion of CFU visualized on the drug containing medium is compared with that of drug free medium to express the results. Interim results can be reported at three weeks of incubation and final confirmation of results will be made at 42 days (Aziz *et al.* 2003; Forbes *et al.* 2004).

Table 3.1 Critical concentrations of first line anti TB drugs in L-J, 7H10 agar and 7H11 agar medium in proportion method (Forbes *et al.* 2004; WHO, 2003)

Media	Antibiotic concentration (µg/ml)			
	Isoniazid	Rifampicin	Etahmbutol	Pyrazinamide
L-J	0.2	40	2	100
7H10 agar	0.2, 0.1	1	5	ND
7H11 agar	0.2, 0.1	1	7.5	ND

In the resistance ratio method, the ratio between the minimum inhibitory concentration (MIC) of the test strain and the MIC of the drug susceptible reference strain (H37Rv) is considered under the same experimental conditions. In performing the RR method, parallel sets of media containing two-fold dilutions of the test drug are inoculated with a standardized inoculum of both test and reference strain. MIC is defined as the lowest drug concentration showing a colony count of ≤ 20. RR ≤ 2 indicates sensitive strain, and RR $\geq$ 8 represent resistant strains. The RR method is the most expensive method among conventional DST methods due to the use of large quantities of culture media (Canetti *et al.* 1969).

The determination of the growth of an organism in the critical concentration of a certain drug is the basis of the absolute concentration DST method. The conclusion on the drug susceptibility can be performed after 4 weeks of inoculation or at 5-6 weeks in the presence of a poor growth. A strain is considered to be susceptible if the number of colonies on the drug containing medium (in critical concentration) is < 20 with a 3+ or 4+ (confluent) growth on the drug free control. Three additional drug concentrations instead of critical concentration should be used when quality controlling the test method (Heifets, 2000).

Conventional DST methods are widely used especially in resource poor setting as these are simple, inexpensive, sensitive and specific. They also do not require either high level technical expertise or sophisticated instruments or infrastructure and are therefore added

advantages. The major drawback of these methods is the long turnaround time (WHO policy statement, 2008).

3.1.2.2 Rapid phenotypic DST methods

3.1.2.2.1Commercial methods

Presently, there are several commercialized semi and fully automated culture based rapid DST methods for *M. tuberculosis* such as BACTEC 460 radiometric system (Becton Dickinson, Sparks, MD), Mycobacteria Growth Indicator Tube - MGIT (Becton Dickinson, Sparks, MD), and MB BacT/Alert system. The turnaround time for a final conclusion on drug susceptibility is dramatically reduced to 1-2 weeks with the use of these culture systems. However, routine drug susceptibility testing using these commercialized systems are limited in resource-constrained settings because of technical complexity, high cost per test and the requirement for sophisticated laboratory instruments and infrastructure (WHO policy statement, 2010).

BACTEC 460 automated system detects the emission of $^{14}CO_2$ and expressed it as a growth index. The inoculated drug containing vials and drug-free control vials are read by the system automatically during the period of incubation on a daily basis. The resistance is measured by the growth index that calculates automatically and the interpretation of results is similar to that of agar proportion method. The system generates antimicrobial susceptibility results for selected first and second line anti-TB drugs within 4 to 8 days (Heifets and Cangelosi, 1999; Pfyffer *et al.* 1999).

The fully automated MGIT 960 system is based on the detection of emission of fluorescence from culture tubes. The O_2 concentration of the culture medium will be reduced with microbial growth. The Ruthenium salt embedded silicon film at the bottom of the culture tube releases O_2, resulting in emission of fluorescence from the bottom of the tube and it is detected by UV illumination. In DST, fluorescence emitting in drug containing and control tubes are monitored and reported automatically over time (Bemer *et al.* 2002; Lu *et al.* 2002). In the manual MGIT method, emission of fluorescence should be recorded manually (Adjers-Koskela and Katila, 2003). The average turnaround time for the final conclusion on DST is 6.4 days and 6.5 for MGIT 960 and manual MGIT methods respectively (Adjers-Koskela and Katila, 2003). Although a substantial amount of literature is available on the use of automated MGIT for drug susceptibility testing, the published data on the manual MGIT method is limited.

BacT/ALERT 3D is a typical walk-away instrumentation which monitors the culture bottles at 10-min intervals and alerts when they become positive as well as at the end of the incubation period. The CO_2 sensor is impacted by a light and reflected rays are monitored by a photodiode. The CO_2, produced by the metabolism of viable bacteria changes the colour of the sensor from green to yellow. Yellow colour is produced specific changes in the intensity of the reflected light and it report as the positive growth (Nair *et al.* 2009; Palomino *et al.* 2007).

3.1.2.2.2 Colorimetric redox indicator (CRI) methods

The colorimetric methods based on the ability of live bacteria to reduce an indicator that can be detected as a colour change is a promising technique for DST of *M. tuberculosis*. The growth of *M. tuberculosis* in a drug medium is measured by using the intensity of colour change of the medium (Palomino *et al.*2007).

Colorimetric assays, using reagents such as Alamar blue (Franzblau *et al.* 1998), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Abate *et al.* 2004; Martin *et al.* 2005), 2,3-bis (2-methoxy- 4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (De Logu *et al.* 2003) and 2,3-diphenyl-5- thienyl-(2)-tetrazolium chloride (TTC) (Mohammadzadeh *et al.* 2006) have been reported to be suitable for detecting drug resistance of *M. tuberculosis.* However, further evaluation of these methods in different settings is needed (WHO policy statement, 2010).

In the TTC based CRI assay, the growth of *M. tuberculosis* in the drug medium will be indicated by the reduction of TTC to an insoluble red colour TTC formazan crystals (Mohammadzadeh *et al.* 2006). In MTT reduction assay, the MTT solution will be added to a 7 day old culture and incubated overnight for the violet precipitation (formazan), to occur. Then, further 3 h incubation is needed with SDS-DMF solution that changes the colour from violet to yellow indicating presence of bacteria (Martin *et al.* 2005).

Alamar Blue is a proprietary reagent that is blue in the oxidized state and changes to pink once reduced. Thus, the growth of bacteria will be indicated by the change of colour from blue to pink after adding of indicator to an 8 day old culture (Palomino and Portaels, 1999).

3.1.2.2.3 Nitrate reductase Assay

M. tuberculosis is an obligate aerobic microorganism with the ability to persist for years or decades in a clinically latent state without causing any overt disease symptoms (Khan and Sarkar, 2012). Once the bacteria are enclosed in a host tissue, several enzyme activities are stimulated for survival in the hostile environment and the reduction of nitrate to nitrite which serves to provide energy in anaerobic conditions is one of them (Wayne and Hayes, 1998).

Assimilation of nitrate in bacteria is a multistep pathway, which involves a combination of series of enzymes functions. Nitrate is converted into nitrite and subsequently to ammonia by nitrate reductase enzyme. Ammonia is normally assimilated into glutamine. Although the molecular basis of nitrate assimilation in mycobacteria remains unknown, it may be similar to the mechanism described for other bacteria (Moreno-Vivian *et al.* 1999). The anaerobic nitrate reductase activity is encoded by four genes, *narG, narH, narJ and narI,* clustered together as *narGHJI* locus (Khan and Sarkar, 2012).

M. tuberculosis is one of the strongest reducers of nitrate in the genus *Mycobacterium* due to the presence of *narGHJI* locus and *narK2X* operon that is responsible for nitrate reductase activity (Sohaskey and Wayne, 2003). The exogenous nitrogen source for nitrate is transported to the cell through the transport protein NarK2 and it is reduced to nitrite by nitrate reductase enzyme. The *nar*GHJI genes mediated nitrate reductase is expressed not only under anaerobic conditions but also under aerobic conditions. The degree of nitrate reduction under aerobic conditions is much lower than in an anaerobic condition. In aerobic conditions, Nark2 will not be able to transport nitrate to the cell and less amount of nitrate enter by diffusion (Khan and Sarkar, 2012; Escoto and Kantor, 1978).

Virulent *M. bovis* has weak nitrate reductase activity during aerobic growth and no hypoxic induction. Bacille Calmette–Guerin (BCG) lacks nitrate reductase activity in both aerobic and anaerobic status (Sohaskey and Modesti, 2009). This is due to the difference in regulation of both nitrate reductase enzyme and transporter in the two strains (Sohaskey and Modesti, 2009).

Nitrate reductase assay (NRA), another colorimetric method that is based on the ability of the organism to reduce nitrate to nitrite is a simple, specific and sensitive method for performing DST. In this assay, inoculated nitrate incorporated culture media will be tested for colour change by sequential adding of HCl, sulfanilamide, and n-1-naphthylethylenediamine. Development of a pink-red colour indicates the presence of *M. tuberculosis* (Ani *et al.* 2009; Gupta *et al.* 2011; Angeby *et al.* 2002). The WHO has

recommended NRA together with colorimetric redox indicator methods for direct DST of *M. tuberculosis* (WHO policy statement, 2010).

3.1.2.2.4 Microscopic observation of drug susceptibility (MODS)

Microscopic observation for the presence of characteristic strings and tangles of *M. tuberculosis* (typical cord formation) under simple inverted microscopy seems to be a promising method for DST. The presence of cord formation in drug containing media (after 5 day of inoculation) indicates existence of drug resistant strains (Caviedes *et al.* 2000; Shah *et al.* 2011; Lu, 2011). However, the MODS may lead to inaccurate results in direct DST as certain NTM species such as *M. marinum* and *M. abscessus* produce true cord in broth medium (Staropoli and Branda, 2008; Sanchez-Chardi *et al.* 2011).

3.2 Justification and objectives of the present chapter

In Sri Lanka, DST is performed only at the National Reference Laboratory-Welisara (state sector) and is still dependent on the conventional L-J proportion method that requires a minimum of 28 days to obtain results. This significantly delays the detection of drug resistance and appropriate treatment. Further, it may lead to increase transmission of drug resistant strains and may reduce the quality of the TB control programme of the country.

The commercialized automated liquid rapid culture systems for diagnosis of MDR-TB are beyond the reach of laboratories in Sri Lanka due to the high cost and need for complex

infrastructure and sophisticated instruments. Thus, the evaluation of rapid inexpensive culture based DST methods is vital for controlling of MDR-TB and prevention of XDR-TB in the country. Thus, the objectives of the present chapter are,

1. To determine the RIF resistance of *M. tuberculosis* isolates using the agar proportion method (APM).
2. To evaluate manual Mycobacteria Growth Indicator Tube (MGIT) and nitrate reductase assay (NRA) for rapid identification of RIF resistant *M. tuberculosis.*

3.3 Research methodology

3.3.1 Study population

Three hundred and ninety (390) *M. tuberculosis* isolates collected during the study described in chapter 2 of the thesis were used for the present study. H37Rv, a susceptible laboratory reference stain and a known rifampicin resistant *M. tuberculosis* isolate confirmed by the National Tuberculosis Institute, Bangalore, India were used as quality control strains.

3.3.2 Preparation of RIF solution

Rifampicin stock solution (10 mg/ml) was prepared by dissolving 10 mg of RIF powder (Sigma, USA) in 1 ml dimethyl sulphoxide (Sigma, USA). Filter sterilized aliquots of stock RIF solutions were stored at -70 °C until use. A working solution (1 mg/ml) was

prepared by diluting the stock solution with sterile double distilled water and was used only once (Forbes *et al.* 2004).

3.3.3 Drug susceptibility testing by APM

The agar proportion method was carried out on Middlebrook 7H10 agar (Difco, US) plates as per CLSI guidelines (Forbes *et al.* 2004). Agar Medium was prepared following manufactures guidelines (Appendix 2.2) and final RIF concentration of test medium was 1μg/ml. Randomly selected RIF containing (test) and RIF free (control) plates were incubated at 37 °C for 48 h for testing contamination.

The inoculum for APM was prepared using 14 day old fresh *M. tuberculosis* colonies grown on solid media (primary cultures were used rather than sub cultures whenever possible). Loopful of bacteria were suspended in 3 ml of sterile Middlbrook 7H9 medium with 10 sterile glass beads contained in sterile 15 ml plastic tubes. The content of the tube was homogenized by vortexing for 2 min. Then, the tube was allowed to stand for 30 min for large clumps of organisms to settle. Supernatant was transferred into a new sterile 15 ml plastic tube and turbidity of the solution was adjusted to McFarland No.1 turbidity standard. This bacterial suspension was further diluted (1:100 and 1:10000) with sterile saline and both drug containing and drug free media were inoculated separately with 100 μl of each dilution. After the inoculum got absorbed into the agar medium, the plates were sealed with a strip of parafilm and incubated at 37 °C in an atmosphere of 5% CO_2 partial pressure.

The plates were observed weekly and the number of CFU was counted. The susceptibility of the isolates was reported after 28 days of inoculation. If the growth was sparse in the drug free medium, plates were reincubated further for an additional period of two weeks (a total of 42 days). The control plates (inoculated with 1:100 or 1:10000 dilution of bacterial suspension) that yielded ≤ 50 colonies were used for the counting. If the growth was inadequate (no. of colonies ≤ 5) or if over growth (> 50) occurred in both control plates, the test was repeated.

The proportion of organisms growing in the presence of RIF was calculated using the following equation. Strains that showed $\geq 1\%$ proportion of colony count between drug containing and drug free media were considered as resistant to RIF.

$$\% \text{ proportion} = \frac{\text{No. of colonies on drug containing medium}}{\text{No. of colonies on drug fee control medium}} \times 100$$

3.3.4 Evaluation of nitrate reductase assay in broth medium as a DST

3.3.4.1 Culture medium and inoculation

Middlebrook 7H9 broth medium with 0.1% $NaNO_3$ was used for NRA (Appendix 2.3). The final concentration of RIF in the test medium was 1µg/ml (Angeby *et al.* 2002; Affolabi *et al.* 2008). Drug free media bottles were prepared in the same manner without addition of RIF solution. Several randomly selected culture bottles (contained 4 ml of medium) were incubated at 37 °C for 48 h to test sterility of culture media.

The McFarland No.1 bacterial suspension was prepared from 14 day old fresh cultures grown on L-J medium as per procedure described in 3.3.3. Drug containing medium was inoculated with 100 µl of McFarland No.1 bacterial suspension and drug free control medium was inoculated with 100 µl of a tenfold dilution of McFarland No.1 bacterial suspension. Two parallel sets each of the control and test media were inoculated per isolate and incubated at 37 °C in an atmosphere of 5% CO_2 partial pressure for 12 days (Angeby *et al.* 2002).

3.3.4.2 Interpretation of drug susceptibility by NRA

After 6 days of inoculation, one set of cultures were tested for colour change by sequentially adding of Griess reagents (10 µl of 50% HCl, 20 µl of 0.2% sulfanilamide and 20 µl of 0.1% N-napthtylethylene-diamine) to the culture medium(Angeby *et al.* 2002). Readings were interpreted as per prescribed colour standards in section 2.3.6.2. The colour change comparable with +5 to +3 of the standard colour series was considered as resistant to RIF. If the drug free control medium of the first set was negative, the second set was tested at 12 days of inoculation in a similar manner. If the drug free control showed a colour change within the positive range of the standard colour series and the drug containing medium did not show a colour change after 12 days of incubation the isolate was considered as susceptible to RIF.

In order to detect nitrite that may have been further reduced to nitric oxide (which cannot be detected by the reagents used above), a small amount of powdered zinc was added to each culture media that did not show colour change. Thereafter, it was tested with Griess

reagents. This avoids reporting of false-negative results as culture medium should be dark pink with absence of *M. tuberculosis* growth (Syre *et al.* 2003).

Presence of AFB and purity of the culture was confirmed microscopically for all tested isolates as bacterial contamination may produce positive results in NRA. Further, to ensure that the media were free of contaminants, the inoculated NRA broths were streaked on blood agar plates to detect any non-specific bacterial growth before testing for colour change.

3.3.5 Evaluation of Manual MGIT for DST of *M. tuberculosis*

3.3.5.1 Culture medium and inoculation

Commercially available 4 ml MGIT (BD diagnostic, USA) were used for the study (Figure 3.1) and100 µl of PANTA, 500 µl of OADC and 40 µl of RIF working solution was added to each tube for preparation of RIF containing medium (final RIF concentration was 1µg/ml) as per manufacturers guidelines. Drug free control medium was prepared in the same manner without addition of RIF solution.

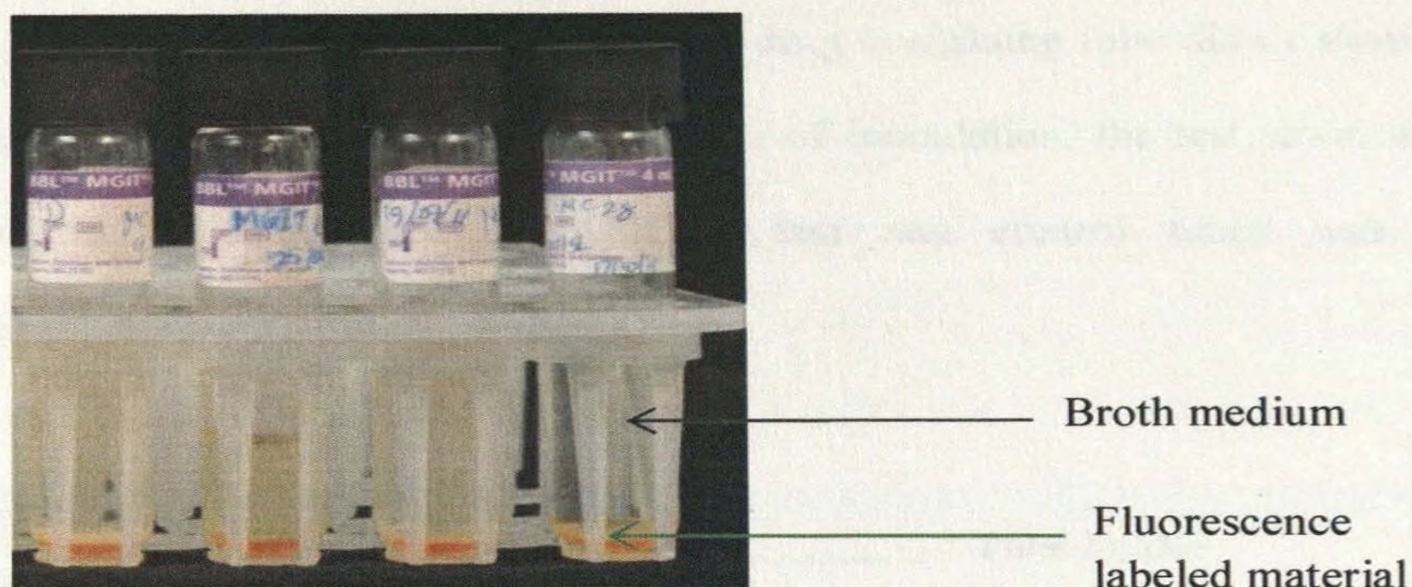


Figure 3.1 Mycobacteria growth indicator tube containing 4 ml Middlebrook 7H9 broth medium and fluorescence labelled material at the bottom of the tube.

McFarland No.0.5 bacterial suspension was prepared by diluting of McFarland No.1 bacterial suspension prepared as described in section 3.3.3. The rifampicin containing MGIT were inoculated with 500 µl of a 1:5 dilution of a McFarland No: 0.5 bacterial suspension and rifampicin free control MGIT were inoculated with 500 µl of a 1:500 dilution of same bacterial suspension as per manufacturer's guidelines and incubated at 37 °C in an atmosphere of 5% CO_2 partial pressure.

3.3.5.2 Interpretation of drug susceptibility by MGIT

The emission of fluorescence was measured using the manual MGIT reader (Figure 3.2) daily from day 2 of inoculation. The tube was placed in the tube slot of the MGIT reader and the reading was taken in the presence of the UV light housed in the reader. If the drug free control tube did not give a reading between 14-20 (positive range-red colour region of reader) (Figure 3.2) within 13·days of inoculation, the test was repeated. If drug free

control tube gave a positive reading and the drug containing tube didn't show a reading between 14-20 (positive range) after 15 days of inoculation, the test strain was read as sensitive to RIF. Presence of AFB in test and control tubes was confirmed microscopically for all tested isolates.

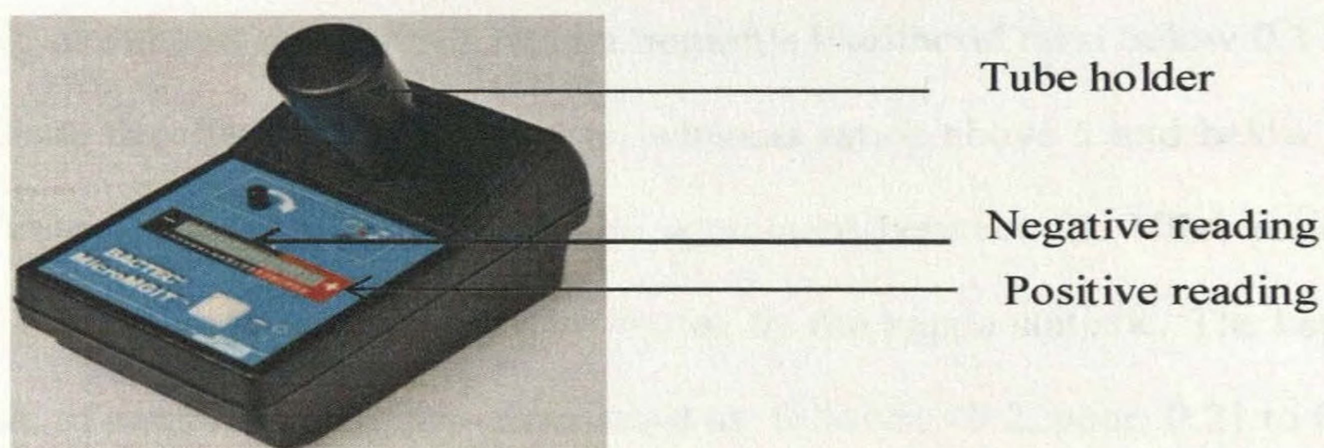


Figure 3.2 View of manual MGIT reader. A reading from 1-14 indicate the absence of growth in the MGIT and a readings from 14-20 (red colour region) indicate the presence of growth

3.3.6 Minimum inhibitory concentration (MIC) of RIF resistant isolates

The MIC of the RIF resistant *M. tuberculosis* isolates retrieved from the present study was determined using a series of 7H10 agar media incorporated with different RIF concentrations from 256 µg/ml to 0.5 µg/ml. Each medium was inoculated with a two-fold dilution of McFarland No. 0.5 bacterial suspension and incubated at 37 °C in an atmosphere of 5% CO_2 partial pressure for 42 days. The growth was observed weekly (Ohno *et al.* 1996).

3.3.7 Data analysis

The suitability of the manual MGIT and NRA in comparison with the APM was evaluated in terms of sensitivity (the ability to detect true drug resistance), specificity (the ability to detect true drug susceptibility), positive likelihood ratio and negative likelihood ratio. A positive likelihood ratio above 10 or a negative likelihood ratio below 0.1 was considered to indicate excellent test-performance, whereas ratios above 5 and below 0.2 were taken to indicate adequate performance. The agreement between the NRA results, the manual MGIT results, and the AMP were estimated by the kappa statistic. The kappa value (k), a measure of test reliability, was interpreted as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent (Sim and Wright, 2005; Kirkwood and Sterne, 2003; Deeks, 2001). The consumable costs per test were calculated in determining the cost for each test method using purchase records.

3.4 Results

3.4.1 Identification of RIF resistance by APM

In counting of the colonies on control media, the plates inoculated with 1:100 dilution of bacterial suspension were used for 68% of MTC isolates. In remaining isolates (32%), the control plates inoculated with 1:10,000 dilution of bacterial suspension were used for the counting as 1:100 dilution produced > 50 or uncountable number of colonies. All resistant isolates except one produced ≤ 50 colonies on plates inoculated with both dilutions and MIC was 1 µg/ml (Figure 3.3). The exception resistant isolate yielded > 50 colonies on

plate that inoculated with 1:100 bacterial dilution and showed higher MIC (8 μg/ml) than other isolates.

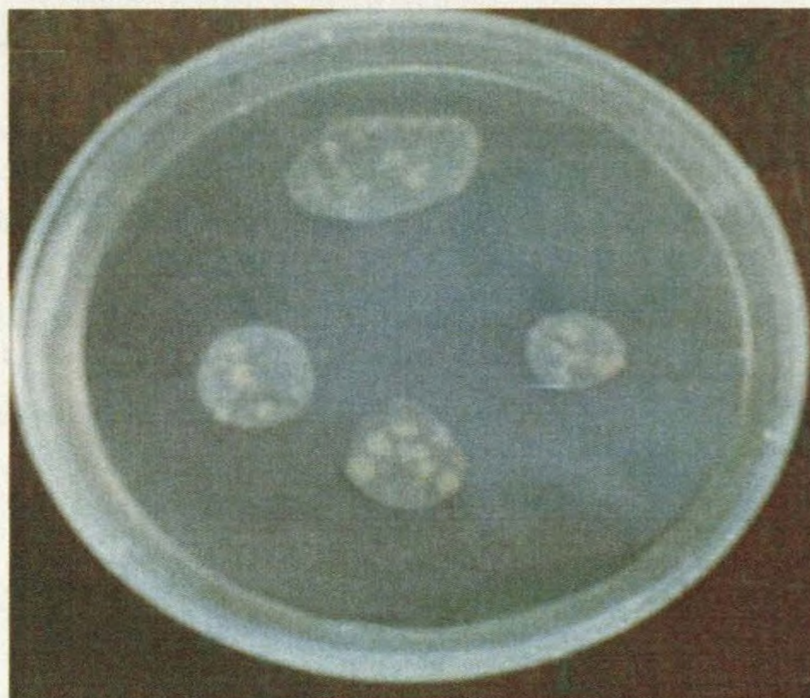


Figure 3.3 Colony forming units of *M. tuberculosis* on 7H10 agar plates in agar proportion method.

Following 42 days of incubation, 9 out of 390 *M. tuberculosis* isolates that failed to produce more than 5 colonies on drug free control medium were removed from the study. Twenty seven (27) out of 381 *M. tuberculosis* strains were identified as RIF resistant isolates by APM. Remaining 354 isolates were confirmed as sensitive to RIF. The final conclusion on RIF susceptibility by the APM was achieved at 32 days (mean) of incubation. Three percent (3%) of isolates were contaminated mainly with yeasts and no contamination was observed when the test was repeated.

3.4.2 Validity of manual MGIT for DST of *M. tuberculosis*

Of 381 *M. tuberculosis* isolates, 26 were identified as resistant to RIF and remaining 355 isolates as sensitive by manual MGIT. There was one discordant result between MGIT and APM as one isolate that was resistant to RIF by the manual MGIT was susceptible by APM. The repeated testing (once) of this isolates confirmed the same results. An excellent agreement was also observed between the manual MGIT and APM (k=0.94) (Table 3.2) with 93 % sensitivity and 100 % specificity for determination of RIF susceptibility. The average turnaround time was 08 days (mean) and the contamination rate was 2% for manual MGIT.

Table 3.2 Sensitivity, specificity, positive and negative likelihood ratio for the MGIT method compared to agar proportion method (n=381) in determination of rifampicin sensitivity.

APM	MGIT		Sensitivity %	Specificity %	Likelihood ratio	
	No. of resistant isolates	No. of susceptible isolates			Positive	Negative
Resistant (27)	25	2	93	100	328	
Susceptible (354)	1	353				0.07

3.4.3 Validity of NRA assay as a DST method

Among 381 *M. Tuberculosis* strains, 26 were determined as resistant to RIF while the remaining 355 isolates were discriminated as susceptible by NRA. The colour change of culture media in NRA is illustrated by the Figure 3.4.

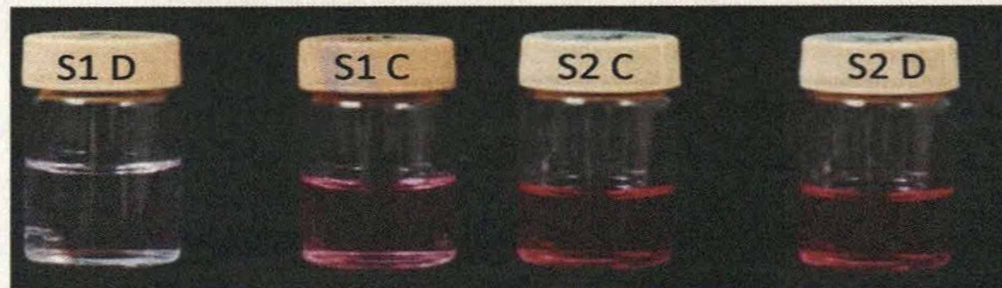


Figure 3.4 Colour change of culture media in NRA for selected isolates. C-RIF free control, D-RIF containing medium, S1- RIF susceptible *M. tuberculosis* isolate, S2- RIF resistant *M. tuberculosis* isolate.

There were 3 discordant results between NRA and APM. Repeated testing (once) of these 3 isolates confirmed the same results. The NRA in 7H9 broth medium showed excellent agreement (k=0.87) with AMP for determination of RIF susceptibility of *M. tuberculosis* with 86 % sensitivity and 99% specificity (Table 3.3). The average turnaround time for NRA in broth medium was 10 days (mean) with a 4% contamination rate.

Table 3.3 Sensitivity, specificity, positive and negative likelihood ratio for NRA compared to agar proportion method (n=381) in determination of rifampicin sensitivity

APM	NRA		Sensitivity	Specificity	Likelihood ratio
	No. of resistant isolates	No. of susceptible isolates	%	%	Positive Negative
Resistant(27)	23	4	86	99	101
Susceptible(354)	3	351			0.13

Among 381 *M. tuberculosis* isolates, 31 were identified as RIF resistance by one or more testing methods (APM, manual MGIT and NRA) used for the study. Remaining 350, isolates were differentiated as sensitive to RIF. Of the 31 RIF resistant isolates, 26 were identified by all 3 methods (APM, manual MGIT and NRA). The DST method/methods confirmed the rifampicin resistance of each isolates is shown in Table 3.4.

Table 3.4 *M. tuberculosis* isolates showing resistance to different drug susceptibility testing methods.

Strain no (Lab No.)	**DST method/s that confirmed rifampicin resistance**
C4, C6, C7, C8, C9, C10, C20, C73, C83, C86, C88, C115, M60, M127, M15, C27, C22 C23,C163, C254 C150, C135, C110 (n=23)	APM, MGIT and NRA
M9, M33 (n=2)	APM and MGIT
PCR 88,PCR 57 (n=2)	Only APM
C120 (n=1)	Only MGIT
M46 , C25, M22 (n=3)	Only NRA

Table 3.5 shows the assessment on major investment, instruments, reagents, cost per test and turnaround time for the APM, manual MGIT and NRA for determination of RIF resistance. The cost per test was calculated by purchase orders collected during the study period.

Table 3.5 Requirement of resources, cost per test and turnaround time for the APM, manual MGIT and NRA.

Tested Phenotypic test	Major investment /infrastructure	Reagent and consumables	Turnaround time (mean no. of days)	Cost per test after installation ($)	Requirement of BSC		Technical requirements
					DST Inoculation	DST reading	
APM	• BSC*- class 2 • Centrifuge • Incubator • Autoclave • Fridge • freezer	• Agar powder • PANTA • OADC • Rifampicin • Disposable petri Plates	32	4	Yes	Yes	• Cumbersome to prepare media • Reading is complicated • Interpretation is complicated
Manual MGIT	• BSC- class 2 • Centrifuge • Incubator • Fridge • Freezer • MGIT reader • Autoclave	• MGIT (4ml) • PANTA • OADC • Rifampicin	8	7	Yes	No	• Easy to set • Reading is not complicated • Interpretation is easy
NRA	Similar to APM	• 7H9 broth • PANTA • OADC • Rifampicin • Bijou bottles • Griess reagents	10	3	Yes	Yes	• Cumbersome to prepare media • Reading is not complicated • Interpretation is easy

* BSC- Bio safety cabinet

3.5 Discussion

Rapid and accurate detection of drug resistance is a prerequisite for initiating effective anti-TB treatment. In Sri Lanka, liquid culture based or molecular based DST methods for rapid detection of drug resistance are not available. Establishment of a more rapid culture based DST method would positively impact the management of a patient harbouring a drug resistant strain as appropriate therapy can be instituted earlier. This will curtail the spread of drug resistance and lead for better clinical outcomes. Thus, the study described in this chapter evaluated the 2 phenotypic methods, manual MGIT and NRA in liquid media as indirect DST for determination of rifampicin resistance. These 2 methods have been proposed as feasible options for detection of drug resistance and the selection of these methods for validation as DST in Sri Lanka were based on several reasons such as rapidity, cost effectiveness and minimum infrastructure requirements.

In contrast to solid medium based DST methods, NRA in broth medium and MGIT use an indicator to detect growth in the liquid medium, eliminating the need for visualization of growth as colonies. This reduces the turnaround time of the assay dramatically and the results of susceptibility testing will be available in less than 2 weeks. In the present study, there was a good agreement between APM and NRA in broth medium ($\kappa = 0.86$) or MGIT ($\kappa = 0.94$) for the detection of RIF resistance of *M. tuberculosis*. Additionally, the level of sensitivity and specificity of the tests are in line with similar type of studies reported previously from different countries (Adjers-Koskela and Katila, 2003).

In the evaluation of the manual MGIT for identification of RIF resistance, an in-house preparation of RIF solution was used instead of the commercially available rifampicin drug preparation kit (BD diagnostics, US). The excellent agreement between the manual MGIT and APM for determining RIF resistance confirms the suitability of using in-house preparation of RIF instead of commercially available drug kits ($ 450) that increases the cost of the test.

The final concentration of the drug in the culture medium is a critical factor that can affect the accuracy of the DST. Thus, dissolving of the correct amount of RIF powder completely in appropriate solute and abstaining from reusing once thawed solution is important for achieving correct potency of the drug. RIF is only partially (< 30%) soluble in distilled water. Therefore, DMSO or ethanol should be used for achieving accurate concentration of the RIF in drug solution.

The manual MGIT reader is a reliable and suitable instrument for use in low resource countries as no housing is required. The time spent in obtaining a reading is about 30 seconds. The cost of a MGIT reader is approximately US$ 3000 and it will be a long term investment. Alternatively, in the absence of a manual MGIT reader a simple ultra violet (UV) lamp (365nm) may be used to detect growth (Chitra and Prasad, 2001).

In the NRA, a standard colour series was used to interpret test results. In the case of intermediate results the test should be repeated for accurate interpretation. The intermediate results in NRA may occur due to low inoculum of the bacteria or when the primary culture represented a mixed growth of both susceptible and resistant strains. Contamination of the test medium of NRA can also lead to erroneous results as several

other bacteria can reduce nitrate to nitrite. As an example, *Gordonia sihwensis,* free living bacteria in water (Kim *et al.* 2003) and *Haemophilus influenzae,* pathogenic bacteria in the lung (Fleming and Fiere, 1977) have the ability to reduce nitrate. Thus, performing a purity test by sub culturing a blood agar plate with a loopful of the test medium will prevent reporting of false positive results due to contamination. In certain cases nitrate can further reduce into ammonia that cannot be detected by Griess regents. Therefore, Zn powder was used for the identification of false negatives. In the absence of bacterial growth, the nitrate should be reduced by Zn powder and produce pink colour with Griess reagents.

Nitrate reductase assay is easy to handle than other colourimetric methods such as MTT that use microplate format. The use of a liquid medium in a microplate format may increase complexity in handing and also constitutes a biohazard risk for healthcare workers.

The consumable cost per test for APM and NRA is around US$ 4.00 and US$ 3.00 respectively. Comparatively, the manual MGIT is more expensive (~US$ 7.00). However, both the NRA and the manual MGIT methods can be initiated with low technical expertise and initial cost. Additionally, both methods are more rapid than the APM as the results of susceptibility testing will be available in less than 2 weeks.

The evaluation of direct NRA and manual MGIT will dramatically reduce the turnaround time of the assay as primary isolation of the *M. tuberculosis* is not needed. However, in direct NRA and manual MGIT, the microbiological techniques should be addressed strictly to prevent contaminations.

Chapter 4

Molecular basis of rifampicin resistance of *M. tuberculosis*

4.1 Background

4.1.1 Rifampicin as an antibiotic

Rifampicin (RIF) is a semi-synthetic derivative of rifamycin B that is derived from *Nocardia mediterranei*. It is one of the most broad-spectrum antibiotics against staphylococci, streptococci, *Neisseria* sp. and *Legionella pneumophila* (Sensi, 1983). RIF is a red-brown crystalline powder with a complicated chemical structure (Figure 4.1) that is poorly soluble in water and freely soluble in chloroform, ethyl acetate and methanol.

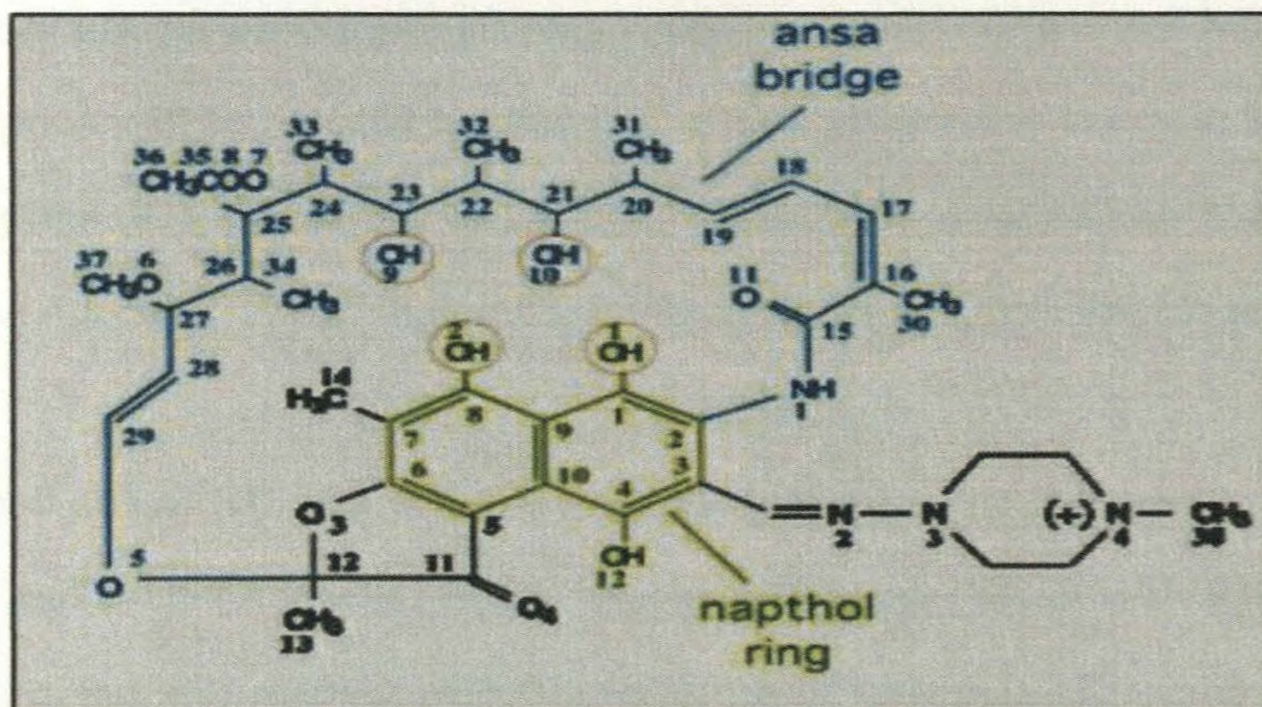


Figure 4.1 Molecular structure of rifampicin. The naphthol ring with oxygen atoms (O1 and O2 at C1 and C8) and ansa bridge with un-substituted hydroxyls (O10 and O9 at C21 and C23) are critical for the activity of rifampicin. (Sensi, 1983; Tella *et al.*2012).

RIF is extremely effective against intracellular pathogens like *M. tuberculosis* as it can diffuse freely into tissues, living cells and bacteria (Sensi, 1983). RIF was introduced into

therapeutic use in 1968 (Sensi, 1983) and is a key component of the short-course chemotherapy regimen for treatment of tuberculosis (WHO, 2010). RIF acts against dividing mycobacteria and hence lesser degree of activity on non-dividing bacilli. The bactericidal activity of RIF stems from its high-affinity to binding and inhibition of the bacterial DNA-dependent RNA polymerase (Campbell *et al.* 2001). However, there is no RIF activity against mammalian RNA polymerase (RNAPs), thus becoming an effective drug for eliminating the bacilli in pathogenic status (Wehrli, 1983)

4.1.1.1 Pharmacokinetics & pharmacodynamics

RIF is readily absorbed from the gastrointestinal tract. After ingesting a dose of 600 mg, the peak plasma concentrations may reach 10 μg/ml within 2 to 4 h (Acocella, 1978) and absorption will be reduced if taken with a meal (American Thoracic Society, 2003). After absorption, RIF that bound to plasma proteins is distributed into body tissues as well as fluids (Acocella, 1978). In pregnancy, it can cross the placenta (Magee *et al.*1996).

The half-life of RIF is 2 to 5 h and is metabolized in the liver and excreted with the bile, feces and urine (Acocella, 1978). Renal failure may result with RIF treatment in some patients, but will recover with the cessation of treatment (Kriscahoyo *et al.* 2000). RIF interacts with certain drugs such as moxifloxacin (by reducing plasma concentration) (Nijland, 2007) and oral contraceptives (by reducing effectiveness) (WHO, 2010).

4.1.1.2 Rifampicin toxicity

RIF is a well-tolerated drug (Kriscahoyo *et al.* 2000; WHO Essential Medicines Library) but diverse alterations in the gastrointestinal tract (abdominal pain, nausea, vomiting), skin, kidney and nervous system may arise (WHO Essential Medicines Library). Other adverse effects such as fever, influenza-like syndrome and thrombocytopenia are more likely to occur with intermittent administration. Clinical monitoring and liver function tests should be performed during treatment of all patients with pre-existing liver disease. RIF will cause a red-orange coloration of body fluids such as urine, tears, saliva, sweat, and sputum (WHO, 2010).

4.1.2 Bacterial RNA polymerase

RNA polymerase (RNAP) is a remarkable molecular machine in gene expression of living cells (Finn *et al.* 2000). The sequence, structure and function of RNAP exhibit unambiguous similarities within eukaryotes, bacteria, archaea and chloroplasts (Finn *et al.* 2000). Bacterial RNAP is a complex oligomer containing four different subunits, α, β, β' and σ and assembled into 2 major forms: a core enzyme ($\alpha_2\beta\beta'$) and a holoenzyme ($\alpha_2\beta\beta'$ plus σ). These sub units α, β, β' and σ are encoded by genes *rpoA*, *rpoB*, *rpoC*, and *rpoD* respectively. The core enzyme can perform RNA polymerisation but requires an σ subunit to initiate site-specific transcription at promoter sites (Ishihama, 1988).

During initiation of transcription RNAP recognizes the promoter DNA. A key function of RNAP is the promoter clearance that is transition from the initial transcribing complex

(ITC) to the elongating complex (EC). In ITC, holo enzyme is stably anchored at the promoter and elongates RNA up to 9 nucleotides in length. Then, initiation (ITC) will be aborted and as a result promoter clearance that involves the release of the σ factor will take place. Then, the commencement of elongation of RNA by the core enzyme will be initiated (Young *et al.* 2002).

4.1.3 The *rpoB* gene of bacteria

The *rpoB* gene (DNA-directed RNA polymerase β-Subunit gene) encodes the β-subunit of RNAP (Adekambi *et al.* 2009). It contains 3,534 nucleotides with 64.2% G + C composition and responsible for encoding a protein with 1,178 amino acids. The β subunit of RNA polymerase in bacteria is involved in chain initiation and elongation in transcription (Miller *et al.*1994).

In molecular studies, the *rpoB* gene is widely used as a molecular marker to define new bacterial species (Adekambi *et al.* 2009) and classification of the genus *Mycobacterium* (Lee *et al.* 2003). Further, the *rpoB* gene of *M. tuberculosis* is used for analysis of epidemiological evolution processes of tuberculosis strains especially among RIF resistant strains (Saeed *et al.* 2009).

4.1.4 Action of RIF on *M. tuberculosis*

4.1.4.1 RIF-RNAP complex

RIF specifically inhibits bacterial RNAP by forming a stable drug-enzyme complex. RIF makes contacts only with the RNAP β subunit by binding in a close complementary fit in a pocket between two structural domains of the RNAP β subunit. There is no direct binding of RIF at the RNAP active site and the closest approach of RIF to the active site (the distance between the active site $Mg2^+$ and Rif C-38) is occurs at a distance of 12.1 A° (Figure 4.2) (Campbell *et al.* 2001).

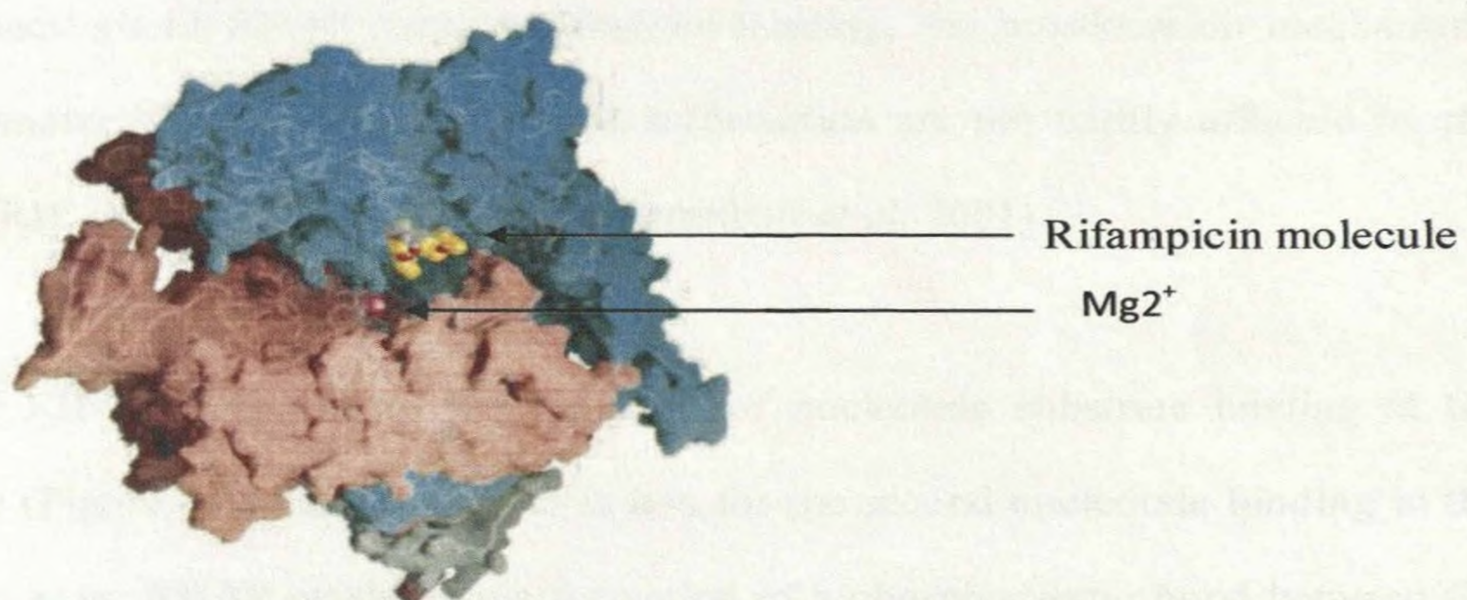


Figure 4.2 Three-dimensional structure of complex of RNAP and RIF. The backbone of the RNAP structure is shown as tubes, along with the colour coded transparent molecular surface. The Mg^{2+} ion chelated at the active site is shown as a magenta sphere. The RIF is shown as CPK atoms (carbon- orange, oxygen- red, nitrogen- blue) (Campbell *et al.* 2001).

4.1.4.2 Mechanism of RNAP Inhibition by RIF

The mechanism of the RIF action on RNAP has been studied mainly on *Taq* (*T. aquaticus*) and *E. coli* genome and the sequence of *rpoB* gene in both *E. coli* and *Taq* is

95% identical to *M. tuberculosis* (Campbell *et al.* 2001). Thus, current assumptions about the mechanism of RIF resistance in *M. tuberculosis* are based on studies of *E. coli* and *Taq*.

RIF binds to RNAP holo enzyme either before or after binding of RNAP to DNA template. Predominant effect of RIF binding on RNAP is direct, complete blockage of elongating RNA transcript. It prevents formation of second or third phosphodiester bond at the 5' end, once the transcript becomes either 2 or 3 nucleotides in length. Thus, RIF inhibits RNAP through a simple steric block of the path of the elongating RNA at the 5' end. The functions of RNAP such as substrate binding, the translocation mechanism, specific promoter binding and open complex formation are not highly affected by the presence of RIF (McClure and Cech, 1978; Campbell *et al.* 2001).

Presence of RIF slightly affects the initiating of nucleotide substrate binding at the RNAP i-site (Figure 4.3). But, the effect is less for the second nucleotide binding in the i+1 site. However, RNAP catalyses the formation of a phosphodiester bond between the two nucleotides. If the initiating nucleoside bears a 5' triphosphate, the subsequent translocation of the RNAP moves to the 2nd nucleotide RNA transcript upstream. Such that, i+1 nucleotide occupies the i-site (-1 position), and the i-site nucleotide moves into the - 2 position. The movement of the 5' nucleotide into the -2 position results in a severe steric clash with the RIF (Figure 4.3). In the end, the RNAP remains at the same template position, the 2nd nucleotide transcript is released, and the futile cycle begins again. The RNAP can translocate normally and the steric clash of the transcript with RIF

occurs during the translocation of the 3rd nucleotide transcript following the synthesis of the second phosphodiester bond (Campbell *et al.* 2001).

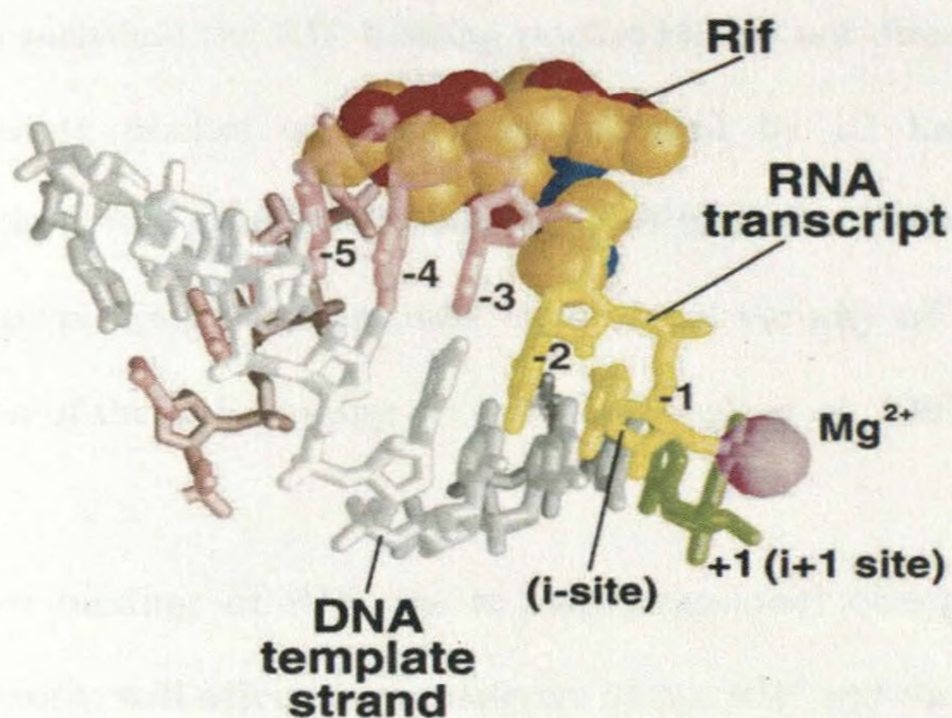


Figure 4.3 The RNAP active site $Mg2^+$ and the 9bp RNA/DNA hybrid (from +1 to -8) from a model of the ternary elongation complex. The incoming nucleotide substrate at the +1 position is coloured green. The -1 and -2 positions, which can be accommodated in the presence of RIF, are coloured yellow. The RNA upstream (-3 to -8), is pink colour. The template strand of the DNA is gray colour. RIF positioned in its binding site on the β subunit (carbon atoms- orange, oxygen- red, nitrogen- blue) (Campbell *et al.* 2001).

4.1.5 Rifampicin resistance due to *rpoB* gene mutations

The amino acid changes in the β subunit of RNAP responsible for RIF resistance (Jin and Gross, 1988). The 12 residues (amino acids Q 390, L 391, Q 393, F 394, D 396, H 406, R 409, S 411, G 414, L 413, I 452, E 445 in *Taq*) of the RNAP are close enough to interact directly with RIF and they are identical between *E. coli, Taq,* and *M. tuberculosis*. Among these 12 residues, the amino acid changes in 11positions are responsible for RIF resistant phenotypes. The 12th position (amino acid E 445 in *Taq*) is highly conserved.

Thus, its substitution would likely to be lethal and consequently not detectable as RIF mutations (Campbell *et al.* 2001). Further, 12 additional positions of RNAP binding pockets have been identified where substitution gives rise to RIF resistance. These residues surround the RIF binding pocket but do not directly interact with RIF. Thus, the RIF binding pocket of RNAP is enclosed by 23 known positions of amino acid substitutions that confer RIF resistance of bacteria. These substitutions would affect the folding or packing of the protein in the local vicinity of the substituted residue, causing distortion of the RIF binding pocket (Campbell *et al.* 2001).

Defective binding of RIF due to conformational changes in the β subunit of RNAP consequently will affect the sensitivity of the RIF and these changes in the β subunit will lead by mutations in the *rpoB* gene. An 81bp region (codons 507–533, according to *E. coli* numbering system) of the *rpoB* gene has been recognized to harbour more than 90% point mutations that lead to RIF resistance in bacteria in different countries. Thus, this region has been named as RIF resistant determining region (RRDR) of *rpoB* gene (Mani *et al.* 2001; Zhang and Telenti, 2000; Matsiota-Bernard *et al.* 1998). However, recent reports have shown that regions spanning the RRDR also contribute to the RIF resistance of *M. tuberculosis* (Lingala *et al.* 2010).

Mutational sites and frequencies of mutations in the RRDR of *rpoB* gene seem to vary between different geographical regions (Qian *et al.* 2002; Pozzi *et al.* 1999; Mani *et al.* 2001). Most common substitution of amino acids has been observed at codons 531, 526 and 516 of RRDR (According to *E. coli* numbering system) (Ramaswamy and Musser,

1998; Ohno *et al.* 1996; Williams *et al.*1998). The minimum inhibitory concentration (MIC) studies suggest that high level of RIF resistance is associated with mutations at codons 526 and 531, whereas substitutions at codons 511,516, 518 and 522 lead to low level RIF resistance (Johnson *et al.*2007). Table 4.1 shows the extensive studies on the *rpoB* gene mutations in RIF resistant isolates of *M. tuberculosis* addressing the nature of amino acids substitutions related to resistance.

4.1.6 Methods for detection of point mutations

During the past several years, many molecular methods such as gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), heteroduplex analysis, RNAse a cleavage method, chemical cleavage method, enzyme mismatch cleavage, hybridization on DNA chips and DNA sequencing have been developed for screening of point mutations, small deletions and insertions of a gene (Nollau and Wagener 1997). However, automated DNA sequencing is currently considered as the gold standard and widely used for detection of point mutations (Hegde and Roa 2006; Nollau and Wagener 1997).

Table 4.1 Mutations in the RRDR of the *rpoB* gene reported up to 2010

Codon No.	Nucleotide Change	Amino Acid Change	Citation
507	GGC→AGT GGC→GGT GGC→GAT GGC→GAC GGC→ GGT	Gly→ Ser Gly→ Gly Gly→ Asp Gly→ Asp Gly → Gly	Bostanabad *et al.*2007 Bostanabad *et al.*2007 Bostanabad *et al.*2007 Yue *et al.* 2003: Kim *et al.*1997 Yue *et al.*2003
508	ACC→GCC ACC→CCC ACC→CAC	Thr→ Ala Thr→ Pro Thr→ His	Bostanabad *et al.*2007 Bostanabad *et al.*2007 Bostanabad *et al.*2007
509	AGC→GAC	Cys→ Asp	Bostanabad *et al.*2007
510	CAG→_AG	Gln→ deletion	Bostanabad *et al.*2007
511	CTG→CCG CTG→GTG CTG→ CTA	Leu→ Ser Leu→ Val Leu→ Lue(Silent)	Bostanabad *et al.*2007 Yue *et al.* 2003: pozzi *et al.* 1999; Bostanabad *et al.*2007 Kim *et al.*1997: Lingala *et al.* 2010
512	AGC→GGC AGC→GCC AGC→ACC	Ser→ Tyr Ser→ Gly Ser →Thr	Bostanabad *et al.*2007 Bostanabad *et al.*2007 Pozzi *et al.*1999
513	CAA→ AAT CAA→ TAA CAA→ GAA CAA→ AAA CAA →deletion CAA → CCA CAA→ CAC	Gln→Asn Gln→Stop Gln→Glu Gln → Lys Gln →Pro Gln →Pro	Bostanabad *et al.*2007 Bostanabad *et al.*2007 Bostanabad *et al* 2007: lingala *et al.* 2010 Yue *et al.*2003.Qian *et al.*2002 Lingala *et al.* 2010 Doustdar *et al.* 2008 Kim *et al.* 1997
514	TTC→ insersion	Phe→ insertion	Hirano *et al.* 1990
515	ATG → GTG	Met → Val	Kim *et al.*1997
516	GAC→CAC GAC→ GGC GAC → TAC GAC→ GTC GAC→ GAC → GCA	Asp→ His Asp → Gly Asp→ Tyr Asp→ val Asp→ Arg Asp→ Ala	Bostanabad *et al.*2007 Yue *et al.*2003 Kim *et al.* 1997; Pozzi *et al.*1999; Doustdar *et al.*2008 Yue *et al.*2003:Kim *et al.* 1997 Lingala *et al.*2010 : Pozzi *et al.* 1999; Doustdar *et al.* 2008 Qian *et al.*2002 ,Lingala *et al.*2010
518	AAC → TAC AAC → CAC	Asn → Tyr Asn → His	Yue *et al.*2003; Lingala *et al.* 2010 Kim *et al.* 1997
519	AAC→AAG AAC → deletion	Asn→Lys Asn→deletion	Bostanabad *et al.*2007 Lingala *et al.* 2010
520	CCG→C_G	Leu→deletion	Bostanabad *et al.* 2007
522	TCG → CCG TCG → TTG	ser →Pro Ser → Leu	Yue *et al.*2003; Kim *et al.*1997 Doustdar *et al.*2008
523	GGG→GCG GGG→GG_ GGG → TGG	Gly→Ala Gly→deletion Gly → Trp	Bostanabad *et al.*2007 Bostanabad *et al.*2007 Pozzi *et al.*1999
525	ACC → ATC	Asp → Try	Pozzi *et al.*1999

526	CAC →GAC	His→ Asp	Yue *et al*.2003; Kim *et al*.1997: Pozzi *et al*. 1999; Doustdar *et al*.2008
	CAC→ CTC	His → Leu	Yue *et al*.2003; Kim *et al*. 1997. Lingala *et al*. 2010
	CAC →CCC	His → Pro	Qian *et al*.2002;Yue *et al*.2003:Yue *et al*.2003
	CAC → GCC	His→ Ala	Bostanabad *et al*.2007;Yue *et al*.2003: Lingala *et al*, 2010 ; Kim *et al*.1997: Pozzi *et al*.1999; Doustdar *et al*.2008,Qian *et al*.2002
	CAC→TAC	His→Tyr	Bostanabad *et al*.2007; Yue *et al*.2003: Lingala *et al*. 2010; Qian *et al*.2002
	CAC→AAC	His→Asn	Bostanabad *et al*.2007
	CAC→_GC	His→deletion	Bostanabad *et al*.2007; Yue *et al*.2003: Kim *et al*. 1997; Pozzi *et al*.1999: Qian *et al*. 2002
	CAC→CGC	His→Arg	Bostanabad *et al*.2007
	CAC→TTC	His→Phe	Bostanabad *et al*.2007
	CAC →CAA	His→Gln	Bostanabad *et al*.2007
	CAC → TCG	FIs → Cys	Soudani *et al*.2007
	CAC → TGC	His → Cys	Kim *et al*.1997
529	CGA → CAA	Arg →Gln	Lingala *et al*.2010
531	TCG→TTG	Ser→ Leu	Bostanabad *et al*.2007; Kim *et al*.1997: Pozzi *et al*.1999; Doustdar *et al*.2008:Qian *et al*.2002
	TCG→TTC	Ser→ Phe	Yue *et al*.2003; Soudani *et al*.2007; Lingala *et al*. 2010; Pozzi *et al*.1999 Doustdar *et al*.2008
	TCG→ TTG	Ser→ Leu	Lingal *et al*.2010
	TCG → TCC	Ser→ Phe	Yue *et al*.2003; Soudani *et al*.2007:Lingala *et al*.2010
	TCG →TGG	Ser→ Trp	Yue *et al*.2003,Qian *et al*.2002
	TCG → CAG	Ser→ Gln	Yue *et al*.2003
	TCG →TAC	Ser→ Tyr	Soudani *et al*.2007
	TCG→ GCG	Ser →Ala	
533	CTG →CCG	Leu → pro)	Yue *et al*.2003; Doustdar *et al*.2008;Qian *et al*.2002

4.2 Justification and objective of the present chapter

The molecular drug susceptibility testing play a major role in control of MDT-TB and prevention of XDR-TB. These methods are based on the detection of mutations that are responsible for each drug. The available commercialised molecular DST methods for detection of RIF resistance, marker of MDR-TB have been customized to detect the world's prevailing mutations in the RRDR of *rpoB* gene. The recent literature have recorded that mutations of *rpoB* gene responsible for RIF resistance are not restricted to the RRDR and vary geographically (Lingala *et al.* 2010; Tan *et al.* 2011). Thus, investigation of the *rpoB* gene mutations in a specific geographical area is vital prior to initiation of a proper molecular DST method for determination of RIF resistance. Molecular DST methods are yet to be established in Sri Lanka. Thus, the objective of the present chapter is to identify the RIF resistant mutations of the *rpoB* gene of *M. tuberculosis* among drug resistant isolates using DNA sequencing.

4.3 Research methodology

4.3.1 Study population

Thirty one (31) RIF resistant *M. tuberculosis* isolates identified by one or more phenotypic methods (APM, NRA and manual MGIT) in chapter 3 were used for the study. H37Rv strain and a known RIF resistant MTB isolate confirmed by the National Tuberculosis Institute, Bangalore, India were used as quality control strains.

4.3.2 Extraction and purification of genomic DNA

Fully grown, fresh, three L-J slants from each *M. tuberculosis* isolate were used for extraction of DNA by phenol-chloroform method. Cultures were heat inactivated by incubating the L-J slants in a pre-heated oven at 80 °C for 1 h (Warren *et al.* 2006). Once cooling to room temperature, 3 ml of extraction buffer (Appendix 3.3) was added to each of the L-J slants. Bacterial colonies were scraped off from the L-J medium (without loosening the culture medium) with a sterile disposable 10 µl plastic loop. The bacterial suspension (in extraction buffer) was added into a labelled, new, sterile 50 ml polypropylene tube containing approximately 20x5 mm glass beads (up to 3 ml). Three suspension of each isolates were pooled and vortexed for 2 min. The vortexed bacterial suspensions were treated with 500 µl of lysozyme (50 mg/ml) and 2.5 µl of RnaseA (10 mg/ml) and incubated at 45 °C in a pre-heated oven. After 16 h of incubation, 5 ml of phenol/ chloroform/ isoamylalcohol (25:24:1) was added. The mixture was gently mixed at every 30 min for 2 h at room temperature. The tubes were centrifuged at 3000 *g* for 20 min at room temperature. The separated top layer was treated with 5 ml of chloroform/isoamylalcohol (24:1) in a new plastic sterile 50 ml tube and centrifuged again using the same conditions. The top phase was separated into new sterile 50 ml tube containing 600 µl 3M sodium-acetate (pH 5.2) and mixed well. Then, the DNA that precipitated by adding 7 ml of ice-cold isopropanol was immediately collected on a thin glass rod. The glass rod with DNA was incubated in a 1.5 ml micro-centrifuge tube containing 1ml of 70% ethanol at room temperature for 10 min for further purification. Then, air dried DNA pellet on glass rod (3 h) was rehydrated in 1.5 ml new micro-centrifuge tube with 200 µl TE (pH 8.0) (Appendix 3.4) and the tube was incubated at 4

°C overnight to ensure complete dissolving of DNA. The purity and the concentration of extracted DNA were determined using the gel electrophoresis. Extracted pure DNA also was quantified using a NanoDrop (2000c/2000) UV-Vis Spectrophotometers (Thermo, USA) and stored at -20 °C).

4.3.3 DNA amplification

4.3.3.1 Primer selection

Three fragments collectively covering approximately 60% of the total length of *rpoB* gene including RRDR (437bp), upstream of RRDR (1395bp) and downstream of RRDR (872bp) were selected for amplification (Figure 4.4).

Specific primers were designed using primer-3 software and published literature (Van-Der-Zanden *et al.* 2003; Rigouts *et al.* 2007). For amplification of the 437bp region including RRDR, rpoBF primer and rpoBR primer were used. TR9 and RRBN primer pair was used to amplify the 872bp region downstream of the RRDR. The 1395bp fragment upstream of RRDR was amplified using FRBN and TR8 primer pair (Figure 4.4 and Table 4.2) (All primers were purchased from Promega, USA).

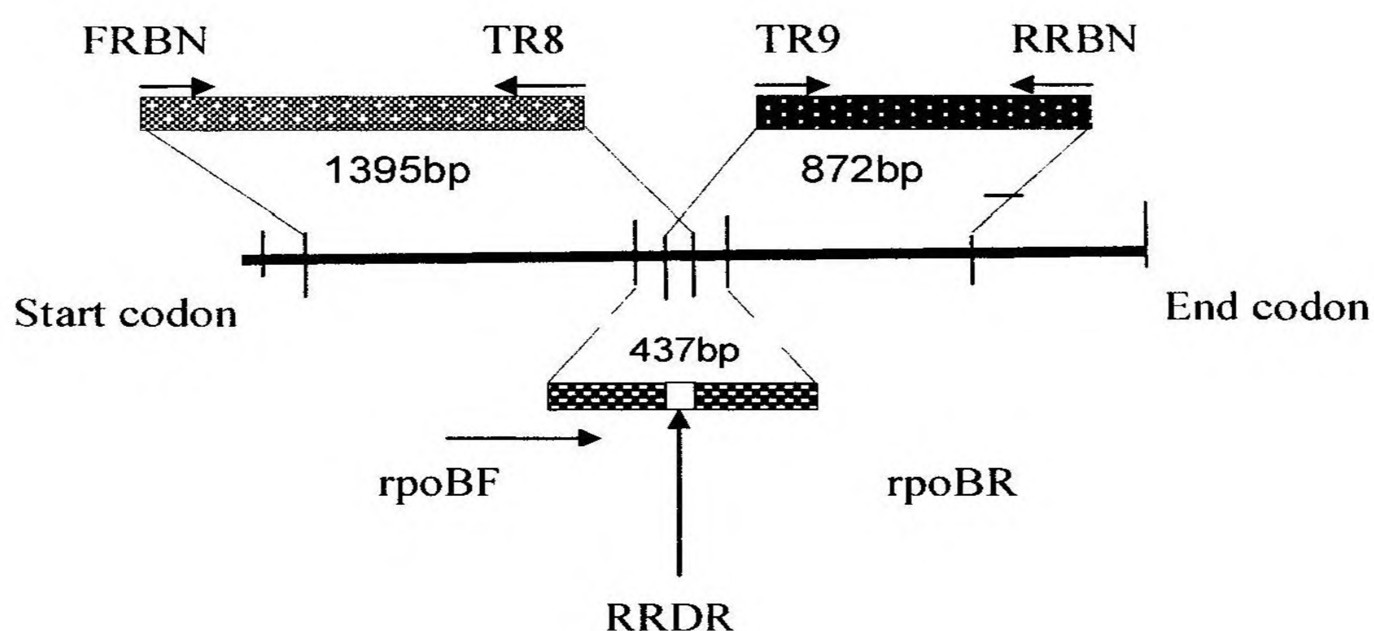


Figure 4.4 Positions of primers used for amplification of selected fragments of the *rpoB* gene of *M. tuberculosis*

4.3.3.2 Optimization of PCR amplification

The PCR reaction mixture and thermal cycle parameters were optimized in order to obtain high quality and quantity (>100 ng/µl as requirement of DNA sequencing) of PCR amplified fragments (437bp, 872bp and 1395bp) of the *rpoB* gene.

The concentration of $MgCl_2$ was varied from 1.0 - 2.5 mM in 0.5 mM increments. The amount of Taq polymerase was varied from 0.5U to 2U in 0.5 U increments. The optimum DNA concentration was determined through a 10 fold series of DNA concentration form 10 ng to 1 pg.

The following thermo cyclic parameters were applied in the PCR amplification. Denaturation temperature and extension temperature were kept at 94 °C and 72 °C while

the annealing temperature was varied around calculated annealing temperatures of primers. In optimization of amplification of the 437bp fragment, the annealing temperature was determined by varying the temperature from 56 °C to 59 °C by 1degree increments. For the optimization of 872bp fragment, the temperature was varied from 56 °C to 65 °C. The 1395bp fragment was optimized by varying the temperature from 56 °C to 62 °C by 1 degree increments.

4.3.3.3 PCR amplification of selected isolates

All isolates (31) were PCR amplified under optimized conditions as follows. The 50 µl PCR mixture containing 1.5 mM $MgCl_2$, 200 µM of deoxynucleotide triphosphates (dNTPs) (Promega, USA), 1U *Taq* polymerase (GenScript), 20 pmol of each primer and 2.5 µl of genomic DNA (10 ng) was used for each PCR reaction. The thermo-cycling parameters used for amplification of three segments of the *rpoB* gene are shown in Table 4.2.

4.3.3.4 Agarose gel electrophoresis

The ethidium bromide (EtBr) stained agarose gel (1.5%) was prepared as described in section 2.3.6.3. The PCR products (2 µl) were mixed with gel loading buffer (4 µl) (Appendix 3.2) and loaded on to the gel. Electrophoresis was carried out at 50 V for 2.5 h and visualized under UV illumination. The concentration of PCR products was determined by using known molecular weight marker.

Table 4.2 Primer sequences and thermo-cycling parameters used for the PCR amplification of selected regions of the *rpoB* gene in RIF resistant *M. tuberculosis* isolates.

Forward primer (5'-3')	Reverse primer (5'-3')	Length Of the fragment	Thermo-cycling parameters
rpoBfor TGGTCCGCTTGCACGA GGGTCAGA	rpoBrev CTCAGGGGTTTCG ATCGGGCACAT	437bp	94°C- 10min – denaturing 94°C-1min 57°C-1min 40 cycle 72°C-1 min 72°C-10 min – extension
TR9 TCGCCGCGATCAAGGA GT	RRBnew GCGCCATCTCGCC GTCGTCAGTACAG	872bp	94°C-10min– denaturing 94°C-1min 62°C -1min 40 cycle 72°C-1 min 72°C-10 min – extension
FRBnew GCAAAACAGCCGCTAG TCCTAGTCCGA	TR8 TGCACGTCGCGGA CCTCCA	1395bp	94°C- 10min – denaturing 94°C-1min 60°C -1min 40 cycle 72°C-1 min 72°C-10 min. – extension

4.3.3.5 DNA sequencing

Each strain was PCR amplified in duplicate and both amplified products (40 μl, ~ 75 ng/μl) were custom DNA sequenced by Macrogen DNA sequencing service in Korea (Capillary sequencing technique - ABI 3730xl Machine). In brief, sequencing reactions

were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using selected primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

The quality control of the DNA sequencing was done with H37Rv strain and a known RIF resistant MTB isolate confirmed by the National Tuberculosis Institute, Bangalore, India.

4.3.3.6 Data Analysis

A search of the GenBank database with the BLAST program was performed to determine the homology of the DNA sequences and homologues sequence were aligned with SeaView software (version 4.2.12) to identify the presence of point mutations. The *rpoB* gene sequence of H37Rv strain of *M. tuberculosis* was used as the reference sequence.

4.4 Results

Point mutations of the *rpoB* gene in *M. tuberculosis* isolates were determined using PCR amplification and DNA sequencing. The majority of isolates were from the Western Province (Colombo, Gampaha, and Kalutara districts) of Sri Lanka.

4.4.1 Optimum PCR parameters

The optimum concentration of $MgCl_2$, Taq polymerase and genomic DNA were 1.5 mM, 1U and 10 ng/µl respectively. The optimum thermo cycling parameters are shown in Table 4.2. Figure 4.5, 4.6 and 4.7 represent photographs of the agarose gel electrophoresis obtained for each amplified fragments of *rpoB* gene.

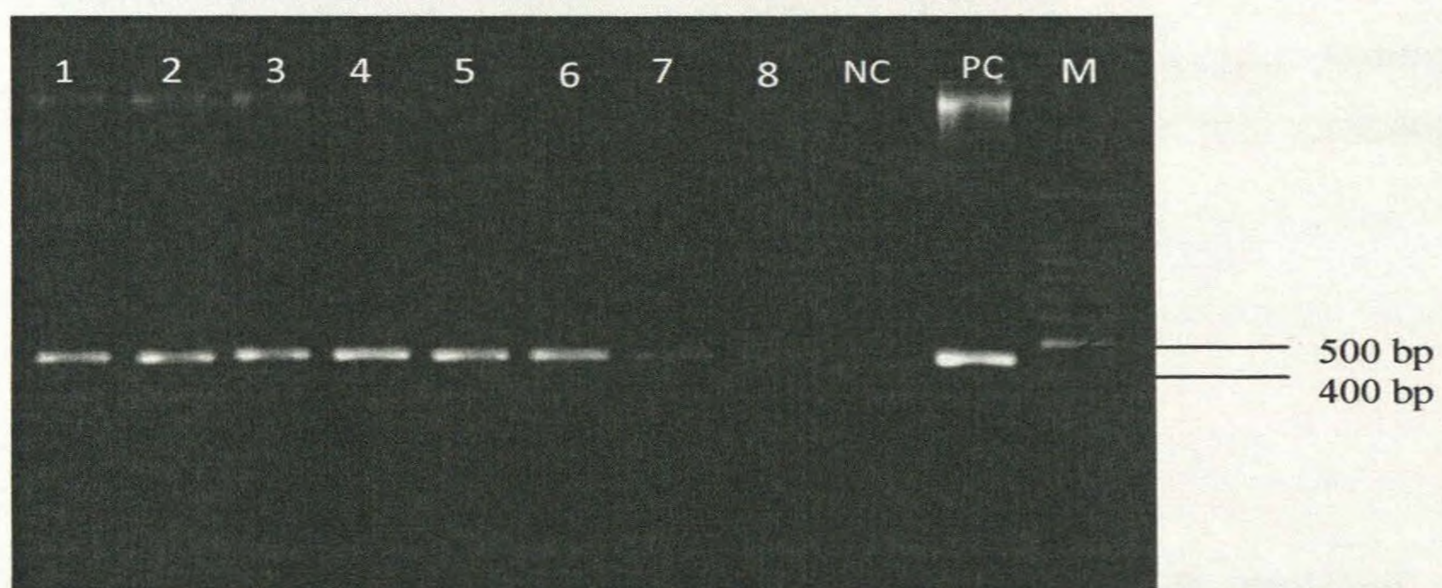


Figure 4.5 Gel photograph showing agarose gel electrophoresis of PCR amplified 437bp fragment of *rpoB* gene for selected isolates. Lanes 1-8 are MTC isolates, NC–Negative control, PC- positive control and M- molecular marker.

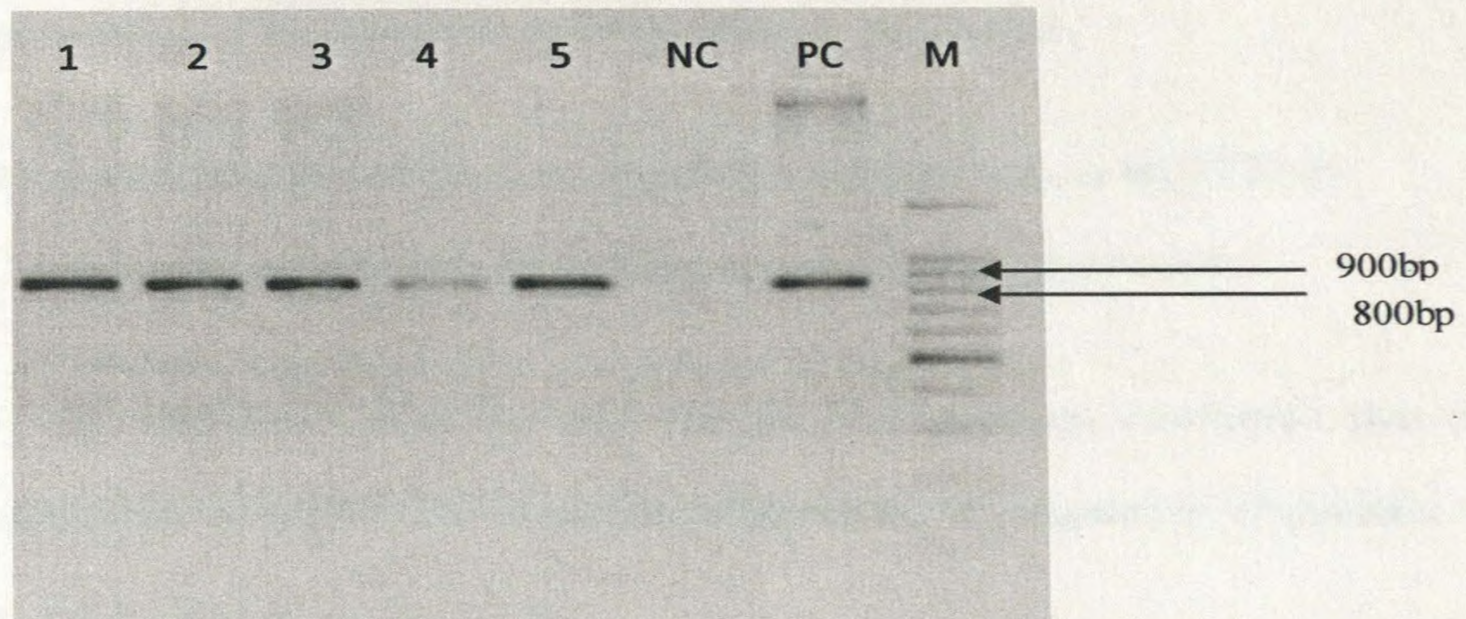


Figure 4.6 Gel photograph showing agarose gel electrophoresis of PCR amplified 872bp fragment of *rpoB* gene for selected isolates. Lanes 1-5 are MTC isolates, NC–negative control, PC- positive control and M- molecular weight marker

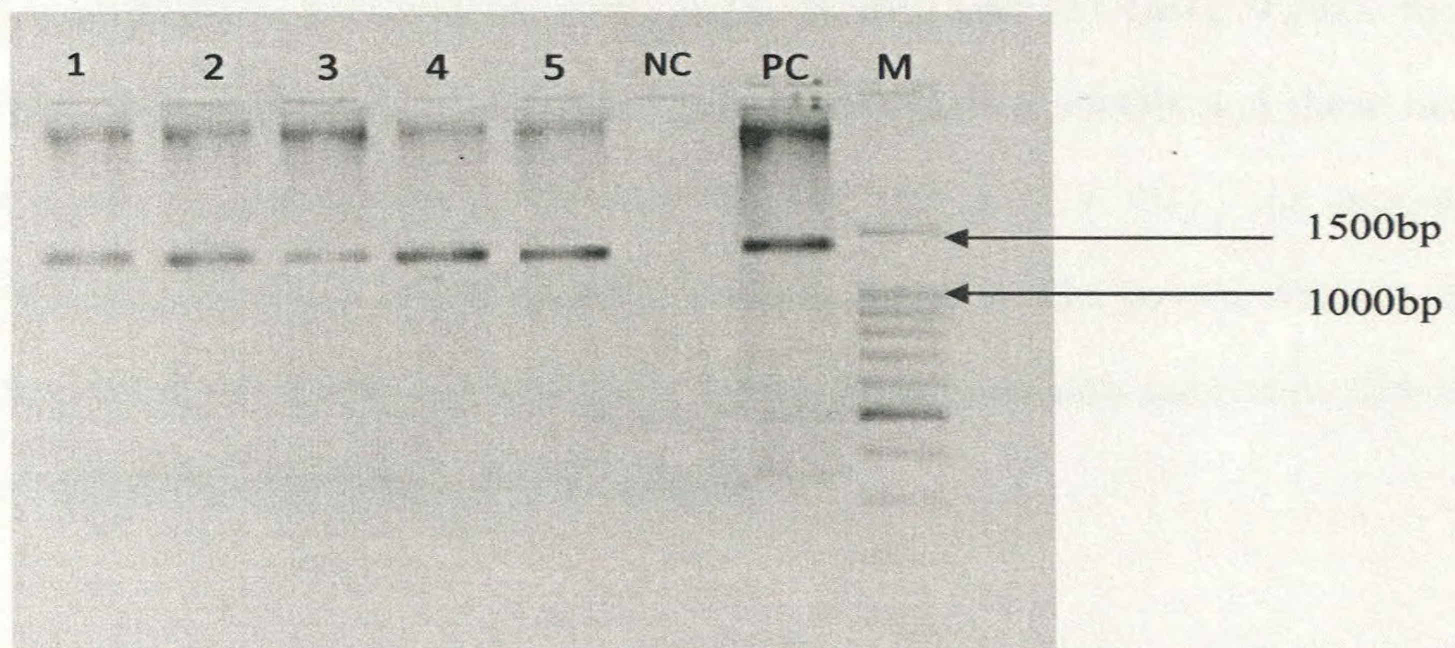


Figure 4.7 Gel photograph showing agarose gel electrophoresis of PCR amplified 1395bp fragment of *rpoB* gene for selected isolates. Lanes 1-5 are MTC isolates, NC-negative control, PC- positive control and M- molecular weight marker.

4.4.2 *rpoB* gene mutations of *M. tuberculosis* isolated from Sri Lanka

The NCBI Accession numbers of the deposited DNA sequences were HQ377336-HQ377338, HQ377340- HQ377345, HQ377351, and JQ314433-JQ314444.

A search of the Gen Bank database with the BLAST program confirmed that the sequences belonged to MTC. The multiple alignments of sequences (Appendix 4) indicated the presence of point mutations.

DNA sequences analysis of the amplified *rpoB* gene fragments revealed that in 54% of drug resistant tuberculosis strains the mutations were in the RRDR region (n=18). These mutation were confined to two codons, 526 (n=15, 48.4%) and 531 (n=3, 9.7%). In the balance isolates, mutations were detected in the regions outer to RRDR and these novel mutations occurred at codons 626 (n=13, 41.9%) and 184 (n=2, 6.4%). As shown in Table 4.3, 29 isolates showed single point mutations at codon 526 (n=14, 45.2%), 626 (n=11, 35.5%), 531 (n=3, 9.7%) and 184 (n=1, 3.2%). Dual mutations (at codon 526+626 and at codon 184+626) were detected in 2 isolates.

Mutations observed at codon 526 (CAC → TAC) and codon 531 (TCG →TTG) was a C to T transition mutation while the novel mutations at codon 626 (GAC → GAG) was a C to G transversion mutation and at codon 184 (GAC → GAT) a transition mutation. All point mutations in this study revealed only a single mutation pattern and there were no multiple patterns of base changes at a single codon.

The Table 4.4 and the Figure 4.8 illustrate the comparison among percentages of most prevailing mutated codons of *rpoB* gene in different geographical areas and those from Sri Lanka.

Table 4.3 Mutation types and frequencies observed for the *rpoB* gene of *M. tuberculosis* isolates in Sri Lanka. The point mutations with corresponding amino acids are indicated.

Mutated codon	Specific mutation	Primary/secondary TB (No.)	Percentage %
526*	CAC (His) → TAC (Tyr)	Secondary-10 Primary -4	45.2
626$^{\$+}$	GAC(Asp) → GAG (Glu)	Secondary-7 Primary -4	35.5
531*	TCG (Ser) → TTG (Leu)	Secondary-0 Primary-3	9.7
184$^{\$+}$	GAC(Asp) → GAT(Asp)	Secondary-0 Primary-1	3.2
626,184	GAC→GAG,GAC → GAT	Secondary-0 Primary-1	3.2
526, 626	CAC→TAC, GAC→ GAG	Secondary-1 Primary-0	3.2
Total			100

*Codons within RRDR, $^{\$}$Codons outer to RRDR, $^{+}$novel mutations

Table 4.4 Comparison among percentages of prevailing mutated codons of *rpoB* gene in 6 different geographical regions

Mutated codon in RRDR	India (Lingala *et al.* 2010) n=30	China (Yue *et al.* 2003) n=72	Sri Lanka n=31	Iran (Bostanabad *et al.* 2007) n=91	Australia (Yuen *et al.* 1999) n=30	Italy (Pozzi *et al.* 1999) n=37
codon 516	13.3	5	0	1.39	10	2.7
codon 526	16.6	40	45.2	16.65	32	29.7
codon 531	46.6	41	9.7	9.56	52	56.7
codon 523	0	0	0	23.63	0	0
codon 626	0	0	35.5	0	0	0
No mutations in RRDR	21	10	41.9	5	4	0

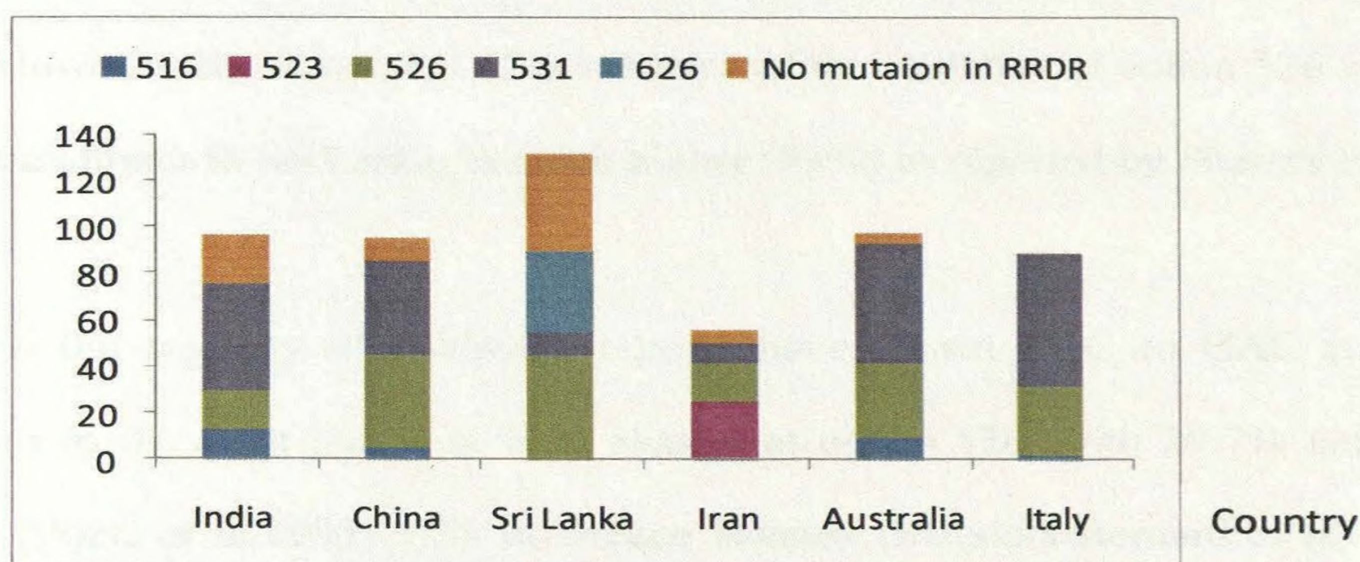


Figure 4.8 Graphical view of the comparisons among percentage of most prevailing mutated codons in *rpoB* gene in 6 different geographical regions. The profile of mutations in Sri Lanka is different from that of other countries and mutation at codon 626 is unique to Sri Lanka. Mutation in codon 523 is unique to Iran.

4.5 Discussion

It is widely reported that *M. tuberculosis* expresses a high degree of geographical diversity in mutations of *rpoB* gene across various parts of the world (Yue *et al.*2003; Ruwen *et al.* 2005; Kim *et al.*1997; Miranda *et al.* 2001; Yuen *et al.* 1999). Mutations at codon 531, 526 and 516 have been reported as the most frequent mutation sites in the mutation hotspot region of the *rpoB* gene in *M. tuberculosis* worldwide (Sajduda *et al.* 2004; Yue *et al.* 2003; Bostanabad *et al.* 2007).

In the present study, the majority of mutations occurred at codon 526 and the mutation frequency (48.4%) observed was slightly higher than frequencies reported by China (40%) (Yue *et al.* 2003), Japan (35%) (Ohno *et al.* 1996) and Korea (38%) (Kim *et al.* 1997). However, the frequency of occurrence of the mutation at codon 526 in India, the closest neighbour to Sri Lanka, is much higher (93%) as reported by Sharma *et al* (2003).

Although the majority of published reports have shown CAC to GAC (transversion mutation) as the most prevalent base change at codon 526, with 29.7% among Italian isolates (Pozzi *et al.*1999) 12% in Greece isolates (Matsiota-Bernard *et al.* 1998), our study revealed the base change was CAC to TAC (transition mutation) in all isolates similar to what has been reported in India (Sharma *et al.* 2003).

The frequency of mutation at codon 531 (9.7%) was much lower in *M. tuberculosis* isolates from this study, compared to frequencies reported by other countries including India (47%) (Lingala *et al.*2010), Australia (52%) (Yuen *et al.* 1999), Vietnam (31%)

(Caws *et al.* 2006) and Poland (41%) (Sajduda *et al.* 2004). However, the base change observed at codon 531 was confined to TCG to TTG (transition mutation) as reported from several Asian countries (Japan (Ohno *et al.* 1996) China (Yue *et al.* 2003), Korea (Kim *et al.* 1997), India (Mani *et al.* 2011) and other countries such as Greece (Matsiota-Bernard *et al.* 1998) and Italy (Pozzi *et al.* 1999). Previous studies have reported that a mutation at codon 531 is frequently associated with the codon 526 mutation in certain regions of the world (Ruwen *et al,* 2005). However this phenomenon was not observed in our study.

The mutation at codon 626 (n=13, 41.9%) GAC (Asp) → GAG (Glu) was the second major mutation found in the present study. Codon 626 is placed downstream of RRDR and has not been previously reported. This novel mutation was present in 13 isolates.

Two isolates showed a silent mutation at codon 184 GAC (Asp) → GAT (Asp) that is placed upstream of RRDR. In one isolate, mutation at codon 184 was associated with a mutation at codon 626. Several mutations in the upstream region of RRDR also have been reported from India (Lingala *et al.* 2010).

Mutations at codon 516 have been reported as a prominent mutation in several geographical areas such as India, 13% (Lingala *et al.* 2010) and Iran, 10% (Doustdar *et al.* 2008) and therefore, commercially available rifampicin resistance diagnosis kits have been optimized to detect mutation at codon 516 among other mutation in the RRDR. However, no mutations were observed at codon 516 in the present study.

Several publications have stated that ~ 4% - 10% of their phenotypically resistant *M. tuberculosis* isolates did not show any mutations within the RRDR (Caws *et al.* 2006; Hirano *et al.* 1999; Yue *et al.* 2003). According to Lingala *et al.* (2010) 21% of phenotypically rifampicin resistant isolates did not show any mutations when a 255bp region of RRDR and 350bp region of non-RRDR of the *rpoB* gene were sequenced. This may be due to mutations occurring in the region outer to RRDR, and this phenomenon was observed in 41.9% of rifampicin resistant isolates of *M. tuberculosis* in the present study. Also a single discordant result was observed between phenotypic and genotypic results in the present study as a isolate showed a silent mutation at codon 184. This isolates was confirmed as resistant to rifampicin by APM and MGIT. It is possible that an additional point mutation is present in this isolate and it may be at a codon elsewhere in the *rpoB* gene not investigated in the present study.

Current molecular diagnostic methods such as INNO-LiPARif are designed to detect known *rpoB* mutations at codon 531, 526 and 516 that are commonly reported in *M. tuberculosis* isolates. Although the mutations in our strains are limited to a few types, the occurrence of a high percentage of mutations outside the RRDR presents important implications for the use of currently available rapid molecular methods which target the mutations in the RRDR region. Molecular tests used for rapid detection of drug resistance need to be customized to enable detection of novel mutations in different geographical areas. Therefore, identification of drug resistant mutations and their relevant frequencies is an important first step prior to introducing the molecular drug susceptibility testing in a particular geographical setting.

Chapter 5

PCR-Enzyme-Linked Immunoabsorbent Assay (PCR–ELISA) for determination of rifampicin resistance of *M. tuberculosis*

5.1 Background

5.1.1 Nucleic acid hybridization

Duplex DNA can be separated into their complementary strands by heat or increasing pH and these strands will gradually reanneal to form the double stranded DNA molecule. This ability is possible only with the interaction of single-stranded molecules that have a homologues base sequence to denatured DNA. This is known as nucleic acid hybridization, a fundamental tool in molecular genetics assay. The rationale of the hybridization assay is identification of the target DNA by using a known DNA sequence called a probe (Strachan and Read, 1999).

5.1.1.1 Nucleic acid probe

Nucleic acid probes are single-strands and can be DNA, RNA (Wolcott, 1992) or oligonucleotide probes. Conventional DNA probes, 0.1 kb to hundreds of kilobases in length are isolated by cloning or by PCR amplification. RNA probes conveniently can be generated by cloning (Melton *et al.* 1984). Oligonucleotide probes are chemically synthesized single-stranded pieces of DNA with 15–50 nucleotides.

5.1.1.1.1 Probe labelling

Incorporation of probes with the reporter molecule is known as probe labelling. The label can be uniform or attached to the 5' or 3' end of the nucleotide sequence. In labelling of DNA by *in vitro* DNA synthesis, DNA or RNA polymerase is used to make labelled nucleotide copies and at least one of the four nucleotide precursors should carry a labelled group. Labelling of DNA by synthesis is normally accomplished using nick-translation, random primed labelling or PCR-mediated labelling (Strachan and Read, 1999).

Nick translation (Rigby *et al.* 1977) is a uniform labelling method and used the combined activity of two enzymes. The nick-translation procedure involves introducing single-strand breaks (nicks) in the DNA, leaving exposed 3′ hydroxyl termini and 5′ phosphate termini.

The random primed DNA labelling method (Feinberg and Vogelstein, 1983) is based on hybridization of a mixture of all possible hexanucleotides. The starting DNA is denatured and then cooled slowly to bind individual hexanucleotides with complementary sequences in the DNA strands. Synthesis of new complementary DNA strands is primed by the bound hexanucleotides and is catalysed by the Klenow subunit of DNA polymerase I. Random primed DNA labelling produces labelled DNAs of high specific activity as all sequence combinations are represented in the hexanucleotide mixture, binding of primer to template DNA occurs in a random manner, and labelling is uniform across the length of the DNA (Strachan and Read, 1999).

The end labelling is provided in the form of a $\gamma\ ^{32}P$ – ATP and single stranded oligonucleotides are labelled at the 5' or 3' end using polynucleotide kinase. The enzyme polynucleotide kinase transfers a labelled phosphorus group from $\gamma\ ^{32}P$ – labelled ATP to the free hydroxyl group (Hilario, 2004).

5.1.1.1.2 Radioactive and non-radioactive probes

The radioactive probes that are labelled by either α or the γ position of different radioisotopes such as ^{32}P, ^{3}H, ^{125}I and ^{131}I are commercially available. Most commonly used isotope is ^{32}P as it gives high specific activity with optimal sensitivity and resolution in hybridization assays. However, as the half-life of ^{32}P is 14 days, the labelled probes cannot be stored for a long period (Keller and Manak, 1989).

In autoradiographic detections, radioactive probes are versatile, simple and highly sensitive. However, it is of restricted use in routine diagnosis due to the inhabitant limitations such as instability of the labelled probe, short half-life of the isotopes, high cost, biohazard risks in handing and need for special waste management systems (Strachan and Read, 1999).

The non-radioactive probes such as biotin labelled probes has overcome the limitations associated with radioactive probes (Pereira, 1986). Most non-radioactive labelling procedures target the amino group substituent on the purine or the pyrimidine ring of the nucleotide bases. Fluorescein (Eshaghpour *et al.*1979), horseradish peroxidase (Renz and

KurZ, 1984) and alkaline phosphate (Reyes and Cockerell, 1993) are the most common reporter groups employed for labelling. The most commonly use haptens are the vitamin biotin (Leary *et al.* 1983) and the plant steroid digoxigenin (Heiles *et al.* 1988) that can be covalently linked to the nucleotide base. Biotin is attached to position C-5 of the pyrimidine ring through an alkaline linker arm. The biotin labelled nucleotides are excellent substrates for DNA polymerase and they could be incorporated into DNA by nick translation (Langer *et al.* 1981), random priming (Takahashi *et al.*1989) and tailing of the 3' end (Kumar *et al.* 1988). Thus, biotin has become the choice for indirect, non-radioactive labelling. Further, long shelf life of prepared probes and ability to detect by various methods increase the usefulness of the biotin labelled probes. Presently, synthetic non radioactive labelled oligonucleotide probes are widely used due to the simple preparation methods and stability over a period of the time (Hill *et al.* 1991).

5.1.1.2.3 Detection of non-radioactive probes

Non-radioactive probes are visualized in various ways such as colourimetry, fluorescent and chemiluminescent detection systems (Mansfielda *et al.*1995). Biotin has a strong affinity toward the avidin and its microbial analogue streptavidin (Diamand and Christopoulos. 1991). The biotin and avidin interaction form the basis for enzyme linked detection methods and hybridized biotin labelled probe react with streptavidin or avidin conjugated to an enzyme. The resulting complex can be visualized by the action of enzyme on the substrate (Hill *et al.* 1991). Similarly, digoxigenin labelling can be detected by using Anti-Digoxigenin-Peroxidase (Gill *et al.* 2006).

Alkaline phosphate, horseradish peroxidase, β galactosidase and urease can be used a reporter enzyme in the colour development process. However, alkaline phosphate and horseradish peroxidase are the most common enzymes used in non-radioactive membrane based DNA detection systems (Wilson, 1991).

5.1.1.3 Hybridization parameters

The hybridization rate is dependent on several factors such as the probe length, complexity of the probe, temperature, ionic strength of the solution, viscosity and the pH of the solution (Gong and Levicky, 2008). Further, the length, conformation and abundance of the probes determine the capture efficiency of the solid phase (Gong and Levicky, 2008; Levicky and Horgan, 2005). Longer probes (600-1000 bp) exhibited lower subtraction efficiency and this may be due to secondary structure and steric interference. (Archer *et al.* 2010). However, longer probes produce higher fluorescent hybridization signals than shorter ones in microarrays (Letowski *et al.* 2004).

In hybridization assay, the signal can be produced in 3 different situations. Firstly, the desired interaction between the probe and target, secondly, the mismatched annealing between the probes and related DNA that will give rise to non-specific signals and thirdly, the non-specific interactions of the probe with the solid support that will results in background signals. Thus, hybridization parameters should be chosen to optimize the desired interaction between the probe and target.

The stringency of hybridization is referred as the degree of the mismatches that can be tolerated in a hybridization reaction still remains a duplex double to produce a positive hybridization signal (Tenover, 1988). Higher salt concentration improves the kinetics of the hybridization, but in the meantime reduces the stringency of the binding (Bajaj, 2000). Higher temperatures provide better dissociation between mismatches by denaturing the less stable mismatched sequences. On the other hand, higher temperatures may also cause the dissociation rate to increase and thus lead to a decrease hybridization amount.

5.1.1.4 Hybridization formats

Various formats such as filter hybridization, sandwich hybridization and solution hybridization can be used for the hybridization of probes to target DNA (Cansiz, 2010). In most common hybridization methods, the target nucleic acid is immobilized on to a solid support while probe is free in the solution. Such mixed phase hybridization methods often provide a convenient format for detection of nucleic acid hybrids as unhybridized probes can be washed away during post hybridization washing. The solid support where the DNA is immobilized can be membrane filters, latex, or microtitre plates. Nitrocellulose and nylon membranes are commonly available filter membranes (Khandjian, 1987). Cationic nylon membrane (nylon membrane that contains cationic groups) binds DNA *via* covalent bonds giving much stronger binding than nitrocellulose membrane (Herzer and Englert, 2001). Nylon membrane is considered to be superior to nitrocellulose membrane as they are more sensitive (Khandjian, 1987) resilient, easier to handle and withstand

multiple hybridization without tearing off (Herzer and Englert, 2001). Southern (1975), was one of the earliest scientists who demonstrated solid phase hybridization and it is important in the study of genetic disease and DNA fingerprinting by restriction fragment length polymorphism (Hwang *et al.* 2003).

The sandwich hybridization consists of a solid bound capture probe, the target DNA and a signal generating substance. The target DNA links to both capture probe and signal generating or reporter probe. Further, in the absence of target DNA, the signal generating molecule does not bind to the capture probe and therefore a signal is not generated. Thus, interference is minimal (Ranki *et al.* 1983) and decreases the background interference.

5.2.2 Applications of DNA hybridization in DST of *M. tuberculosis*

Prevention and control of multi drug-resistant tuberculosis is almost synonymous with timely detection of drug resistance. The treatment of TB requires a prolonged course of expensive drugs and hence the total cost of treating a single case is high. The hidden costs that are incurred due to delayed diagnosis of MDR-TB affects the growing economies especially in developing countries (O'Riordan *et al.* 2008).

DNA based DST methods of *M. tuberculosis* have been directed towards detection of previously identified mutations in the RRDR of *rpoB* gene (for RIF resistance) or simultaneous identification of mutations in both *rpoB* gene and *katG* gene (for INH

resistant) (O'Grady *et al.* 2011). Most of the methods are based on DNA amplification followed by detection of mutations by hybridization based methods. However, the high costs, the need of more than average technical expertise and complex, expensive and sophisticated equipment are major challenges preventing the routine use of commercialised DNA based DST methods especially in resource poor settings (Chegou *et al.* 2011).

5.2.2.1 Line probe assay (LPA)

Line probe assays were endorsed by the WHO in 2008 as a molecular method for detection of drug resistance from direct sputum smear-positive specimens as well as from primary culture isolates of *M. tuberculosis* complex. However, direct use of line probe assays on smear-negative clinical specimens is not recommended to date (WHO, 2008 policy statement). In Line probe assay, PCR amplified products are hybridized to MTC DNA probes specific for most prevalent mutations associated with drug resistance (WHO policy statement, 2008).

INNO-LiPARif.TB test (INNOGENETICS, Belgium) and GenoType MTBDR*plus* test (Hain Lifescience, Germany) are commercially available LPA for rapid detection of drug resistance of TB. INNO-LiPARif.TB is based on the reverse hybridization and detect of RIF resistance by 10 oligonucleotide probes that are immobilized on nitrocellulose paper strips (Morgan, 2005). One probe specific for the MTC, 5 wild-type probes that cover codons 509 to 534 of RRDR and remaining 4 probes specific for point mutations, D 516

V, H 526 Y, H 526 D, S 531 L of *rpoB* gene that are frequently responsible for rifampicin resistance are used in the test (Figure 5.1) (WHO policy statement, 2008). Thus, hybridization of amplified DNA of a clinical sample to the strip allows determination of either resistance or susceptibility by visual observation of colour changes of the respective bands on the strip (Hauck, *et al.* 2009; WHO policy statement, 2008).

The Genotype MTBDR test is also based on the same general principle used in the INNO-LiPA Rif. TB test with the advantage of detecting the presence of mutations in *inhA, katG* and *rpoB* gene simultaneously. Thus, MTBDR test detects *M. tuberculosis* complex, mutations in the *rpoB*, *katG* (responsible for high-level INH resistance) (Bang *et al.* 2006; Ling *et al.* 2008) and *inhA* (confers low-levels of INH resistance) (Ling *et al.* 2008) genes. Further, 4 probes detect *rpoB* gene mutations (D 516 V, H 526 Y, H 526 D, and S 531 L) and 8 wild-type probes covering the region from codon 505 to 533 of the *rpoB* gene. For identification of isoniazid resistance, it includes probes for *katG* mutation, S 315 T1 and S 315 T together with 4 probes for mutations in *inhA* C 15 T, A 16 G, T 8 C and T 8 A (Hauck *et al.* 2009).

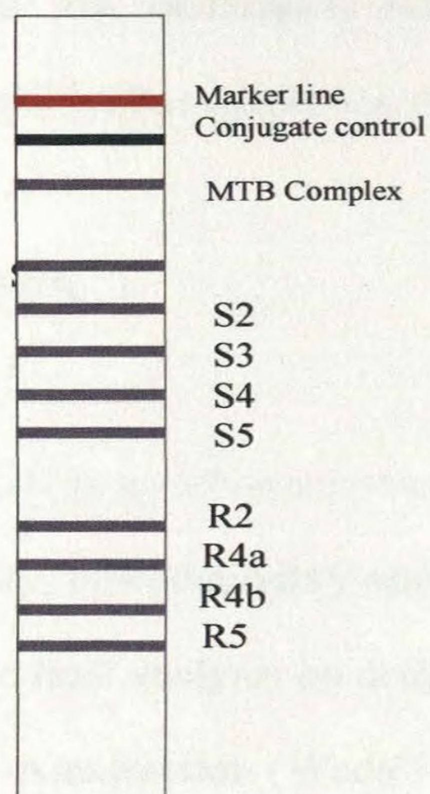


Figure 5.1 Location of different probes immobilized on a INNO-LiPA Rif.TB strip. A red Marker Line is drawn on top of the strip for orientation. The Conjugate Control line provides an internal control for the colour development reaction. The MTB line is a specific probe for *M. tuberculosis* complex. S1- S5 is a wild type probe and R2, R4a, R4b and R5 are specific mutant probes.

The GenoType MTBDR*sl* test, another line probe assay that has been designed to test resistance of second-line anti-TB drugs (fluoroquinolones, ethambutol, aminoglycosides and cyclic peptides) in addition to testing of MDR-TB, for determination of XDR-TB (Brossier *et al.* 2010).

Line probe assays are highly sensitive (≥97%) and specific (≥99%) for the detection of RIF resistance alone or in combination which effectively determine INH resistance (sensitivity ≥90%; specificity ≥99%) from primary isolates of *M. tuberculosis* and smear-positive sputum specimens (WHO policy statement, 2008). The major advantages of

LPAs are the low biohazards risk and availability of final results on DST within approximately 5 h after extraction of DNA (WHO policy statement, 2010).

5.2.2.2 Real-Time PCR

Real-Time PCR is an advantageous method for detection of resistance due to its rapidity high sensitivity, reproducibility and low contamination risk (Rathore *et al.* 2011; Espy *et al.* 2006). The final analysis on drug resistance using real-time PCR is possible within 3 h following DNA extraction (Wada *et al.* 2004). The drug-resistant mutations are detected by two main methods. In the first method, a fluorescent signal is generated by hybridization of a probe to the target sequence at the end of the each PCR cycle. The use of TaqMan probe (Sajduda *et al.* 2004) and molecular beacons (Yesilkaya *et al.* 2006) for allele discrimination of *M. tuberculosis* belong to this category. However, the detection is limited to the most common mutations, such as those in codon 315 of *katG* and codon 531 of *rpoB* genes as these assays require the use of two different fluorophore-labeled probes for differentiation of a single allele (Yesilkaya *et al.* 2006; Sajduda *et al.* 2004; Espasa *et al.* 2005). Additionally, this method requires a real-time PCR instrument with multiple channels.

Melting curve analysis is a second method of mutation detected using real-time PCR. The high-resolution melting curve (HRM) analysis has been successfully applied to detect drug-resistant mutations in *M. tuberculosis* (Ong *et al.* 2010; Chen *et al.* 2011). However, their applications are limited as the HRM assay requires highly advanced real-time PCR

instruments (Luo *et al.* 2011). More recently, several new probe-based melting curve analysis technologies, using unlabelled probes (Erali *et al.* 2008), dual labelled probes (Luo *et al.*2011), and molecular beacons (Chakravorty *et al.*2011) have been developed for analysis of gene mutations. However, a limited number of studies are reported on their application in the detection of drug-resistance in *M. tuberculosis* (Luo *et al.* 2011).

5.2.2.3 DNA microarrays

Microarray is a technique that allows the analysis of several genetic markers by simple hybridization of DNA on miniature glass microchips containing oligonucleotide probes (Volokhov *et al.* 2002). Thus, this is a valuable tool for detecting genetic variations responsible for MDR-TB. Microarray allows detection of 80 % of INH resistant isolates, based on the *katG* gene and > 90 % of RIF resistant isolates based on *rpoB* gene within 12 h (Gryadunov *et al.* 2005). Also, microarray is vital for rapid screening of PZA resistance of *M. tuberculosis* that face challenges with culture based susceptibility testing methods due to the requirement of an acidic medium for drug activity in the culture medium (Denkin *et al.* 2005).

5.2.2.4 Xpert *Mycobacterium tuberculosis*/Rifampicin Assay

GeneXpert (Cepheid, Sunnyvale, California, USA) is a more recently developed fully automated molecular method for identification of MTB and RIF resistance. This assay detects *M. tuberculosis* and RIF resistance by PCR amplification of RRDR fragment of

the *rpoB* gene (specific for *M. tuberculosis*) and subsequent probing of this region for mutations. Testing is carried out on the MTB/RIF test platform (GeneXpert, Cepheid), which integrates sample processing, DNA amplification and hybridization in a disposable plastic cartridge. The cartridge contains all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The only manual step is the transfer of a defined volume of sputum sample mixed with a bactericidal buffer into the cartridge. The conclusion on presence or absence of tuberculosis and drug resistance tuberculosis are available within 2 hours with very low biohazard risk (Helb *et al.*2010).

5.2 Justification and objectives of the present chapter

The literature reviewed so far, indicated the usefulness of DNA markers in detecting MDR-TB and individual analytical capability of molecular applications that have been developed as DST methods. During the last decade, various molecular tools that are directed towards detection of previously identified mutations in the RRDR of the *rpoB* gene have been commercialised for identification of RIF resistance in tuberculosis. However, most of these molecular methods are not utilized in settings where resources are limited due to high cost involved in initial setting up as well as running of the test. The expenditure incurred for purchasing instruments and consumables, infrastructure development and training personnel on specific technical knowledge are some concerns regarding the cost (Morgan *et al.* 2005; Bang *et al.* 2006; Ling *et al.* 2008; Espy *et al.* 2006; Sajduda *et al.* 2004). Further, gene mutations responsible for RIF resistance, the marker of MDR-TB, may vary in different geographical regions (Zaczek *et al.* 2009;

Adikaram *et al.* 2012). Thus, available techniques based on frequently identified mutations in RRDR (at codon 516, 526 and 531) may not achieve the required sensitivity in certain geographical regions. These factors highlight the need for development and evaluation of an economical DNA based method for detection of drug resistance in tuberculosis that can be customized as per user requirement.

Currently, there is no molecular approach used for detection of drug resistance in Sri Lanka, resulting in considerable delay in determination of drug sensitivity patterns and appropriate treatment for MDR-TB. Thus, the study described in the present chapter was aimed at developing and validating a Polymerase Chain Reaction–Enzyme-linked Immunosorbent Assay (PCR-ELISA) also known as enzyme-linked oligosorbent assay as a low cost, simple and rapid molecular method for determining drug resistant tuberculosis in Sri Lanka.

5.3 Research methodology

5.3.1 Study population

M. tuberculosis isolates (n=42) and non tuberculosis mycobacteria (NTM) isolates (n=3) collected from the Chest Hospital and Central Chest Clinic Colombo-Sri Lanka were used for the study. H73Rv was used as the quality control strain.

5.3.2 Methodology

DIG DNA labeling by PCR reagent kit and DIG detection reagent kit with streptavidin coated micro-titre plates, were purchased from Roche Applied Sciences, Germany.

5.3.2.1 PCR based digoxigenin labelling

DNA was extracted from selected *M. tuberculosis* and NTM culture isolates as described in section 4.3.2. Two fragments of the *rpoB* gene, 437bp and 872bp fragments were dig labelled using PCR digoxigenin labelling kit. PCR was prepared in 20 μl reaction volume containing 1.5 mM $MgCl_2$, 0.5U *Taq* polymerase, 2 mM dATP, dCTP, dGTP, 5.7 mM dUTP and 0.3 mM DIG-dUTP, 20 pmol of each primer and 1 μl containing either 100 fg or 1 pg of genomic DNA for amplification of 437 and 872 bp fragments respectively. The primers and thermo-cycling parameters used for amplification were similar to described in Table 4.2 in chapter 4.

5.3.2.2 Selection of capture probes for ELISA

Six, 5' biotinylated allele specific oligonucleotide capture probes (F1-F6) corresponding to point mutations at codons 526, 531 & 626 were custom synthesized (Promega, USA). Allele specific capture probes F1 to F4 were hybridized with the 437bp PCR amplified fragment and capture probes F5 and F6 were hybridized with 872 bp fragment (Table 5.1). A search of the GenBank database with the BLAST program was performed to confirm the specificity of the capture probes for the *M. tuberculosis* complex.

Table 5.1 Sequences of capture probes designed for PCR-ELISA (F1-F6). The highlighted letters indicate the allele specificity of probes.

Probe Name	Sequence (Bio 5'- 3')	Represent mutation site of the rpoB gene
F1	GTTGACCCACAAGCGCCG ACT	Wild type (codon 526)
F2	GTTGACCTACAA GCG CCG ACT	Mutant type(codon 526)
F3	GAC TGT CGG CGC TGG GG	Wild type(codon 531)
F4	GAC TGT TGG CGC TGG GG	Mutant type (codon 531)
F5	CGA TGCGGACGG TCG CTT CG	Wild type(codon 626)
F6	CGATGCGGAGGG TCG CTT CG	Mutant type(Codon 626)

5.3.2.3 Optimization of hybridization and signal detection

PCR-ELISA was optimized with the digoxigenin detection kit. Each capture probe (15 pmol/µl) was immobilized on to a streptavidin coated microtitre plate by incubating with hybridization buffer at room temperature (25 ^{0}C) for 20 min with shaking at intervals according to manufacture's guidelines.

Thereafter, each well was washed 5 times using wash buffer. The digoxigenin labelled PCR amplified product was denatured in a 1.5 ml microcentrifuge tube containing 20 µl of denaturation solution at 22 °C for 15 min followed by the addition of hybridization buffer to a final volume of 250 µl. Thereafter, 200 µl of this mixture was pipetted out into each well of the microtitre plate coated with capture probes and hybridized. The optimum

temperature was determined through a series of temperature variations (37 °C, 45 °C and 55 °C) and the optimum incubation time for hybridization was selected through time variation (1 h, 1.5 h, 2 h and 2.5 h). The plate was washed using wash buffer (x 5) and 200 µl of 1:99 dilution of anti-digoxigenin-peroxidase (ADP) was added to each well. The plate was sealed and incubated in the dark with shaking at 37 °C for 30 min. The excess conjugate was removed by washing (x5) and 200 µl of colour development substrate, ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) was added to each well and incubated in the dark at 37 °C for 15 min with shaking. The colour development was carried out at 5 min, 7 min, 10 min, 15 min and 20 min to determine the optimum incubation time. The optical density (OD) of colour development was measured at 405 nm (reference wave length 492 nm – for normalization) using an ELISA plate reader (Awareness technology, USA). PCR grade water was used as the negative control and H37Rv strain was used as the positive control. The systematic view of the procedure for PCR-ELISA (with the optimum temperature and the time duration in each step) is illustrated in Figure 5.2.

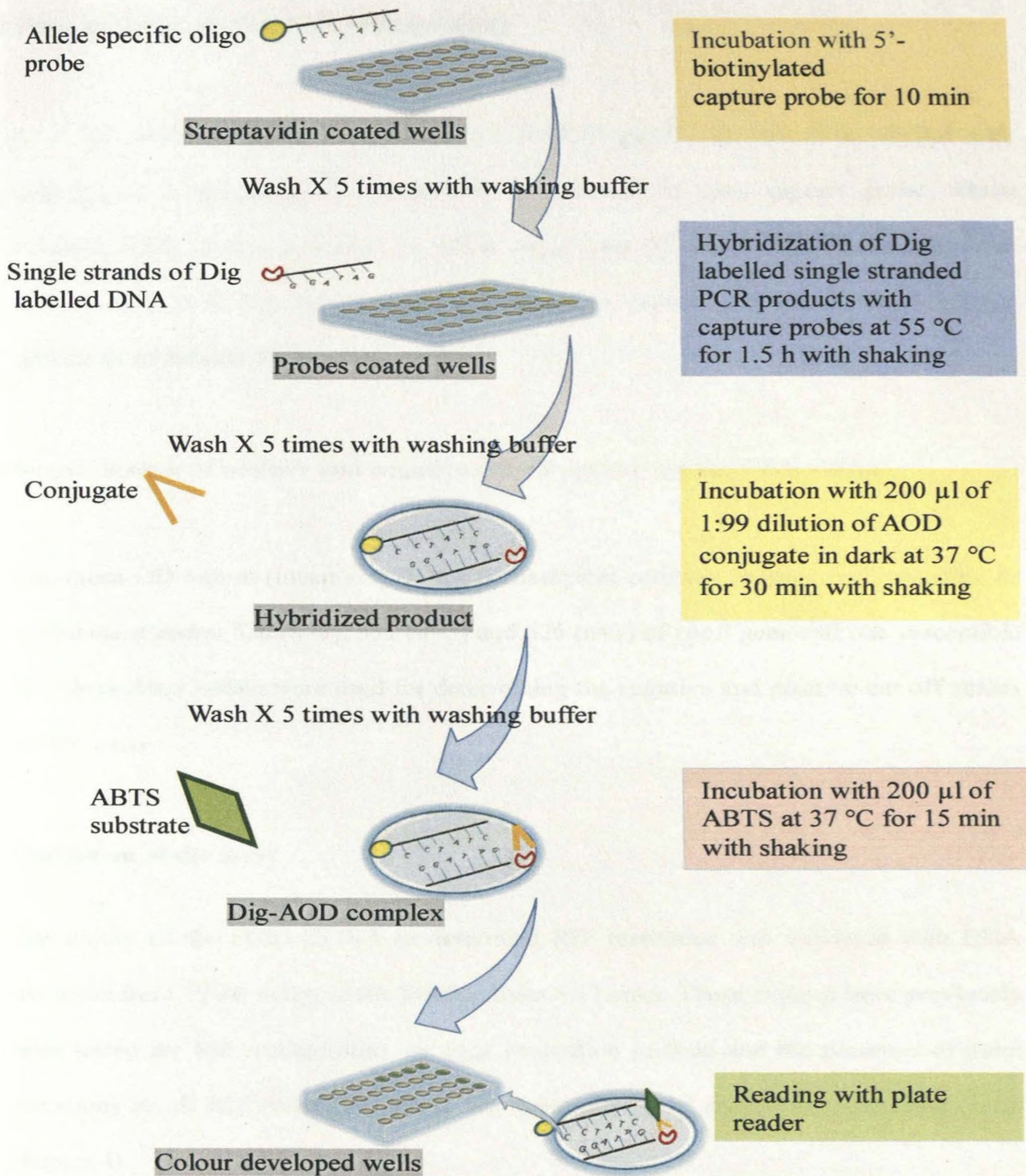


Figure 5.2 Flow diagram showing the PCR-ELISA process (the optimum temperature and the time duration for hybridization and colour development are indicated in relevant steps).

5.3.2.4 Determination of sensitivity and specificity

A 10 fold serial dilution of *M. tuberculosis* DNA (1 pg to 1 fg) was PCR labelled with digoxigenin to determine the sensitivity of detection of each capture probe. Three different NTM strains confirmed by DNA sequencing (*M. abscessus, M. fortuitum, M. avium*) were PCR dig labelled and hybridized to capture probes to determine the specificity of these probes.

5.3.2.5 Determination of positive and negative cut off optical density (OD) values

The mean OD values (mean ± S.D) for 9 rifampicin resistant isolates corresponding to mutations at codon 526 (n=3), 531 (n=3) and 626 (n=3) of *rpoB* gene and one susceptible *M. tuberculosis* isolate were used for determining the negative and positive cut off values for the assay.

5.3.2.6 Validation of the assay

The ability of the PCR- ELISA to determine RIF resistance was validated with DNA extracted from 32 *M. tuberculosis* isolates from Sri Lanka. These isolates have previously been tested for RIF susceptibility by agar proportion method and the presence of point mutations in all RIF resistant isolates has been confirmed by DNA sequencing (refer chapter 4).

5.3.2.7 Statistical Analysis

Data were processed with SPSS statistical software package 15.0 for Windows. The test reliability, sensitivity, specificity and cut-off OD value of the PCR-ELISA for identification of rifampicin resistance (as an indirect DST method) were determined using two different statistical methods.

Receiver Operating Characteristic (ROC) curve analysis

In ROC curve analysis, the sensitivity (the ability to detect true drug resistance) and the specificity (ability to detect true drug susceptibility) of each probe were calculated against the agar proportion method. The optimum cut off point (at the highest sensitivity and specificity) for each capture probes was determined in order to distinguish positive and negative results.

Kappa analysis

In kappa analysis, the suitability of the PCR-ELISA as a diagnostic test was evaluated in terms of sensitivity and specificity compared to the agar proportion method. The positive and negative cut off OD values for the overall test were expressed as arithmetic mean and standard deviation. The kappa value (k), a measure of test reliability, was interpreted as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent (Sim and Wright, 2005; Kirkwood and Sterne, 2003; Deeks, 2001).

The cost of establishment and cost per test for PCR-ELISA methods were compared with frequently used commercialized molecular DST methods using price quotations (based on cost in Sri Lanka as of 2012 January).

5.4 Results

5.4.1 Optimum parameters for the hybridization process

The stringency temperature and time of probe- DNA hybridization were 55 °C and 1.5 h. The optimum incubation time for the colour development was 15 min. Colour development in PCR-ELISA (under optimized conditions) for selected isolates are shown in Figure 5.3.

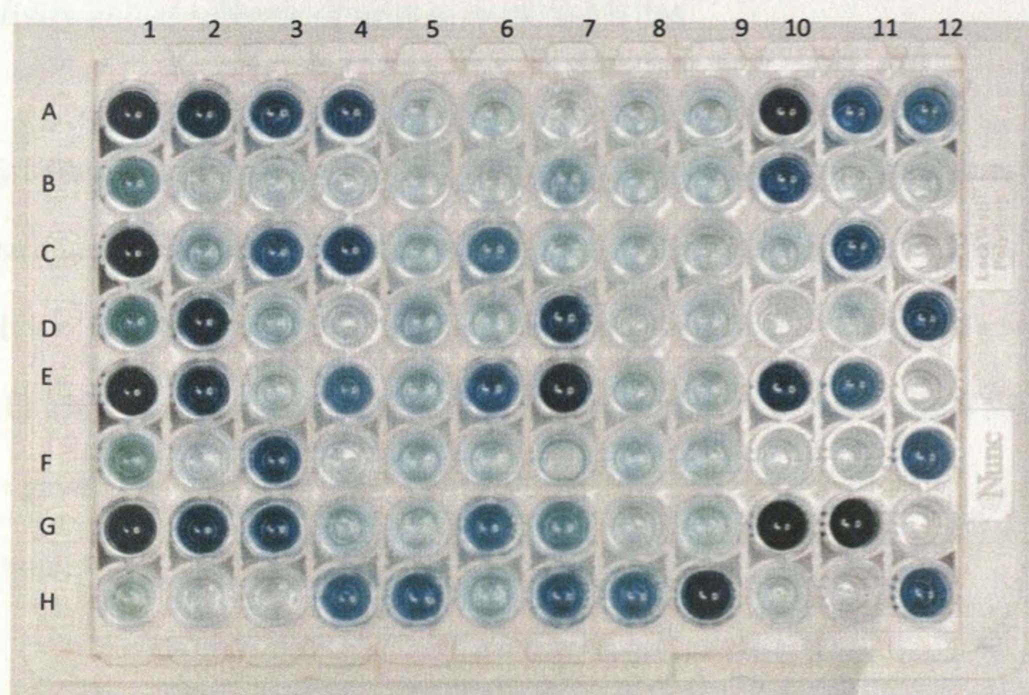


Figure 5.3 Photograph showing the colour development in PCR-ELISA. A1-F1 shows colour development of H37Rv for probe F1-F6. A5-F5 shows colour development of probe F1-F6 for negative control (water). A8- F8 shows colour development for *M. abscessus* (NTM). Renaming wells represent *M. tuberculosis* isolates.

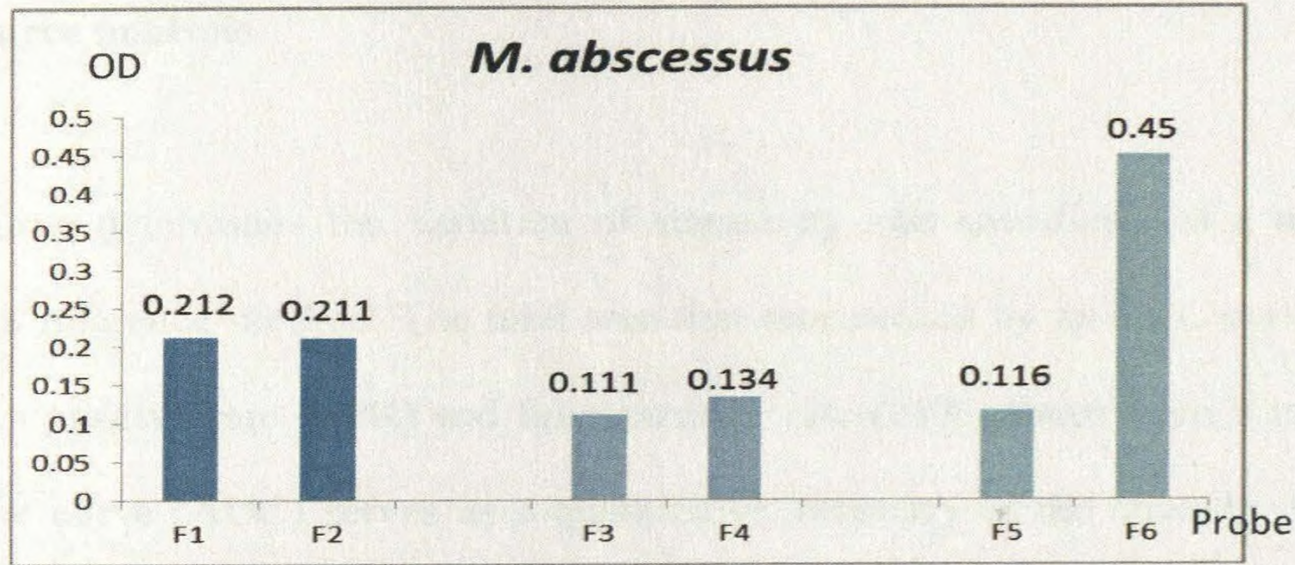
5.4.2 Test criteria

To increase the accuracy of the assay, the following criteria were used during interpretation of results. The results were valid, if at least one of the probes were able to produce an OD of ≥ 1 and if none of the probes gave an OD value between positive and negative cut off values.

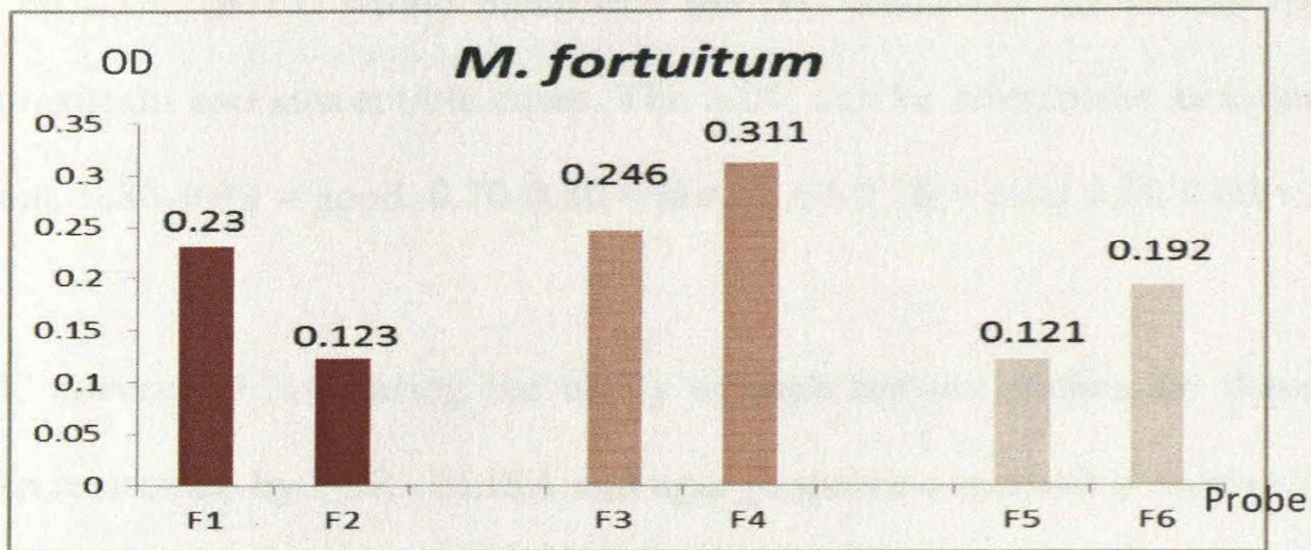
5.4.4.2 Sensitivity and specificity of probes in PCR-ELISA

A serial dilution of PCR amplified *M. tuberculosis* DNA was hybridized to capture probes to determine the sensitivity of the PCR-ELISA. All capture probes did not show equal sensitivity. Capture probes F1 and F4 were able to detect 100 fg of genomic DNA (OD>1) while the other probes (F5 to F6) produced an OD >1 with 1 pg DNA. The NTM strains gave an absorbance value below the mean negative cut off value ($\leq 0.4 \pm 0.12$), confirming the specificity of the assay (Figure 5.4).

a)



b)



c)

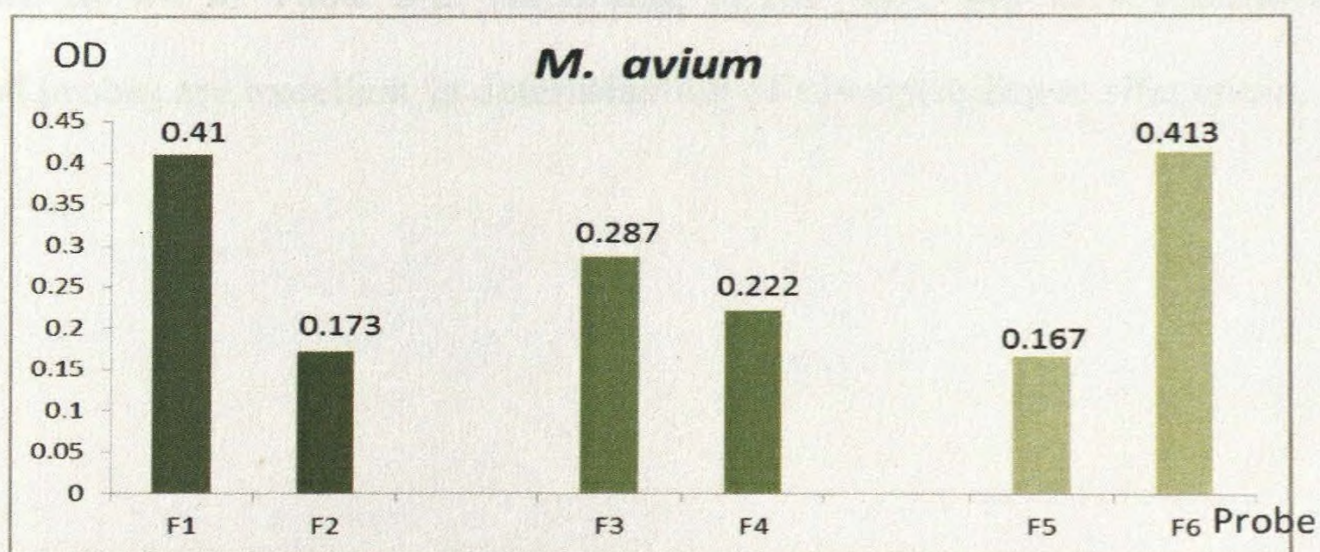


Figure 5.4 Optical density values for selected NTM strains in RCR- ELISA assay. All OD are below the negative cut off value ($\leq 0.4 \pm 0.12$).

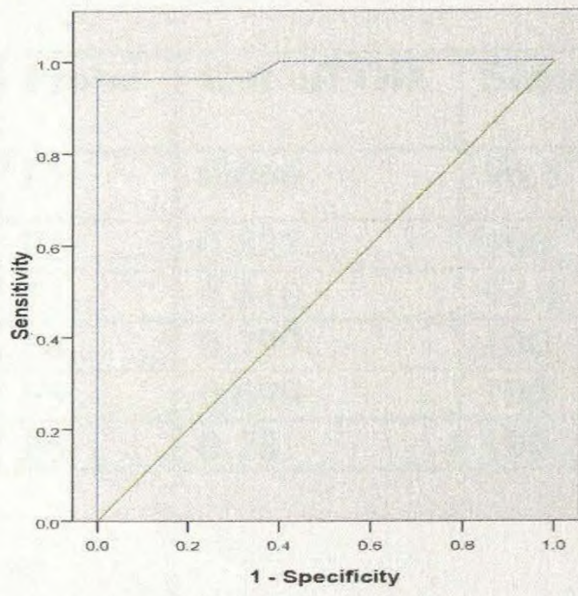
5.4.3 PCR-ELISA as an indirect DST for determination of rifampicin resistance

5.4.3.1 ROC curve analysis

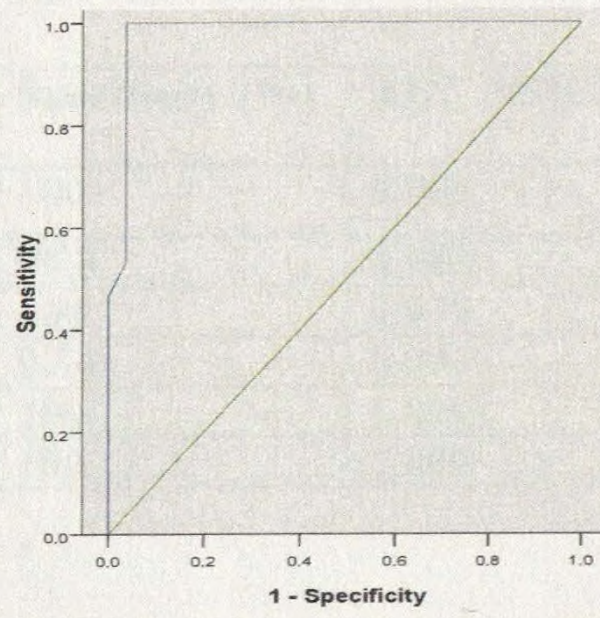
ROC curve determines the variation of sensitivity and specificity of a tested method against a reference method. The total area that represented by an ROC curve is 1. Thus, both true positive rate (TPR) and false positive rate (FPR) range from 0 to 1. The area under the curve (AUC) serves as a quantitative summary of the strength of association between the underlying test statistics and disease status (whether resistant or susceptible). Further, an AUC of 1.0 would mean that the test could use for perfect discrimination between resistant and susceptible cases. The AUC can be interpreted as follows (0.90-1.0 = excellent, 0.80-0.90 = good, 0.70-0.80 = fair, 0 .60-0.70 = poor 0.50-0.60 = fail).

The ROC curves for comparing the utility of each capture probes for determination of rifampicin resistance by PCR- ELISA and agar proportion method is shown in Figure 5.4. The cut off (at the highest sensitivity and specificity) for each probe derived from ROC curves are shown in Table 5.2. According to the AUC (at 95% confidence interval) values, all probes are excellent in determination of susceptibility to rifampicin.

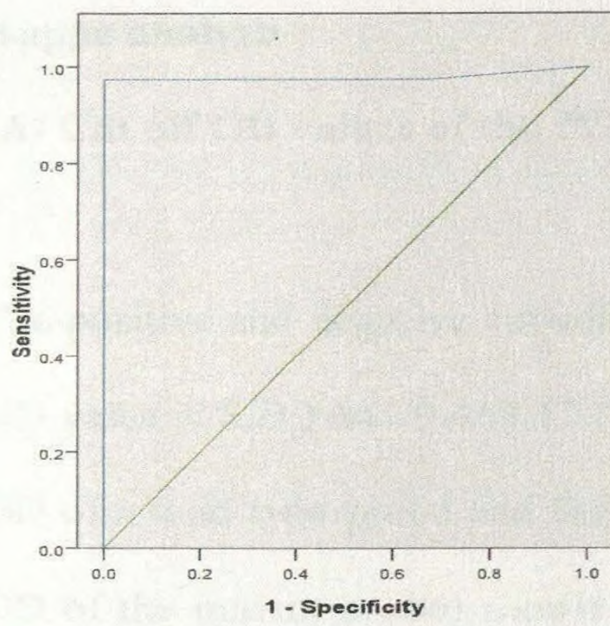
a)



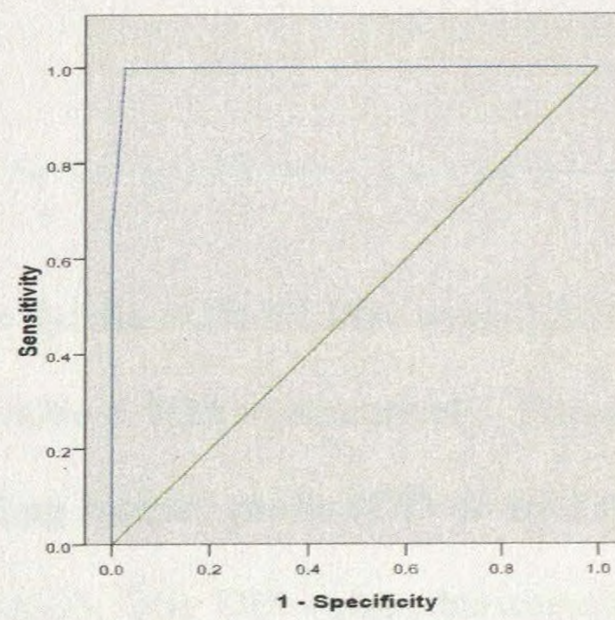
b)



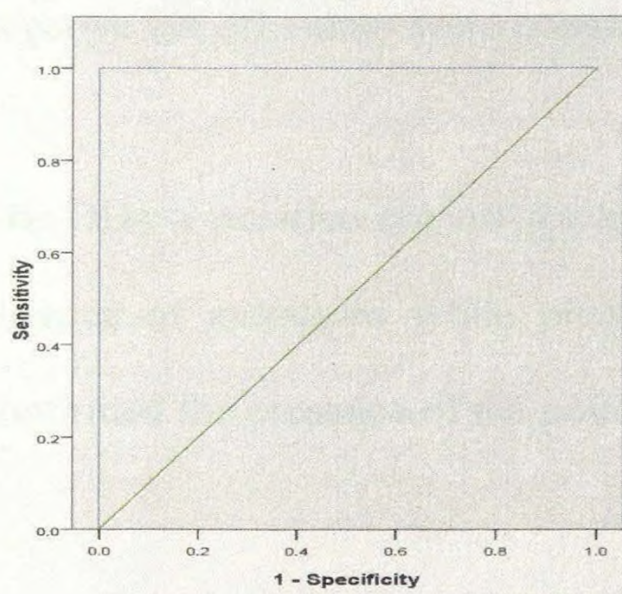
C)



d)



e)



f)

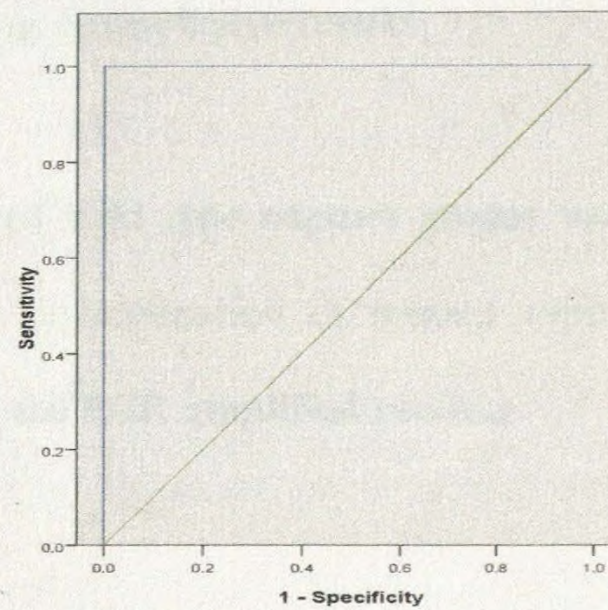


Figure 5.5 ROC curve analysis for each probe. (a) probe F1 (b) probe F2 (c) probe F3 (d) F4 (e) F5 (d) F6

Table 5.2: AUC, sensitivity, specificity and cut off OD for each probe in ROC curve analysis

Probe	**Cut off OD**	**Sensitivity (%)**	**Specificity (%)**	**AUC (95% CI)**
F1	0.666	96.3	100	0.986
F2	0.827	100	96.3	0.981
F3	0.610	97.4	100	0.979
F4	0.799	100	97.4	0.996
F5	0.699	100	100	1.000
F6	0.781	100	100	1.000

5.4.3.2 Kappa analysis

(A) Cut off OD values of the PCR-ELISA

The positive and negative cut off OD values for the PCR-ELISA were 1.25±0.37 (Mean OD value ± S.D.) and 0.4±0.12 (Mean OD value ± S.D.) respectively. The difference in OD of a wild type probe and the corresponding mutant probe (OD of wild type probe – OD of the mutant probe) should be ≥ 0.82±0.33. The OD values between positive and negative cut off values were considered as giving inconclusive results.

The ODs > positive cut off for hybridization of wild type capture probes indicated the absence of mutations while positive ODs for hybridization of mutant capture probes confirmed the presence of the point mutation in the PCR amplified product.

(B) Sensitivity and specificity of the PCR-ELISA test

The PCR-ELISA was able to correctly determine RIF susceptibility in 29 of 32 *M. tuberculosis* isolates (RIF sensitive isolates = 08 and rifampicin resistant isolates = 21) that were used for the validation of the assay. Three isolates, gave inconclusive results with the F3 capture probe (OD between positive and negative cut off values). Repeated testing (2 times) of these 3 isolates revealed the same results.

PCR-ELISA showed good agreement (κ = 0.8,) with agar proportion method. The sensitivity and specificity of the PCR-ELISA was 86% and 100% (95% confidence intervals) respectively in determining RIF susceptibility of *M. tuberculosis*. Further, PCR-ELISA was able to detect all mutations that were identified by DNA sequencing resulting in excellent agreement (κ =1) between PCR-ELISA and DNA sequencing.

The comparative analysis on cost (based on price available in Sri Lanka in 2012 January), technical expertise and the need of sophisticated instruments for the commercial molecular DST methods and PCR-ELISA methods is demonstrated in table 5.2.

Table 5.3 Comparative Analysis of estimated cost for establishment of commercial molecular drug susceptibility testing method

Molecular DST method	Cost for installing	Cost per test after initiation	Special Instrument	Major consumables	Technical expertise
INNO-LiPA Rif.TB test	$7900	$45	Water bath with shaking platform Orbital, Thermal cycler	INNO-LiPA Rif.TB Kit (20 strips) INNO-LiPA Rif.TB amplification kit	PCR , Use of instruments
GenoType MTBDRplus	$12000	$45	TwinCubator , Sonicating Incubator Centrifuge, Thermal cycler	GenoType MTBDRplus kit Reagents for PCR amplification	PCR, use of instruments
Microarray	$ 8500	$ 70	Microarray chip Chip detector portable fluorescence analyzer Thermal cycler	PCR amplification reagent kit	PCR, probe Hybridization, use of instruments
GeneXpert	$ 66,000	$ 55	GeneXpert Diagnostic System Thermal cycler	Single-use disposable cartridges	Use of fully automated instrument
RT-PCR COBAS® TaqMan® MTB Test	$29,000	$75	Real-Time PCR System	Real-time PCR kits	Use of fully automated instrument
PCR-ELISA	$ 5000	$40	Plate reader, plate shaker, Thermal cycler	Dig labeling kit Dig detection kit	PCR, probe Hybridization

5.5 Discussion

The sensitivity observed for the 437 bp fragment was 10 times greater (100fg) than that observed for the longer fragment (1pg). Liu *et al.* 2007 have also reported that the sensitivity of PCR-ELISA varies with the length of the amplified fragment. Although the 872bp fragment requires a 10 fold increase in DNA concentration for hybridization, this fragment covers all three codons tested for mutations, making it more cost effective and time saving as a single PCR product can be used for the assay. Furthermore, 1 pg DNA can be obtained from a few colony forming units unit (CFU) of bacteria, eliminating the necessity of sub culturing of isolates.

5.5.1 Validity of PCR –ELISA assay

To avoid misinterpretation of results, the dig labelled PCR amplified products were hybridized with the wild type and mutant capture probes as a double check procedure. For example, if the absorbance value for hybridization of an amplified product to a wild type capture probe is in the accepted negative range it should not be interpreted as positive for the mutation or RIF resistant as reported by Garcia *et al.* (2001). It could be due to a technical error such as failure in PCR amplification, probe hybridization or digoxigenin detection. To be accurate, the amplified product should give an absorbance in the positive range when hybridized to a mutant capture probe. Therefore, in the present study three wild types capture probes were also used with the corresponding three mutant probes and the criteria that, at least one probe should be able to produce ODs of ≥ 1 under optimized

test conditions was used to increase the accuracy of the assay, preventing misinterpretation.

The validity of the PCR-ELISA as an indirect drug susceptibility testing was determined by 2 methods. In ROC curve analysis, a cut off OD value, sensitivity and specificity for each probe was determined. In the present study, both sensitivity and specify of all probes were $\geq$ 96% and there was an excellent discrimination (AUC > 0.9) between RIF susceptible cases and resistant cases. However, in ROC curve analysis method, 6 different cut off OD values were used in interpretation of the test results.

According to kappa analysis, the developed PCR-ELISA showed good agreement, with 86% sensitivity and 100% specificity to the agar proportion method. In this analysis, there is a single set of cut-off OD (positive cut off and negative cut off) for the overall PCR-ELISA assay (for all 6 probes). The use of both negative and positive cut off OD values increase the accuracy of the test. With regard to intermediate values (between positive and negative cut off OD), the test should be repeated. However, if the repeated test results are also inconclusive, ROC curve analysis is recommended where the individual cut off for each probe is used.

The ROC curve analysis increase the sensitivity in PCR-ELISA for the determination of RIF resistance as the cut off OD values for each probe is less than the overall positive cut off value (1.25±0.37 (Mean OD value ± S.D.)) in kappa analysis. Thus, the degree of sensitivity of the PCR- ELISA generated by the kappa analysis (86%) is less than that was

generated by ROC curve analysis ($\geq$ 96%). However, use of a single set of cut off OD values is more practical than the use of individual cut off for each probe.

5.5.2 PCR-ELISA is an indirect molecular DST method for low resource settings

PCR-ELISA technique has been successfully applied in diagnosis of bacterial (Sailsa *et al.* 2001) and parasitological diseases (Hodgkinson *et al.*2003; Gomes *et al.* 2010). This technique has also been used in determination of mutations in diseases such as cancer (Ward *et al.* 1995) and thalassemia (Gil *et al.* 2007).

In most DNA based DST methods of *M. tuberculosis*, the initial PCR amplification of the selected gene target will be followed by specific mutation detection methods that require various instrumentations and infrastructure add high cost to the assay. Thus, the development of simple, cost effective, DNA based DST method that can be customized as per user requirement would be very useful in the prevention of MDR-TB. Further, the diagnostic tests designed to detect MDR-TB would have to satisfactorily meet four essential criteria; safety, high sensitivity, high specificity and low cost (WHO, 2010).

The biohazard risks are minimal following extraction of DNA in PCR-ELISA. Thus, PCR-ELISA can be performed even at a routine laboratory work bench by managing PCR contaminations. Use of dUTP in the dig labelling is an additional advantage as it will not contribute to carryover contamination that occurs frequently in routine PCR based assays (Pang *et al.* 1992). The initiation cost of PCR-ELISA is limited to purchasing of a PCR machine and a plate reader, the instruments necessary to perform DNA amplification and

quantification analysis for colour development during the ELISA respectively. Plate reader is a routinely used instrument in most diagnostic laboratories even in low resource settings. In the absence of a plate reader, the qualitative analysis by visual observation of colour development is possible for determination of RIF sensitivity to some extent. However, a reference colour series should be used in the qualitative analysis of PCR-ELISA. Further, the technical expertise required is minimal as laboratory health care workers are familiar with both PCR and ELISA to a great extent. Thus, the test initiation cost of PCR-ELISA as a molecular DST method is manageable even for peripheral TB diagnosis centres. The consumable cost of PCR-ELISA ($40 per test) is less than other established DNA based DST methods. The PCR dig labelling and digoxigenin detection kit is sufficient to analyse 30 PCR-ELISA reactions using 6 probes. Additionally, this technique is potentially automatable, and up to 96 or 384 reactions can be done simultaneously. The turnaround time of the test is 2 days after isolation of primary *M. tuberculosis* cultures. Therefore, the indirect PCR-ELISA is an excellent alternative as a molecular DST method, especially in resource limited settings.

Chapter 6

Analysis of transmission of rifampicin resistance of *M. tuberculosis* using DNA fingerprinting

6.1 Background

6.1.1 Molecular epidemiology of tuberculosis

Molecular epidemiology of TB is the study of occurrence of TB in human populations using molecular techniques (Narayan, 2004). In essence, molecular epidemiology focuses on the role of genetic and environmental risk factors for transmission of disease, at the molecular, cellular or biochemical level. It is a blend of epidemiology and molecular biology as well as clinical medicine and statistics (Mathema *et al.* 2006). Molecular epidemiology is a multidisciplinary approach used to study the spread of tubercle bacilli in mini epidemics and outbreaks. It analyses the transmission dynamics and determine the risk factors for transmission in a community. Molecular epidemiology has a great role in distinguishing exogenous re-infection from endogenous reactivation as well as identification of laboratory cross contamination (Narayan, 2004).

The mid-1980s revealed the first effect on molecular applications for discriminating clinical isolates of *M. tuberculosis*. Previously used methods such as colony morphology, comparative growth rates, susceptibility to selected antibiotics and phage typing were unable to provide sufficient discrimination. Thus, developing appropriate molecular tools for genotyping of *M. tuberculosis* was essential. The key aspects in choosing a satisfactory

molecular approach for genotyping was based on the observed rate of polymorphism and the genetic diversity of *M. tuberculosis* strains in a population (Mathema *et al.* 2006).

6.1.2 DNA fingerprinting methods for genotyping of *M. tuberculosis*

Various DNA fingerprinting methods has been developed for genotyping of *M. tuberculosis* and investigation of molecular epidemiology. Numerous repeat sequences in *Mycobacterium* genome, such as insertion elements (ISs), trinucleotide repeats (Wiid *et al.* 1994), variable number tandem repeats (VNRT) (Frothingham *et al.* 1998), mycobacterial interspersed repetitive units (MIRU) (Supply *et al.* 2001) and the direct repeat (DR) regions (Groenen *et al.*1993) are commonly used as genetic markers for genotyping.

6.1.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP is one of the DNA fingerprinting tools that widely used for genotyping of *M. tuberculosis* (Hwang *et al.* 2003; Dymova *et al.* 2011) and it was initiated with the discover of IS6110 insertion element (refer section 1.1.2.3) (Thierry *et al.* 1990b; Cave *et al.* 1991). IS6110 element is a 1361bp sequence and only a few nucleotide variation has been detected among its copies. The characteristics of insertion sequences such as high variable copy number and the great variability of insertion sites in the chromosome allow easy typing of strains (Hermans *et al.*1990, Van Embden *et al.* 1993, Kremer *et al.* 1999,). RFLP facilitate to investigate the international transmission of tuberculosis and identify

specific strains with unique properties such as high infectivity, high virulence or drug resistance (Van Embden *et al.* 1993).

The number of copies of IS6110 element present in the genome is species and strain specific. Generally, *M. tuberculosis* strains carry 0 to 25 copies of IS6110 element in different positions of the genome (McEvoy et al. 2007). The number of IS6110 copies in genome of *M. tuberculosis* varies with the geographic origin also. *M. tuberculosis* strains from African and European origin contain 5 to 15 copies while a higher copy number is present in isolates from Hong Kong. However, many strains from the Far East contain a single IS6110 element (Van Soolingen *et al.* 1993) and 40% of the strains from India have either a single copy of the IS6110 element or none (Das *et al.* 1995). Additionally, several single-copy strains were also identified from Malaysia, Tanzania, Vietnamese (Yuen *et al.* 1993) and Oman (Fomukong *et al.* 1994).

The degree of IS6110 polymorphism among the descendants of a particular clone in a population is a reflection of the time that has elapsed, since their divergence as the transposition of IS6110 is a time-dependent process. Thus, analysis of the molecular epidemiology of *M. tuberculosis* by IS6110- RFLP may provide information about the evolutionary history and the dissemination of particular clones in a given geographic region (Van Soolingen *et al.* 1995).

IS1081 is another insertion sequence (Collins and Stephens, 1991) that is used as a genetic marker of *M. tuberculosis* (Park *et al.* 2000). It contains 1324bp flanked by 15 bp inverted repeats (Collins and Stephens, 1991). IS1081 has a lower degree of polymorphism than IS6110, and transpositional activity is low (Van Soolingen *et al.* 1993). The differentiation power of RFLP using IS1081 is limited due to its low copy number compared to IS6110 element. As an example, the genome of H37Rv, the reference strain of *M. tuberculosis* contains 16 copies of IS6110 insertion sequence (Philipp *et al.* 1996) and 6 copies of IS1081 insertion element (Van Soolingen *et al.* 1992). Thus, the RFLP analysis of IS6110 is widely used as a gold standard strain-typing technique of *M. tuberculosis* (Van Soolingen *et al.* 2001) due to its apparent mobility, average copy number, and highly discriminatory power at population level (Kremer *et al.* 1999).

The discovery of alternative genetic markers and typing methods are promoted due to certain drawbacks associated with analysis of insertion sequences by RFLP. One of the major limitations of RFLP-based typing is the requirement of large amount of pure DNA. In certain cases, epidemiologically unrelated *M. tuberculosis* strains that harbour one or two IS6110 copies give same banding pattern in RFLP. Thus, additional typing techniques have to be considered when dealing with strains having low IS6110 copy numbers (Van Soolingen *et al.* 1993).

6.1.2.2 Spoligotyping

Spacer oligonucleotide typing or spoligotyping is based on the direct repeat (DR) regions in direct repeat locus of the *M. tuberculosis* genome (Figure 6.1). Two types of genetic rearrangements have been shown in DR regions. In one type of polymorphism, variation can be seen in one or a few discrete, contiguous DRs plus spacer sequence that is probably driven by homologous recombination between adjacent or distant DRs regions. The other type of rearrangement is probably driven by transpositional events of the IS6110, which is almost invariably present in the DR cluster of *M. tuberculosis* complex strains (Groenen *et al.*1993).

Spoligotyping is a PCR based method that analyses the polymorphism in the well-conserved 36bp DRs interspersed with 35- to 41- bp non-repetitive spacer sequences in MTC genome (Kamerbeek *et al.* 1997). Both the number of DR (ranging from 10-50 copies) and the presence of spacers vary from strain to strain and serve as an epidemiological marker (Van Soolingen *et al.* 1995). DRs are amplified using one set of primers. Spoligotype patterns are produced by hybridization of amplified sample DNA to multiple synthetic spacer oligonucleotides (43 oligonucleotides) (Figure 6.1). Each genotype is represented by a binary string and analyses the presence or absence of 43 distinct direct variant repeats in the DR (Kamerbeek *et al.* 1997). The digitally represented data can be compared with international spoligotyping database and it allows for grouping of isolates as clades or strain families (Driscoll *et al.* 2009).

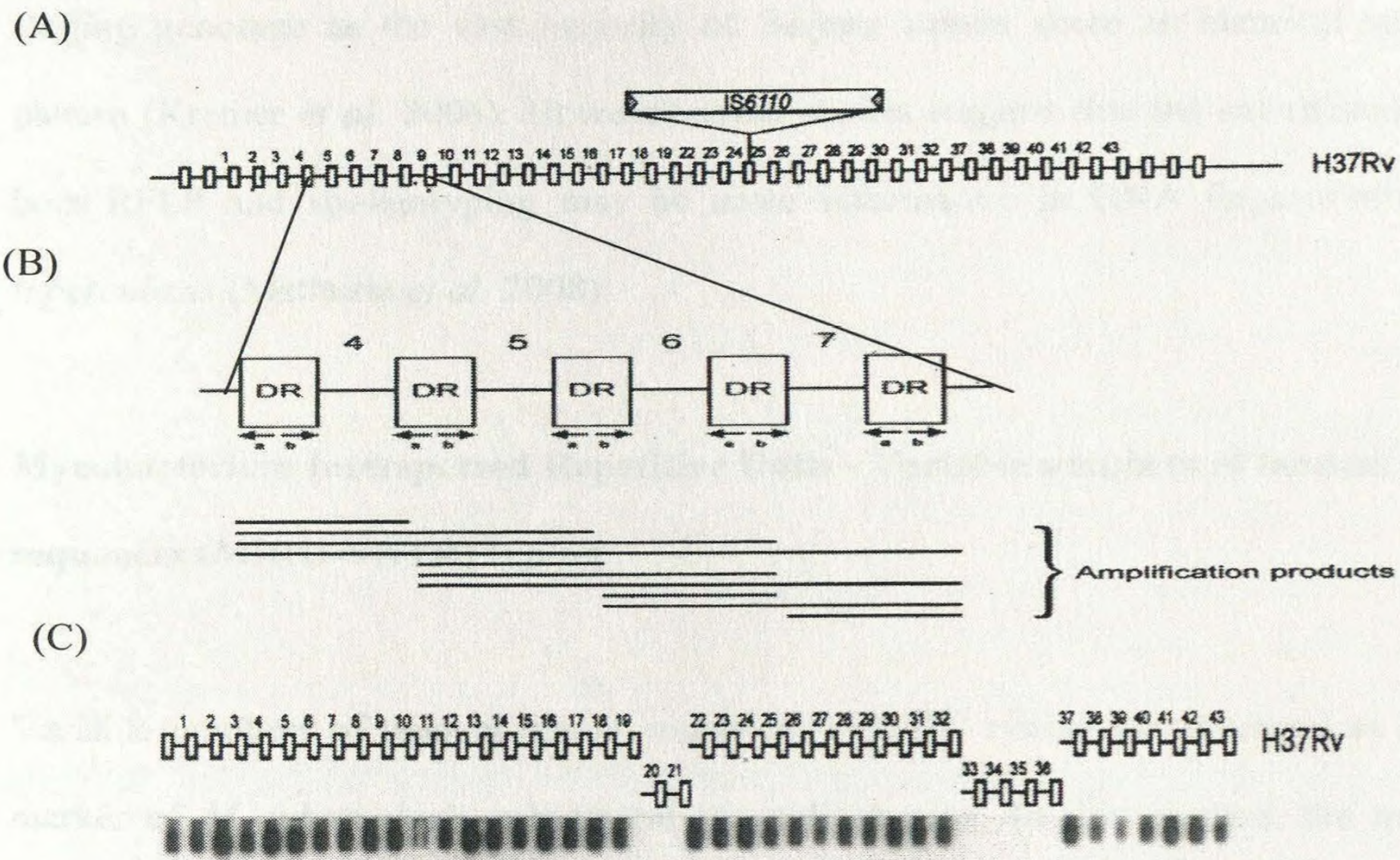


Figure 6.1 (A) Structure of the DR locus in the mycobacterial genome. The chromosomes of *M. tuberculosis* H37Rv contain 48 DRs (depicted as rectangles), which is interspersed. The site of integration of insertion element IS6110 is depicted. (B) Principle of *in vitro* amplification of the DR region by PCR. Any DR in the DR region may serve as a target for these primers; therefore, the amplified DNA is composed of a mixture of a large number of different-size fragments. (C) Hybridization patterns (spoligotypes) of amplified mycobacterial DNAs of *M. tuberculosis H37Rv*. The order of the spacers on the filter corresponds to their order in the genome (Kamerbeek *et al*. 1997).

The method is highly reproducible and can be applied for large epidemiological projects as compared to RFLP, spoligotyping does not require high amount of pure DNA. It reduces time between suspicion of the disease and typing to 1 or 2 days as it requires only a few bacteria that can be obtained from clinical specimens or slides of ZN staining (Vander Zanden *et al.* 1998; Kamerbeek *et al*. 1997). The discriminatory power of spoligotyping is generally poor and may not be able to provide sufficient discrimination between isolates in

Beijing genotype as the vast majority of Beijing strains share an identical spoligotype pattern (Kremer *et al.* 2004). However some studies suggest that the simultaneous use of both RFLP and spoligotyping may be more informative in DNA fingerprinting of *M. tuberculosis* (Mathuria *et al.* 2008).

6.1.2.3 Mycobacterium Interspersed Repetitive Units - Variable numbers of tandem repeat sequences (MIRU-VNTR) typing

Variable numbers of tandem repeat sequences (VNTR) typing has emerged as a genetic marker of *M. tuberculosis* subsequent to spoligotyping. In this method, the number of repeats present at a particular tandem repeat locus in the bacterial genome is analysed. This is also a PCR-based method which analyses the 24 loci using gel electrophoresis, automated capillary systems or high performance liquid chromatography. These 24 loci are interspersed with *Mycobacterium* Interspersed Repetitive Units (MIRUs), Exact Tandem Repeats (ETRs) and Queen's University of Belfast (QUB) loci (De Beer and Kremer 2010). As the length of the repeat is known, the sizes of the PCR product reflect the number of repeats in each VNTR locus. MIRU-VNTR is faster than RFLP and its results can be compared easily (Mazars *et al.* 2001; Supply *et al.* 2006). MIRU-VNTRs have been adopted in combination with spoligotyping for large-scale high-throughput genotyping of *Mycobacterium tuberculosis*. However, even the combination of these two methods is yet less discriminatory than IS6110 fingerprinting by RFLP (Sypply *et al.* 2006).

6.1.3 Genotypes of *M. tuberculosis*

Mycobacterium tuberculosis complex has been categorized into 3 principal genetic groups by analysis of several genetic markers. Group 1 includes 3 lineages namely, the East African-Indian (EAI) lineage, the Beijing lineage, the Central-Asian (CAS) or Delhi lineage (Palomino *et al.* 2007). The EAI lineage is characterized by a low number of IS6110 copies and is frequent in South-East Asia, India, and East Africa (Kremer *et al.* 1999). Presently, it has been renamed as Indo-Oceanic lineage (Gagneux *et al.* 2006). The Beijing lineage was recently renamed as East Asian Lineage (Gagneux *et al.* 2006). It contains high virulence strains and is recognized to be frequently associated with multi drug resistance (Anh *et al.* 2000; Pfyffer *et al.*2001). Beijing strains are characterized by the presence of an inverted IS6110 copy within the direct repeat (DR) region, an IS6110 element at a particular insertion site and one or two IS6110 copies in NTF locus (Plikaytis *et al.* 1994; Kurepina *et al.* 1998). The CAS lineage is specific for India (Bhanu *et al.* 2002,), Sudan, other sub-Saharan countries and Pakistan (Brudey *et al* .2006). The Haarlem family (highly prevalent in Northern Europe) (Kremer 1999), the Latin American and Mediterranean (LAM) family (Sola *et al.* 2001), the X family (most prevalent in South Africa) (Sebban, 2002), the T families (Marmiesse, 2004) and others belong to the principal genetic groups 2 and 3 (Palomino *et al.* 2007).

6.1.4 Transmission of MDR-TB

Genotyping of *M. tuberculosis* has been extensively used for investigating epidemics of MDR-TB. There is much debate about the degree of transmissibility as a specific characteristic of drug resistant strains. Further, this focuses on whether MDR-TB strains are more easily transmissible or whether the mutations that confer drug resistance also impair the reproductive function of the organism (Sougakoff, 2011).

Application of molecular epidemiological methods was central to the identification and description of all drug resistant outbreaks. The most extensive MDR-TB outbreak reported was from New York, among 267 patients who were infected by Beijing/W genotype (Frieden *et al.* 1996). This cluster of cases included drug resistant isolates that were resistant to all first line anti TB drugs. Molecular methods were used to identify, how this initially drug susceptible strain expanded to result in a MDR-TB phenotype by sequential acquisition of resistance conferring mutations in several genes (Bifani *et al.* 1996). Beijing/W genotypes can be identified by their characteristic multi-banded IS6110 restriction fragment-length polymorphism (RFLP) patterns, a specific spoligotype pattern characterized by the presence of spoligotype spacers 35–43 (Bifani *et al.* 2002) and resistance conferring gene mutations. Although these data led many to propose that Beijing/W strains behaved differently from other strains, more recent work suggests that MDR-TB outbreaks are not limited to the Beijing/W genotype. Smaller outbreaks involving other MDR-TB genotypes have been reported in other settings such as the Czech Republic, Portugal and Norway (Kubin *et al.* 1999).

6.2 Justification and objective of the present chapter

Much of the MDR-TB burden falls on developing countries that are not engaged in routine molecular epidemiological surveillance and little is known about the characteristics of circulating drug resistant strains. Literature reviewed within the chapter revealed that drug resistant outbreaks may not be associated only with world prevailing strains such as Beijing/W. Furthermore, the genotype of the *M. tuberculosis* may vary with the geographical region. Thus, it is possible to find hitherto unreported MTB-TB strains with specific characteristics such as those responsible for transmission of MDR-TB and MDR-TB outbreaks. Therefore, routine surveillance of molecular epidemiology of MDR-TB is essential to prevent and control drug resistant tuberculosis (Portugal *et al.* 1999).

In Sri Lanka, there is very little data on epidemiological characteristics of *M. tuberculosis* strains, i.e. whether recurrent tuberculosis is due to exogenous reinfection, current rate of active transmission in different strains, emergence of new strains and possibility of extensive transmission or outbreaks of MDR-TB. Thus, the study described in this chapter will address the genetic diversity and the transmission patterns of RIF resistant *M. tuberculosis* strains in Sri Lanka, using molecular fingerprinting to achieve the following objectives.

(a) To determine the RFLP pattern of 31 RIF resistant *M. tuberculosis* isolates and 46 RIF susceptible *M. tuberculosis* isolates

(b) Investigate the transmissions of RIF resistance of *M. tuberculosis* in Sri Lanka using a dendrogram based on the RFLP banding patterns

6. 3 Research methodology

All reagents were of molecular biology grade and purchased from Sigma Company, St. Louis, MO, USA (unless otherwise stated). A Amersham™ ECL Direct™ Nucleic Acid Labeling and Detection System were purchased from GE Healthcare, USA.

6.3.1 Study population

Phenotypically and genotypically confirmed 31 RIF resistant *M. tuberculosis* isolates and phenotypically confirmed 46 RIF susceptible *M. tuberculosis* isolates were used for the RFLP analysis. H37Rv was used as the control strain.

6.3.2 Restriction endonuclease digestion

Genomic DNA from selected *M. tuberculosis* strains were extracted as described in section 4.3.2. Restriction enzyme digestion was performed according to the method described by Warren *et al.* (2009), in a total volume of 100 µl by sequential addition of PCR grade water, 10 µl of 10X restriction buffer, 6 µg of DNA and 30 units of *Pvu*II restriction enzyme. The mixture was vortexed well and incubated at 37 °C for 16 h. The *Pvu*II enzyme was heat inactivated by incubating the mixture at 65 °C for 10 min. The digested DNA (8 µl) was mixed with loading buffer (4 µl) and was separated by electrophoresis in EtBr stained 1% agarose gel at 1.5 V/cm for 16 h and visualized under the UV light. Remaining digested DNA (92 µl) was precipitated by adding 9 µl sodium acetate (pH 5.2) and 300 µl ice cold absolute ethanol. The mixture was kept at -20 °C for 16 h. DNA was

pelleted by centrifugation at 10,000 x *g* for 30 min at 4 °C. Supernatant was discarded. The pellet was resuspended in 500 µl ice cold 70% ethanol and centrifuged as above. After removing the supernatant, DNA was dried at room temperature (~ 25 °C) for 24 h to 30 h. DNA was re-dissolved in 1X loading buffer (volume was determined according to the concentrations of DNA presence on the test gel) that include molecular weight maker X (Roche diagnostics, USA) (Appendix 3.5). The mixture was allowed to dissolve over night and 10 µl of DNA mixture (DNA + loading buffer) was separated on 0.8% agarose gel by electrophoresis at 2 V/cm for 16 h and visualized under UV light.

Electrophoresed DNA was denatured by immersing the inverted gel in 500 ml denaturing buffer (Appendix 3.6) at 25 °C for 30 min with shaking. The denatured gel was neutralized in 500 ml neutralizing buffer (Appendix 3.7) at 25 °C for 30 min with gentle shaking. The nylon membrane (GE healthcare, USA) was marked by spotting with orientation marker (Appendix 3.8) at 6 different positions on upper and lower margin of the membrane. Then, the membrane was hydrated in 500 ml sterile distilled water for 30 seconds and equilibrated in 500 ml 20x SSPE buffer (Appendix 3.9) for 5 min. Southern transfer set up was arranged and allowed capillary transfer for 16 h. Thereafter, the Southern transfer set up was dismantled and the nylon membrane was baked at 80 °C for 2 h in vacuum oven and stored at 4 °C.

6.3.4 Probe hybridization

6.3.4.1Preparation and labelling of IS6110 probe

The 240bp fragment of IS6110 insertion sequence of *M. tuberculosis* was selected as the probe and amplified by PCR (as described in section 2.3.6.3). The PCR amplified product was purified using PCR amplification clean-up kit (Promega, USA) as per manufacture`s guidelines. DNA concentration of the amplified IS6110 fragment was determined using a NanoDrop (2000c/2000) UV-Vis Spectrophotometers (Thermo, USA). In labelling, 2 µl of prepared probe (200 ng) that mixed with 13 µl PCR grade H_2O was heated at 100 °C for 5 min and snap cooled on ice (4 °C) for another 5 min. Thereafter, 15 µl of horseradish peroxidase and 15 µl of gluteraldihyde (Amersham ECL^{tm} nucleic acid labeling system) were added sequentially. Finally, the mixture was incubated at 37 °C for 10 min.

6.3.4.2 Pre hybridization and hybridization

The procedure described by Warren *et al.* (2009) was followed in pre hybridization and hybridization of labelled probe.

Pre-hybridization of Southern blot was carried out in 48 ml ECL^{tm} Gold buffer (GE healthcare, USA) at 42 °C for 1 h in a sealed bag. Thereafter, 45 µl of labelled probe was added directly to the Gold buffer (48 ml) in the sealed bag. Hybridization was carried out at 42 °C for 16 h with shaking. The hybridized membrane was washed twice in 400 ml primary wash buffer (Appendix 3.10) for 20 min at 42 °C. Then, the membrane was washed twice in 400 ml of 2 X SSC (Appendix 3.11) at room temperature (~25 °C) for 5

min with shaking. Finally, the membrane was sealed in a new plastic bag and subjected to chemiluminescence by incubating in 8 ml (4 ml of detection reagent 1 + 4 ml of detection reagent 2) of Amersham ECL^{tm} detection reagents for 90 seconds. Thereafter, all fluid was removed and sealed the bag. The membrane was exposed to X-ray film in a dark room for 5- 10 min. Thereafter, the X-ray film was developed according to standard methods, at the Department of Radiology of National Hospital of Sri Lanka, Colombo.

After the detection was completed, membrane was incubated in 400 ml boiling SDS for 1 h, at room temperature (~25 °C) with shaking to remove the hybridized probe. Thereafter, the membrane was sealed in a new plastic bag to carry out the hybridization of the molecular weight maker X DNA in that membrane. The probe for the maker X DNA was prepared as described in section 6.3.4.1. The hybridization, and detection was as carried out as described in section 6.3.4.2. Finally membrane was de-probed by incubating in 400 ml boiling SDS for 1 h at room temperature (~25 °C) and stored at 4 °C for further use.

6.3.5 Data analysis

Band positions on the autoradiogram were determined manually. The sizes of IS6110 RFLP fragments were calculated by comparison of their mobility with known molecular sizes of internal marker (Marker X - Appendix 5). The accuracy of the normalization

procedure was controlled by comparing the IS6110 fingerprint patterns of reference strain, H37Rv.

Genetic relationship among 77 isolates was analysed by the presence (1) or absence (0) of specified DNA fingerprints of each isolate compared to the molecular weight marker X on the same lane. A distances matrix was computed in PHYLIP (version 3.69) by the program Restdist (using the distance matrix which was introduced by Nei and Li) and internal branching probabilities were determined by bootstrap analysis using 100 replications. Cluster analysis was carried out with the same programme using Neighbour Joining method (Felsenstein, 1989) and the dendogram was constructed using Phyfi software (Fredslund, 2006).

6.4 Results

As shown in Figure 6.2, the IS6110 fingerprints for both catalogues (RIF resistant and susceptible) were highly variable according to the number of copies and location of bands. The number of copies of IS6110 per isolate varied from 1 to 14, with sizes ranging from 665bp to 10180bp. All isolates had at least one copy of the IS6110 element. Majority of the isolates (n=16, 20.8%) had 11 copies of IS6110 and among them 11isolates were resistant to RIF. None of the isolates had 3 or 4 copies (Figure 6.3). Further, copy number 2, 5 and 14 were shown only in RIF susceptible strains (Figure 6.3 and Figure 6.4).

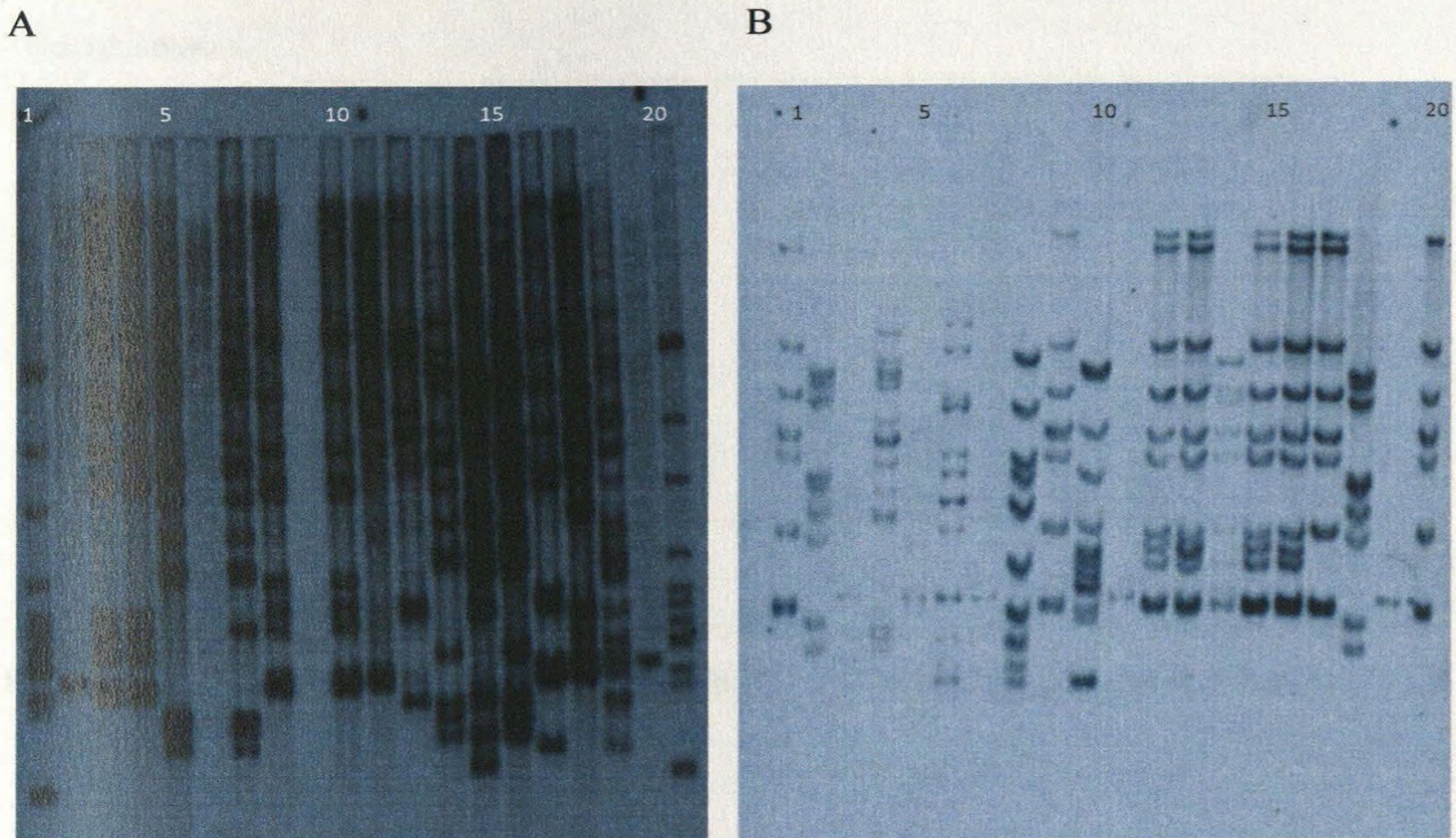


Figure 6.2 (A) IS6110-based RFLP patterns of 17 RIF resistant *M. tuberculosis* isolates. Lanes 1 and 20-reference strain of *M. tuberculosis* (H37Rv); lane 2 to19- RIF resistant *M. tuberculosis* isolates. (B) IS6110-based RFLP patterns of 19 RIF susceptible *M. tuberculosis* isolates. Lane 10- H37Rv; lane 1to9 and 11to 20- RIF susceptible *M. tuberculosis* isolates.

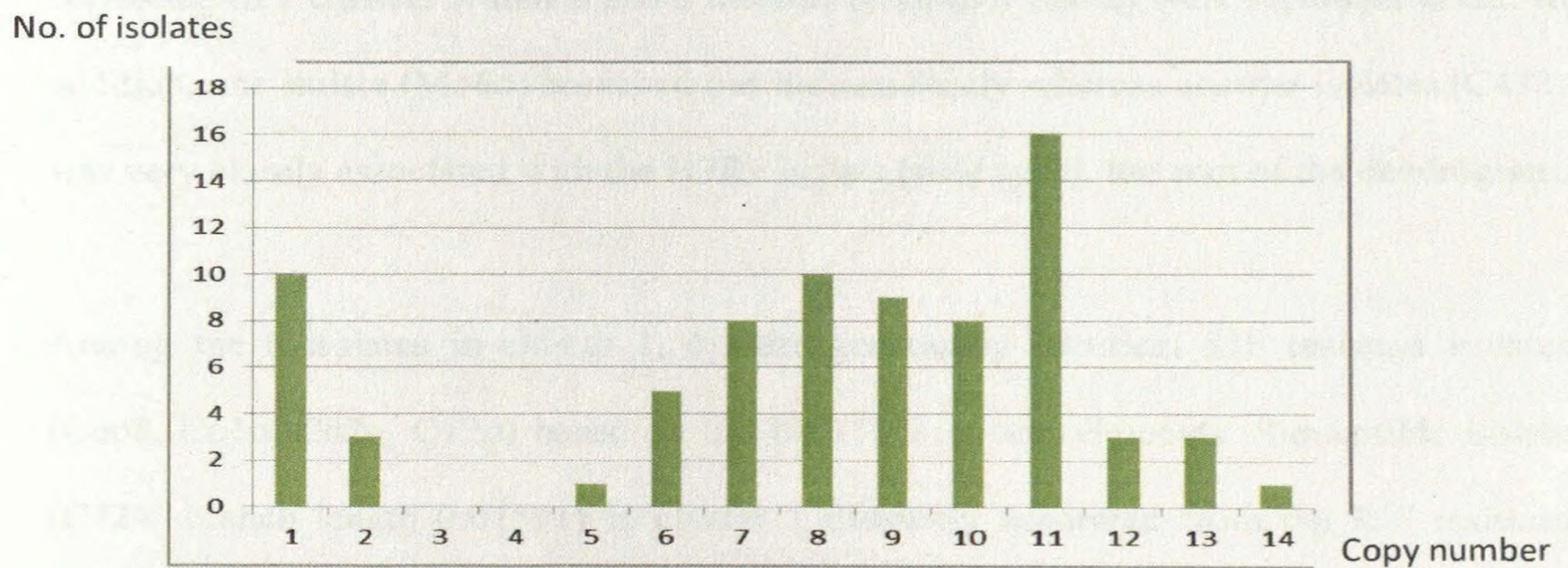


Figure 6.3 IS6110 copy numbers present in *M. tuberculosis* isolates (n= 77)

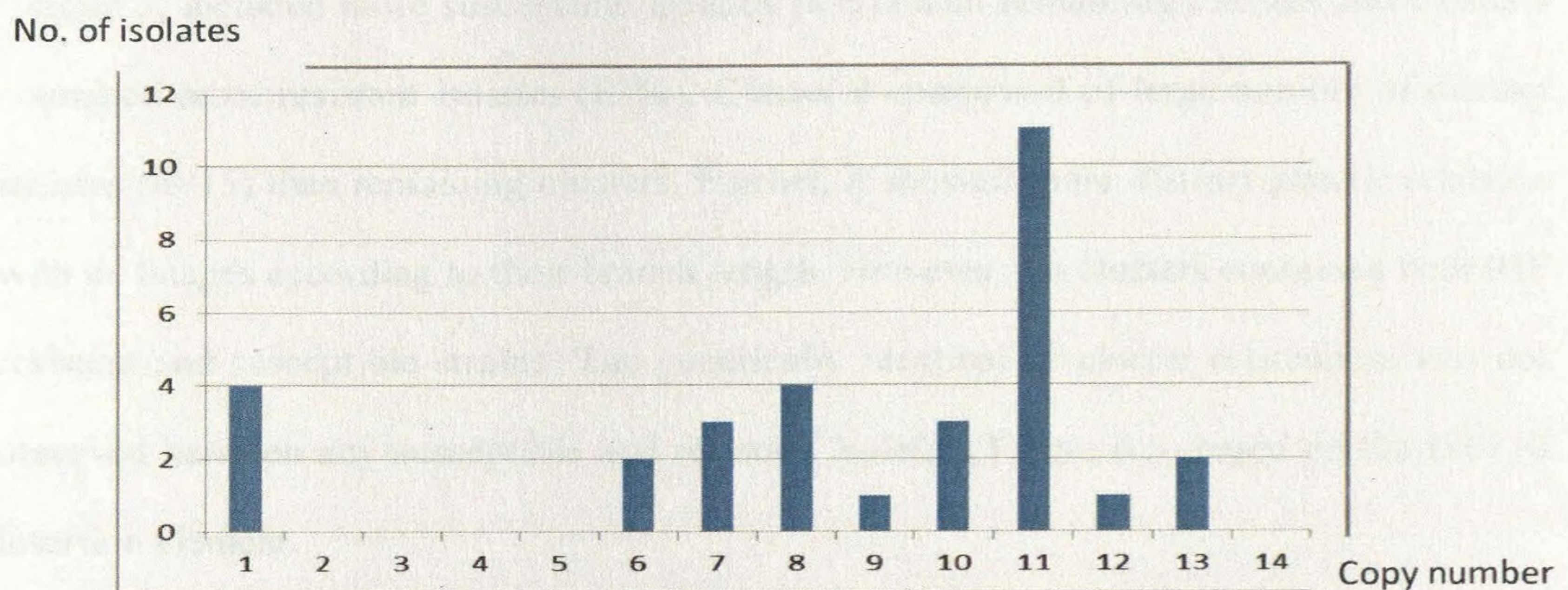


Figure 6.4 IS6110 copy numbers present in RIF resistant *M. tuberculosis* isolates (n=31)

The genetic relatedness among IS6110 fingerprint patterns were analysed using the distance matrix method and the Neighbour Joining dendrogram is shown in figure 6.5. A total of 68 distinct isolates were found among 77 *M. tuberculosis* isolates and they were separated into two groups, G1 and G2 at the node A. The group G1 was the larger group consisting of 7 clusters within it and 2 clusters (Cluster 1 and 2) were separated in G2. In addition, one isolate (Mo6o) branched out independently whereas another isolates (C432) was very closely associated with the H7Rv isolate (wild type), the root of the dendrogram.

Among the 6 isolates in cluster 1, 4 were genetically identical RIF resistant isolates (Coo8, Co1o, Co2o, C12o) based on the IS6110 insertion elements. Susceptible isolate (C424 -branch length 0.01591) in cluster 1 distinctly separated from the RIF resistant isolates.

Cluster 5, included more susceptible isolates (87%) than remaining clusters and cluster I contained more resistant isolates (83%). Cluster 8 comprised of large number of distinct isolates (n=15) than remaining clusters. Further, it showed more distinct genetic relations with its linages according to their branch length. However, all clusters contained both RIF resistant and susceptible strains. The genetically identical or closest relatedness was not observed between any susceptible and resistant isolates (Figure 6.5) based on the IS6110 insertion element.

Figure 6.6 represent the Neighbour Joining dendrogram constructed for 31 RIF resistant *M. tuberculosis* isolates and they showed 28 distinct RFLP IS6110 patterns. They represented 3 clusters and 13 isolates were categorized into cluster 2. Two isolates were distinct and 1 of the distinct isolates (Coo6) was more related to the H37RV (wild type) than others (branch length 0.01084).

Cluster 1 contained 3 genetically identical isolates (Coo8, Co2o, and Co1o). In cluster 3, one clade contained 2 genetically identical isolates (C115 and Co22) while its sister clade also had identical isolates (Co27 and Coo7). The RIF resistant isolates; Coo8, Co2o, Co1o and C12o were genetically identical in the dendrogram that was constructed for 77 *M. tuberculosis* isolates (figure 6.5). However, C12o had separated into a sister clade in the dendrogram constructed for only RIF resistant *M. tuberculosis* isolates (Figure 6.6).

RIF resistant isolates that contained novel mutations at codon 626 (n=13) (refer chapter 4) had 11distinct IS6110 RFLP patterns and they were distributed in all clusters (Figure 6.6).

Among them, Co20 was genetically identical to Coo8 while the isolate C007 was similar to Co27. However, they were belonged to separate clusters (Cluster 2 and 3 respectively) (Figure 6.6).

Isolates PC88 and Mo33 containing the novel mutation at codon 184 belonged to 2 separates cluster (Clusters 2 and 5 respectively (Figure 6.6)

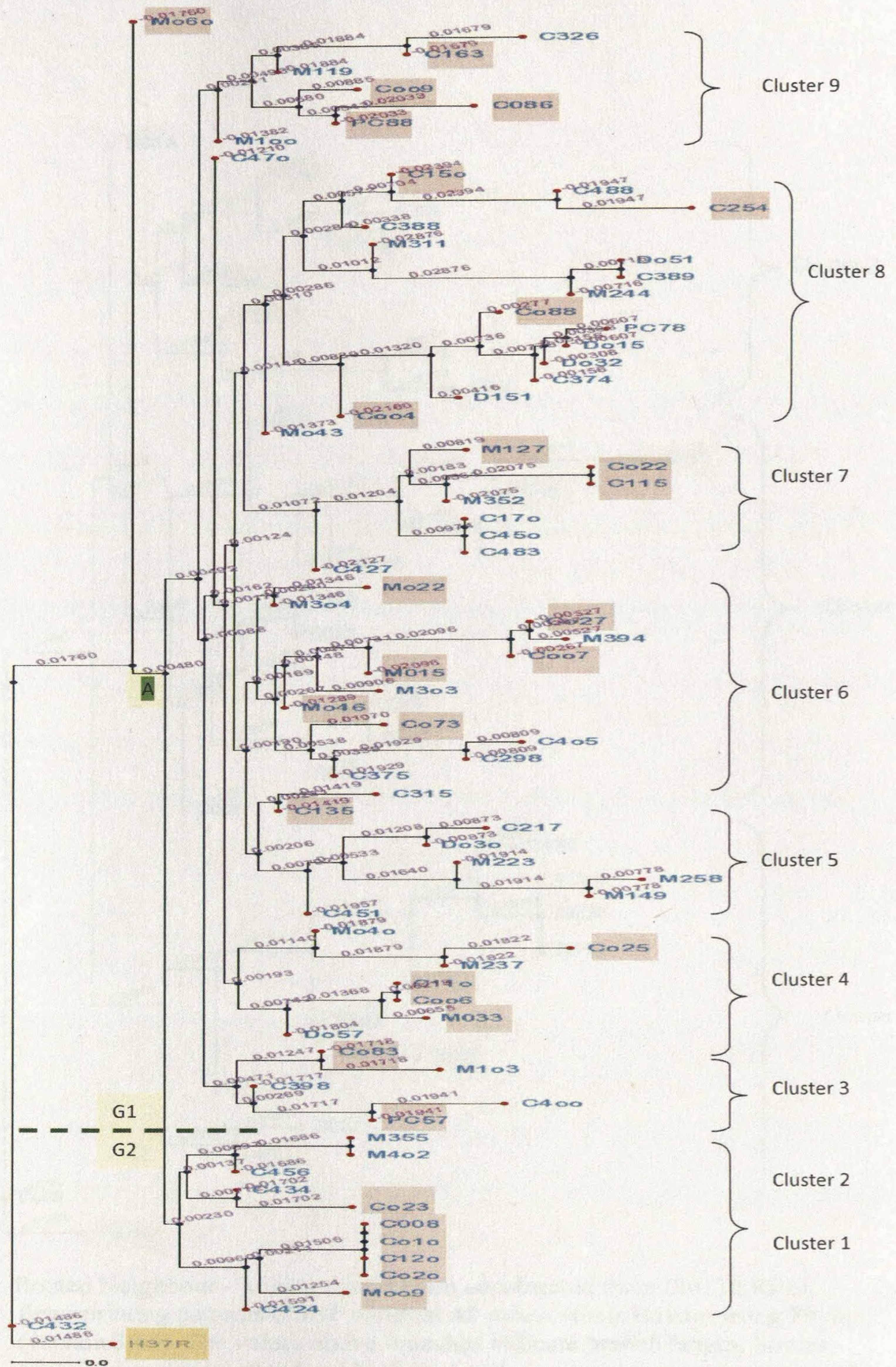


Figure 6.5 Rooted Neighbour - joining dendrogram constructed from IS6110 RFLP fingerprinting patterns of *M. tuberculosis* isolates using Phylip (version 3.69). RIF resistant strains highlighted in pink colour and the reference H7Rv (named as H37R) strain highlighted in orange. The values above branches indicate branch length.

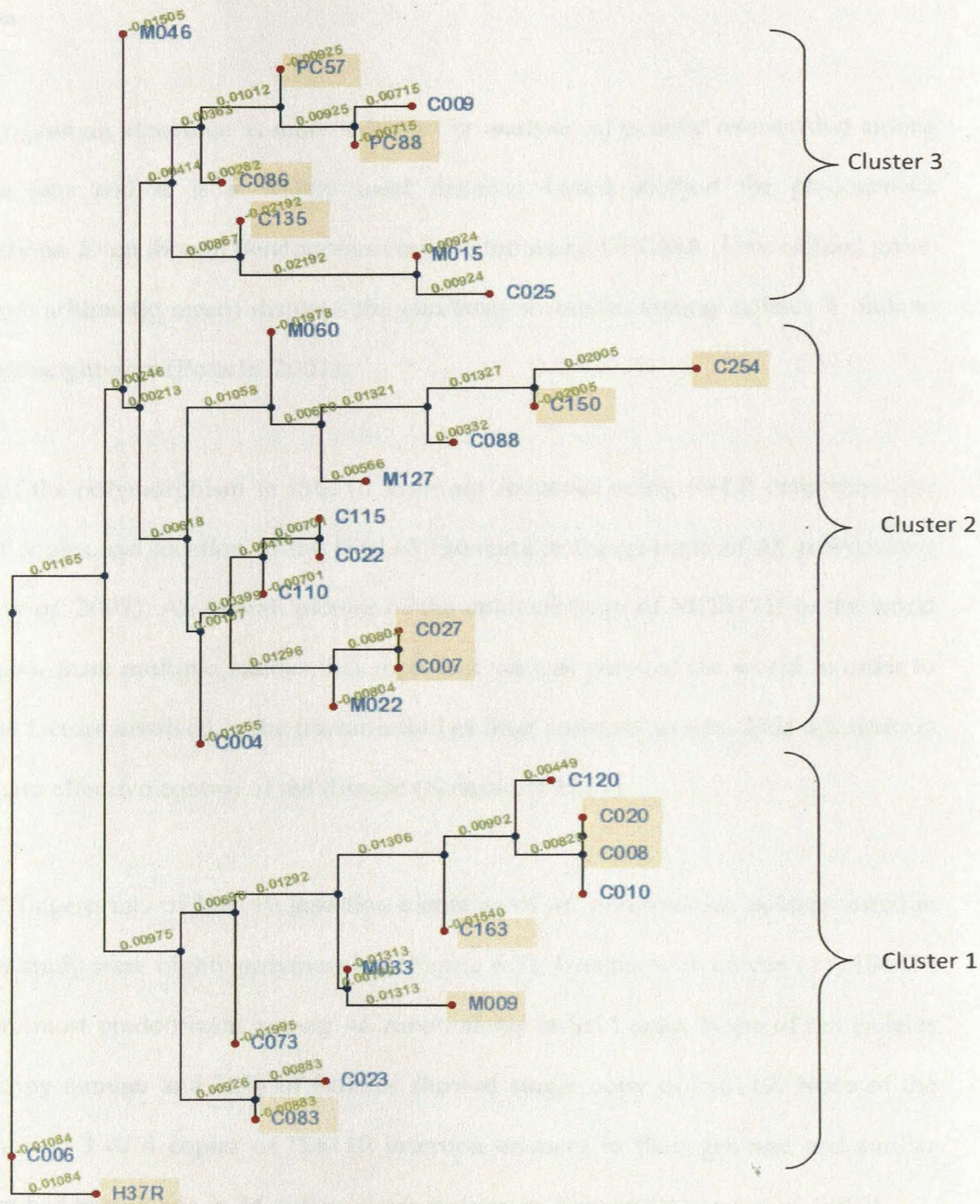


Figure 6.6 Rooted Neighbour - joining dendrogram constructed from IS6110 RFLP fingerprinting patterns of RIF resistant *M. tuberculosis* isolates using Phylip (version 3.69). The values above branches indicate branch length. Strains contained mutation at codon 626 (novel mutation) are in orange colour and H7Rv (named as H37R) strain highlighted in yellow.

6.5 Discussion

Neighbour- joining algorithm is more suitable for analysis of genetic relationship among large data sets and it is a widely used distance based method for phylogenetic reconstructions. Even though dendograms constructed using UPGMA (Unweighted group method with arithmetic mean) displays the similarity distances among isolates it fails to pick closest neighbours (Posada, 2001).

Analysis of the polymorphism in IS6110 insertion sequence using RFLP determines the number of copies and location of the IS6110 elements in the genome of *M. tuberculosis* (McEvoy *et al.* 2007). An overall picture of the epidemiology of MDR-TB in the world can be drawn from multiple studies that represent various parts of the world in order to identify the factors involved in the transmission of drug resistant strains. This information may facilitate effective control of the disease (Sougakoff 2011).

The RFLP fingerprints of IS6110 insertion elements of *M. tuberculosis* isolates tested in the present study were highly polymorphic (Figure 6.5). Isolates with eleven (11) IS6110 copies were most predominant among *M. tuberculosis* in Sri Lanka. None of the isolates had zero copy number and 12% of isolates showed single copy of IS6110. None of the isolates showed 3 or 4 copies of IS6110 insertion element in their genome and similar observation had been made in *M. tuberculosis* isolates in Taiwan (Hwang *et al.* 2003).

Only 18% of tested *M. tuberculosis* isolates had ≤ 5 IS6110 copies. In contrast, Magana-Arachchi *et al.* (2011) has shown that 52% of isolates had 5 or less copies of IS6110 in *M.*

tuberculosis isolates retrieved from Kandy district in Sri Lanka. Further, Das *et al.* (1995) has reported that high number of *M. tuberculosis* isolates (40%) retrieved from India lack or contained only a single IS6110 element.

Among *M. tuberculosis* isolates studied in the present study, 83% had > 6 copies (6-14 copies) of IS6110 insertion elements in their genome. This observation is similar to findings of Tunisia and Denmark where 75% of isolates had 6-10 copies and 50 % of the strains carried 11-15 copies in IS6110 element respectively (Chevrel-Dellagi *et al* 1993; Sola *et al.* 2001). Further, 87% of RIF resistant *M. tuberculosis* strains in the present study had 6-13 copy numbers of IS6110. Many of the strains in EAI family have few copies of IS6110 (< 5) while Beijing family is one of the lineages with the highest number of copies of IS6110 element (Alonso *et al.* 2011, Phyu *et al.* 2009). As Beijing strains are reported to be frequently associated with RIF resistance (Glynn *et al.* 2006; Wang *et al.* 2009) it is possible to suggest that most of RIF *M. tuberculosis* isolates tested in Sri Lanka may belong to the Beijing family. Further, this suggestion is supported by a previous study that used spoligotyping (Rajapaksa *et al.* 2008) which concluded as the majority of *M. tuberculosis* strains in Sri Lanka belong to Beijing family. The lack of a standard Beijing strain in the present fingerprinting analysis is a limitation and findings indicate the importance of using reference strains for major genotypes of *M. tuberculosis* in arriving at definitive conclusions on RFLP analysis.

Among the RIF resistant isolates (Figure 6.5 and 6.6), 87 % (n=27) were genetically distinct. It supports the assumption that RIF resistant *M. tuberculosis* in Sri Lanka is not

related to outbreaks in contrast to those reported from Cape Town, where 52% of drug-resistant tuberculosis cases were caused by transmission of a drug-resistant strain (Rie *et al.* 2000). Further, isolates with novel mutation at codon 626 is distributed in all clades of the dendrogram (Figure 6.6) indicating the presence of epidemiologically nonrelated strains. Thus, based on the IS6110 fingerprinting, it is plausible that *M. tuberculosis* strains with mutation at codon 626 represent epidemiologically different strains.

M. tuberculosis could be transmitted as a drug-susceptible organism, and patients could acquire drug resistance independently. In this case, there should be drug-susceptible isolates with similar RFLP fingerprint patterns to drug-resistant isolates. In the present study, none of the RIF resistant isolates showed genetically uniqueness or close relationship with susceptible isolates (according to branching length) based on IS6110 insertion elements. Thus, the present study highlights the absence of the acquisition of RIF resistance, following an infection with a RIF susceptible strain. Therefore, the RIF resistance of *M. tuberculosis* in Sri Lanka probably due to the transmission of drug resistant strains (primary drug resistance).

The classification of drug resistance in patients as primary or acquired resistance is very important for national tuberculosis control programmes. The degree of acquired drug resistance reflects the quality and the effectiveness of management of individual patients and quality of tuberculosis treatment in the community. Thus, the present study provides evidence for the presence of good management in treatment of TB patients in Sri Lanka as there is no acquired resistance. However, the occurrence of primary drug resistance

highlights the need for definitive laboratory identification of RIF resistance before starting chemotherapy for TB patients in Sri Lanka.

Except in 4 isolates (Co2o, Coo8 and Coo7, Co27), there was no similarity between genetic relationship based on IS6110 insertion element and *rpoB* gene mutations in RIF resistant isolates (Figure 6.6). As an example, strains Co2o and Coo8 that contained mutation at the codon 626 (confirmed by DNA sequencing- refer chapter 4) showed similar fingerprinting patterns (100%) with the Co10 that had a mutation at codon 526. This observation highlights that genetically similar strains based on the IS6110 may have mutations at different sites in the *rpoB* gene. This observation will be important in selecting a molecular based drug susceptibility testing for particular geographical region as genetically similar strains may contain different mutations in *rpoB* gene.

The further studies focused on genotype identification by a combination of typing methods that include IS6110-RFLP typing, spoligotyping and VNRT-MIRU typing would be helpful in order to arrive at a more complete epidemiological investigation of MDR-TB.

Chapter 7

General discussion and conclusion

7.1 General discussions

The study on rifampicin resistance of *M. tuberculosis* in Sri Lanka was presented in several chapters where each chapter was focused on a specific objective of the study. Two different culture based DST methods were evaluated using *M. tuberculosis* isolates retrieved from AFB positive sputum specimens as an alternative to proportion method. The genetic basis of rifampicin resistance of *M. tuberculosis* isolates in Sri Lanka was determined by investigation of *rpoB* gene mutations. The PCR-ELISA was developed as a simple, cost effective, rapid and sensitive DNA based DST method for determination of RIF resistance. Finally, the transmission pattern of RIF resistance was investigated by DNA fingerprinting.

As hypothesized the novel mutations and frequencies of the prevailing mutations described in the present study has substantially contributed to broaden the knowledge on the profile of mutations of the *rpoB* gene. The novel mutation at codon 626 was responsible for significant proportion of RIF resistance (35.5%) of *M. tuberculosis* isolates in Sri Lanka. Even though the point mutation at codon 531 has been reported as a globally prevailing common mutation it contributed only 9.7% of the RIF resistance. The most prevailing mutated (48.4%) codon was at 526 (Adikaram *et al.* 2012). Further, mutations at codon 516 have been reported as prominent in several geographical areas such as India, (13%) and Iran (10%) and therefore, commercially available RIF resistance diagnosis kits have been optimized to detect mutation at codon 516 among other mutation in the RRDR.

However, no mutations were observed at codon 516 in the present study. The majority of published reports have shown CAC to GAC (transversion mutation) as the most prevalent base change at codon 526 in contrast to the present study where it was a CAC to TAC transition mutation in all isolates (n=15) carrying this mutation. It is also reported that in certain regions of the world the mutation at codon 531 to be frequently associated with the codon 526 mutation (Ruwen *et al,* 2005). However this phenomenon was not observed in this study. The present study clearly shows that the mutated codons, types of mutations (transversion or transition) and their frequencies vary geographically and diagnostic methods should take into account such changes in local settings.

A laboratory has to play a critical role in diagnosing TB, MDR-TB and monitoring treatment. The strength of the laboratory network often directly influence the success of TB control programmes. The study described in chapter 2of the thesis challenges the defining of *Mycobacterium* lung diseases using the results based only on the AFB microscopy and it highlights the need of strengthening the laboratory facilities in Sri Lanka. Developed countries have the sophisticated laboratory infrastructure with high tech methods for providing rapid detection and identification of *M. tuberculosis.* In contrast, many developing countries, even those with high TB burden, struggle to provide satisfactory levels of laboratory diagnosis using microscopy with scarce to non-existent culture methods (Drobniewski *et al.* 2003; WHO,2006). As NTM are often resistant to first line anti TB medication, AFB will be detected in sputum continuously in patients with NTM pulmonary infections even after the 6 month treatment of anti TB drugs(Vidal *et al.* 1996). Therefore, these cases may be incorrectly diagnosed as drug resistant TB and subsequently treated with

high cost, less effective and more toxic second line anti TB drugs (Maiga *et al.* 2012). Most patients treated with second line anti TB drugs experience at least one side-effect and two thirds of such patients have had at least one drug terminated temporarily or permanently as a result of adverse effect such as hepatitis and renal failure (Bloss *et al.* 2010).Thus, improper diagnosis of *Mycobacterium* lung diseases leads to severe complications in patients while having a negative impact on the economy of the country.

As described in chapter 2 of the thesis, out of the 401 *M. tuberculosis* isolates identified during the study, 51 isolates were recovered from suspected secondary TB patients. Thus, their anti TB treatment regimen should have been adjusted with the drug susceptibility pattern of the *M. tuberculosis* isolates before continuing the medication with second line anti TB drugs. The drug susceptibility testing is a standard practice in high resource countries. However, the majority of low-resource countries use DST for priority needs such as drug resistance surveillance, extra pulmonary and childhood TB, and MDR-TB (WHO, 2006). Thus, there is a gap between estimated and notified MDR-TB cases. As an example, in 2010, there were 650,000 estimated cases of MDR-TB worldwide. The actual diagnosis of MDR-TB was less than 10% in most of countries except in European region and South Africa (WHO, 2011).

In Sri Lanka, it takes around 1-2 months for a patient to receive the drug susceptibility testing report after isolation of primary *M. tuberculosis* culture. During this period, empirical therapy is started in order to reduce the transmission of disease and especially if the patient is seriously ill or the disease is progressing rapidly. When DST results are available, drug

regimens are adjusted appropriately (WHO, 2009). However, prolonged inappropriate treatment would cause serious complications in patients as well as spread of MDR-TB among others is inevitable. As TB is mostly a disease of poverty, diagnosis should concentrate on more cost effective and simple technologies. The evaluated NRA and manual MGIT are simple cost effective methods that can be easily applied in low resource settings like Sri Lanka. The drug susceptibility pattern may be available within 8 and 10 days by manual MGIT and NRA respectively after primary isolation of *M. tuberculosis*. Therefore, these 2 methods are good alternatives for APM in determining of RIF resistance. Nevertheless, both the NRA and the manual MGIT methods can be initiated with low technical expertise and the initial cost also less. Thus, manual MGIT and NRA methods will certainly assist decentralization of the DST facility within the country.

Drug susceptibility results will take at least a week even with rapid culture based methods. Thus, DNA based methods that arrive at a conclusive DST result within a few hours or days are vital for ensuring early and accurate chemotherapy. In the last century, several DNA based DST methods were commercialised. However, these methods are not freely available for routine use in developing countries. Additionally, these methods may not achieve expected sensitivity in all settings as mutations of *rpoB* vary geographically (Siu *et al.*2011; Tan *et al.* 2011).Considering all the above factors, the PCR-ELISA described in chapter 5 of the thesis was established as a simple molecular DST method that can be customized according to .user requirements.

A good agreement was shown between PCR-ELISA and APM for identification of RIF resistance. The sensitivity and specificity of probes were ≤ 1 pg and 100% respectively. The probes can be designed as per needs of the user and results on drug susceptibility will be available 2 days after primary isolation of culture. Further, sophisticated instruments or infrastructure are not required and these techniques are familiar to most laboratory personnel even in peripheral laboratories. Thus, the initial instrumentation and consumable cost is affordable for settings like Sri Lanka.

Drug-resistant tuberculosis is caused either by transmission of resistant strains of *M. tuberculosis* or by acquisition of resistance through inadequate treatment (Seddon *et al* 2012). Thus, identification of the transmission pattern of drug resistant strains is vital in controlling and prevention of MDR-TB. DNA fingerprinting has become a powerful tool for tracing and recognising the TB transmission chains and outbreaks in a particular geographical area (Barniol *et al.* 2009).Chapter 6 of the study revealed the epidemiological data that are urgently needed for better control of disease and forecasting of the future transmission of RIF resistance of *M. tuberculosis* in Sri Lanka.TheIS6110 fingerprinting pattern of investigated *M. tuberculosis* isolates in the present study, showed significant genetic distance between RIF resistant isolates and susceptible isolates indicating the presence of primary RIF resistant isolates in Sri Lanka. Further, there are no outbreaks of RIF resistance of *M. tuberculosis* in Sri Lanka and a high genetic variation among resistant isolates was observed. Further, the results revealed in chapter 6 indicated the success of the treatment regimen of the country as there was no resistant acquisition by strains. However, the early and accurate definitive laboratory identification of RIF resistance should be

addressed precisely for prevention of MDR-TB in Sri Lanka. Thus, the transmission pattern of RIF resistant *M. tuberculosis* that was investigated in the present study will be important for planning treatment, control and prevention of the MDR-TB in the country.

7.2 Conclusion

India, the closest neighbour of Sri Lanka is one of the major contributors of MDR-TB and XDR-TB burden in the world. If there are no adequate control programmes that address the persistent issues of drug resistant tuberculosis in a timely manner, Sri Lanka will be in a risk of acquiring high MDR-TB in the future.

The information derived on disease etiology and transmission of rifampicin resistance from this study justifies the need for reliable and rapid diagnostic tools to identify rifampicin resistance. Thus, introduction of the evaluated NRA in liquid medium and the manual MGIT will make the appropriate contribution for control of MDR-TB.

The two novel point mutations outer to the RRDR and different frequencies of universally prevailing mutations in the RRDR of the *rpoB* gene of *M. tuberculosis* isolates in Sri Lanka emphasize the need for expanding the geographical database of the mutations for effective application of the *rpoB*-based diagnosis of MDR-TB in public health settings. It may be necessary to customize the *rpoB*-based molecular diagnostic methods so as to make them more suitable to detect locally prevalent mutations.

The PCR-ELISA developed during the study, is a simple, rapid, inexpensive, sensitive and specific DNA based DST method that can be customized as per variation of profile of mutation of *rpoB* gene of *M. tuberculosis* in a particular geographical region. It will certainly contribute to the control and prevention of MDR-TB in Sri Lanka by determining the drug resistance more accurately. Future studies should focus on, validation of indirect PCR-ELISA with a larger number of resistant isolates as well as with different types of mutations. Additionally, evaluation of PCR-ELISA as a direct method would be of much importance, as it will avoid the primary isolation of cultures, reducing the turnaround time of the assay.

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Appendices

Appendices

Appendix 1 - Ethics approval

The Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka approved the study (ERC Number is EC/06/062). The informed consent form patients were obtained prior to sample collection.

Appendix 2 - Data collection questionnaire

2.1 Request form

Drug resistance in relapse and recurrent TB patients

To be filled by medical officer. Please tick the appropriate box.

This patient is a,

(1) Suspected relapse patient ☐

(2) Re-infected patient ☐

(3) Pre MDR treatment patient ☐

(4) Treatment failure patient at the end of CAT 1 ☐

(5) Sputum POSITIVE patients after 3 months of CAT1 treatment ☐

(6) Pre CAT II Patient ☐

Please include any patient who belongs to any of the categories to the research study.

2.2 Questionnaire for the study on "drug resistance in relapse and recurrent TB patients"

Serial number: Place: Date:

General data:

Name: Registration number:

Address: Date of Birth:

Sex: Occupation:

Past history:

1. How many courses of treatment prior to the present episode with dates:

2. Details of each episode

   - **1st episode**: When (year & month):
     Treatment completed/ Cured & treatment completed/Treatment failure/ Relapse/Defaulter/Relapse & Defaulter not distinguishable/ Chronic/Unknown

   - **2nd episode**: When (year & month):
     Treatment completed/ Cured & treatment completed/Treatment failure/ Relapse/Defaulter/Relapse & Defaulter not distinguishable/ Chronic/Unknown

   - **3rd episode**: When (year & month):
     Treatment completed/ Cured & treatment completed/Treatment failure/Relapse /Defaulter/Relapse & Defaulter not distinguishable/ Chronic/Unknown

3. Was there a contact history in the first episode: **YES / NO**
   If positive: household / work place / prison
   Others...............................

4. Outcome of treatment during the last episode (tick the most appropriate one)
   Treatment Completed / Cured & treatment completed/Treatment failure/Relapse/ Defaulter/Relapse & Defaulter not distinguishable/ Chronic/Unknown

5. Co-morbid illnesses:

   Diabetes: + / - Rheumatoid Disease: + / - Epilepsy: + / -
   Psychiatric: + / - Other (Specify)

6. If an regular treatment with a specific drug /s for co-morbid illness /es (e.g. steroids or NSAIDS for rheumatoid arthritis, oral hypoglycemic for diabetes): + / -

   Name the drugs

Appendix 3- Culture media and reagents for microbiological techniques

3. 1 L-J medium (1600 ml)

The anhydride L-J powder (37.5 g) was dissolved in 600 ml distilled water. After adding 12 ml of glycerol, the mixture was autoclaved at 121 °C for 10 min. Then, 1000 ml egg fluid and 5000 u of penicillin were added to the L-J solution at room temperature and dispended in to sterile wide mouth McCarthy bottle (7 ml per each) under aseptic conditions. The L-J medium was coagulated at 85 °C for 1 h on an inspisater. PNB-incorporated L-J slants were made following same procedure with adding of PNB in to sterile L-J medium. L-J slants were stored at 4 °C after examination of the randomly selected cultures for contamination by incubating at 37 °C for 48 h and used before 2 weeks.

3.2 7H10 agar medium (200 ml)

According to the manufactured guidelines, 3.4 g of anhydride 7H10 agar powder was dissolved in 180 ml of distilled water containing 400 µl of glycerol and autoclaved at 121 °C for 10 min. Then, 20 ml of OAD mixture and 720 µl of PANTA antibiotics were added to the agar solution around 50 °C and dispended in to sterile wide mouth McCarthy bottle (4 ml per each). Each bottle was slanted on an inspisater and allowed to settle. 7H10 agar slants were stored at 4 °C after examination of the randomly selected cultures for contamination by incubating at 37 °C for 48 h and used before 2 weeks.

3.3 7H9 broth medium (200 ml)

According to the manufactured guidelines, 0.94 g of anhydride 7H9 broth powder was dissolved in 180 ml of distilled water containing 1ml of glycerol and autoclaved at 121 °C for 10 min. Then, 20 ml of OAD mixture and 720 µl of PANTA antibiotics were added to the solution at room temperature and dispended in to sterile bijou bottle (4 ml per each). Randomly selected bottles were incubated at 37 °C for 48 h to examine contaminations. Bottles were stored at 4 °C until used.

3.4 PANTA antibiotic mixture (for 25 vials)

Polymyxin B	1200 µg
Amphotericin B	120 µg
Nalidixic Acid	480 µg
Trimethoprim	120 µg
Azolicilin	120 µg

The amphotericin B dissolved in DMSO and other antibiotics were dissolved in DDW and made up to final solution of 18 ml. Sterilization was done by filtering and stored at -20 °C until used.

3.5 Ziehl-Neelsen staining

Air-dried smear was heated with 1% carbol fuchsin solution for 5 min. The smear was washed with 3% acid alcohol. The counters stain methylene blue was put on to the decolourized smear and incubated for 2 min at room temperature. Finally, washed with water and allowed to air dry.

3.6 Standard colour series for NRA test

Stock solutions

1) 0.067 M disodium phosphate
2) 0.067 M monopotassium phosphate
3) 0.067 M trisodium phosphate
4) 1% phenolphthalein
5) 1% bromthymol blue
6) 0.01% bromthymol blue

Working buffer solution: Mixed 35 ml of disodium phosphate, 5 ml monopotassium phosphate and 100 ml of trisodium phosphate.

Solution **1** Mixed 0.1 ml of phenolphthalein and 0.2 ml of 0.01% bromthymol blue with 10 ml of working buffer solution.

Eight clean test tubes (same size tubes that used to perform the nitrate reduction test) were labelled as 1- 8. Then, 2 ml of working buffer solution was added into each tube from No. 2 to 8. After that, 2 ml of solution **1** was added to the tube No.1 in the series. Then, 2 ml of solution **1** was added into tube No. 2, mixed well and transferred 2ml to the next tube (tube No. 3). Serial dilutions were made and discarded 2 ml from the tube No.8. Tubes were autoclaved, sealed and stored at 4 °C. The colour standards:

Tube 1 = 5+
Tube 2 = 4+
Tube 3 = 3+
Tube 5 = 2+
Tube 6 = 1+
Tube 8 = +/-

Appendix 4 –Reagents and buffers for molecular biological techniques

4.1 10x TBE (pH 8.0)

To prepare 1 l of TBE, 54.5 g of Tris (0.45 M), 24.73 g of boric acid (0.44 M) and 2.92 g EDTA (10 mM) were dissolved in in distilled water and adjusted the pH using HCl. After made up to 1 l stored at room temperature.

4.2 6x Gel loading buffer (pH 8.0)

Dissolved 60 mg of bromophenol blue and 6 mg of SDS in 30 ml of 100% glycerol and diluted to 100 ml with TE. Stored at room temperature.

4.3 Extraction buffer (pH 7.4)

Dissolved 50 g mono sodium Glutamatic acid (MSG), 9.3 g EDTA and 6.06 g Tris- HCl in 900 ml distilled water and made up to 1 l. Adjusted pH with HCl and stored at 4 °C.

4.4 TE (pH 8.0)

Dissolved 1.57 g of Tris - HCl (100 mM) and 2.92 g of EDTA (10 mM) in 100 ml distilled water and adjusted pH. Autoclaved and stored in room temperature.

4.5 Loading buffer /internal molecular weight maker X

Mixed 6 ml TE (pH 8), 2 ml loading buffer and 6.6 μl Marker X (1650 ng) in a tube and stored at -20°C.

4.6 Denaturing buffer

Dissolved 87.66 g of NaCl and 20 g of NaOH in distilled water and made up to 1 L. Stored at room temperature.

4.7 Neutralizing buffer (pH 7.5)

Dissolved 87.66 g of NaCl and 60.57g of Tris-HCl in distilled water and made up to 1 l. Stored at room temperature.

4.8 Orientation marker

Mixed 2 µl marker X (250 ng/ µl), 20 µl H37RV DNA (1.25 µg/ µl), 45 µl 0.8 M NaOH and 23 µl TE (pH 8.0) and stored at -20°C.

4.9 20 x SSPE buffer (pH 7.4)

Dissolved 175.32 g of NaCl, 24 g of NaH_2PO_4, 5.8 g of EDTA in distilled water and topped up to 1 l. pH was adjusted with NaOH pellets and stored at room temperature.

4.10 Primary Wash buffer

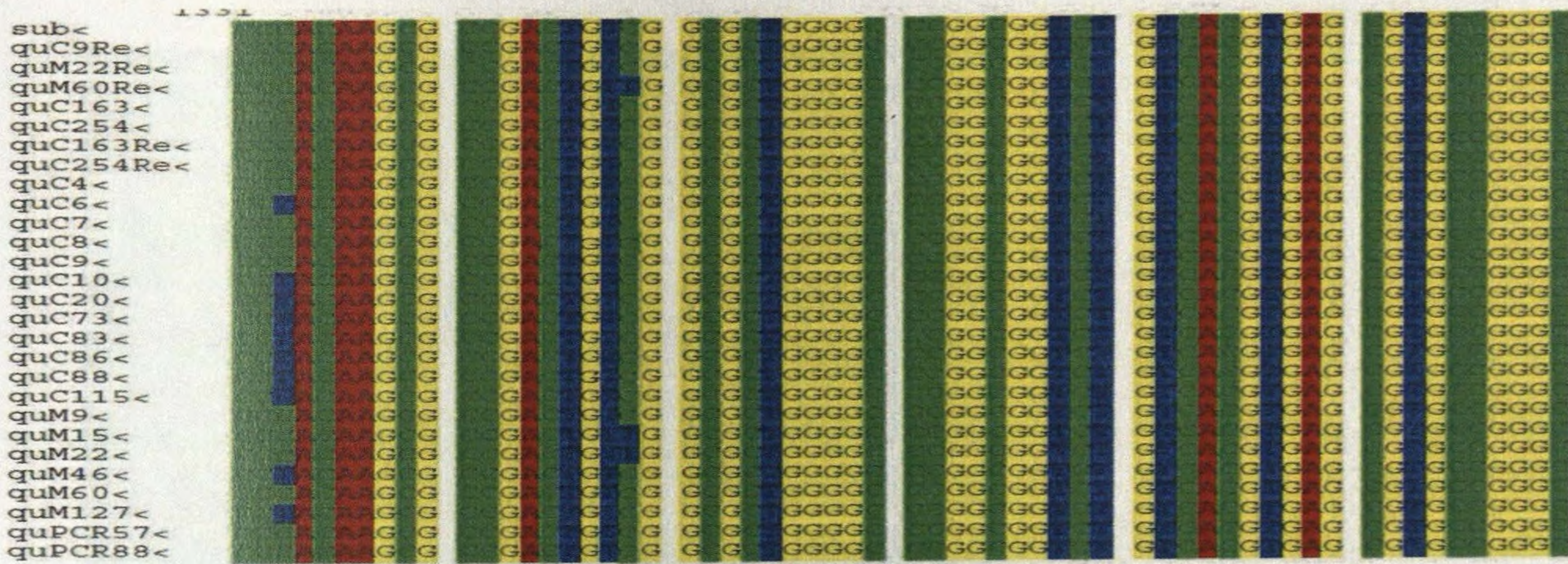
Dissolved 720 g of urea, 8 g of Sodium dodecyl sulphate (SDS) and 25 ml of 20X SSC in distilled water and made up to 2 l. Stored at room temperature.

4.11 20x Standard saline citrate (SSC) pH 7.0

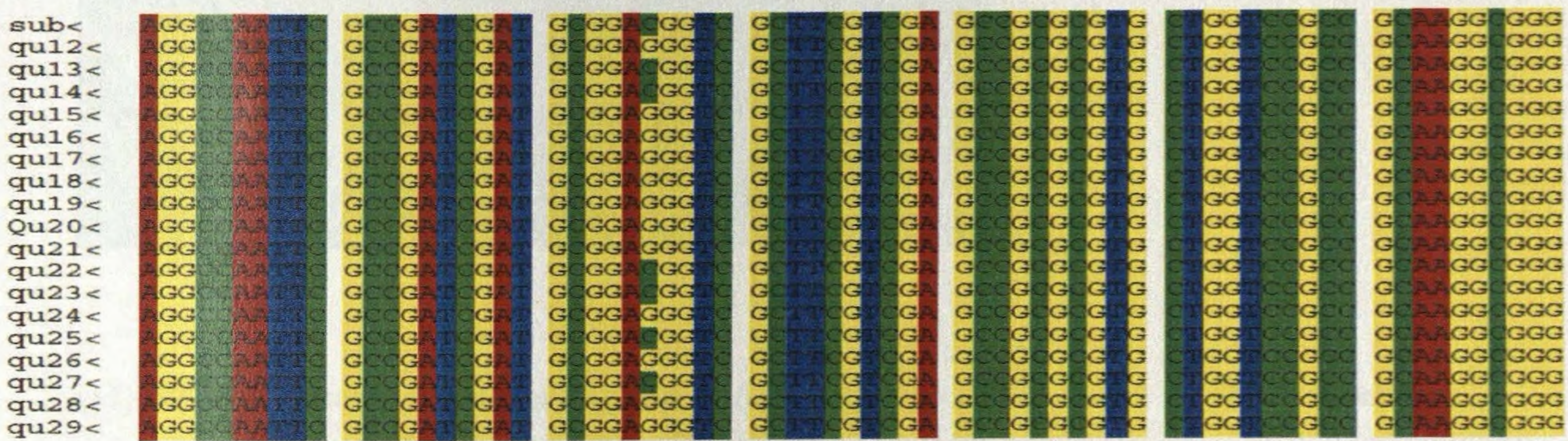
Dissolved 175.32 g of NaCl and 77.4 g of Tri-sodium citrate in distilled water and topped up to 1 l. Stored at room temperature and diluted with distilled water to 2x as required.

Appendix 5 –DNA sequence alignment

5. 1 Part of multiple alignment of DNA sequences of 437 bp fragment of *rpoB* gene



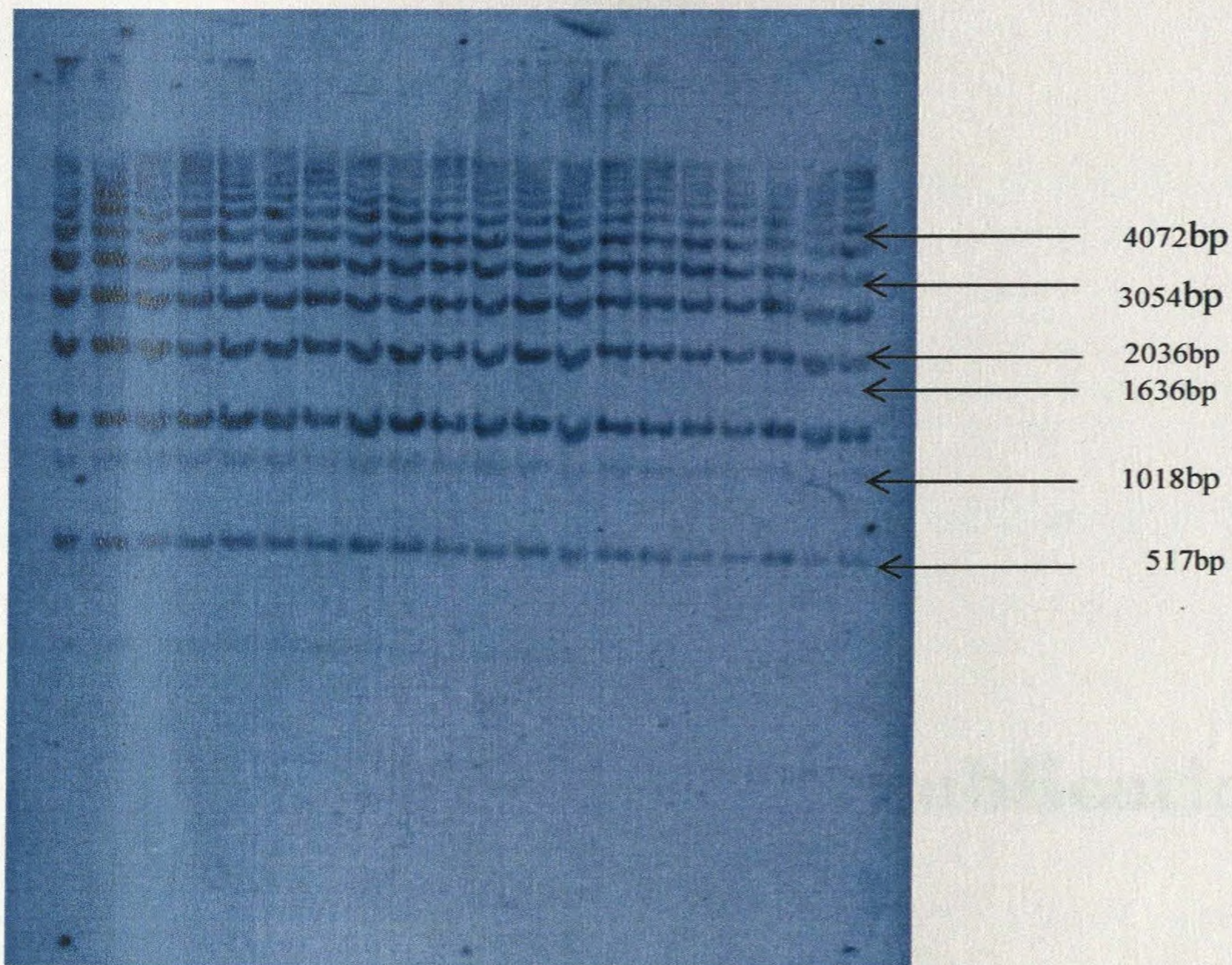
5.2 Part of multiple alignment of DNA sequences of 872 bp fragment of *rpoB* gene



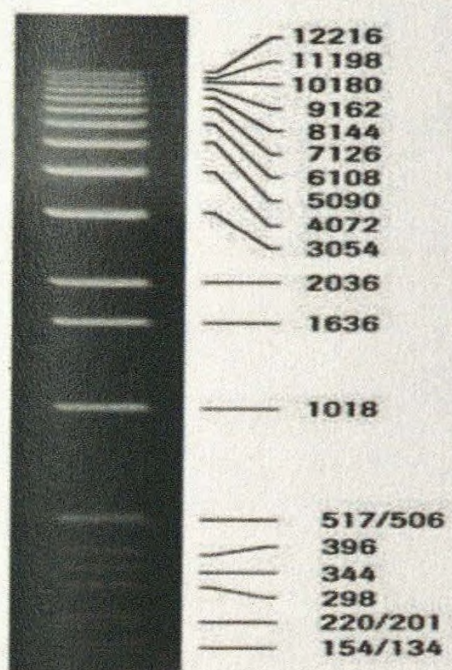
5.3 Part of multiple alignment of DNA sequences of 1392 bp fragment of *rpoB* gene

Appendix 6 - DNA molecular weight marker X (0.07 - 12.2 kbp)

(A) Autoradiogram develop for DNA molecular weight marker X



(B) DNA Molecular Weight Marker X (2% agarose gel) - From product catalogue



Publications

MICROBIAL DRUG RESISTANCE
Volume 18, Number 5, 2012
© Mary Ann Liebert, Inc.
DOI: 10.1089/mdr.2012.0031

DISEASE

Geographical Profile of *rpoB* Gene Mutations in Rifampicin Resistant *Mycobacterium tuberculosis* Isolates in Sri Lanka

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The nature and frequency of mutations in the *rpoB* gene of rifampicin (RIF) resistant *Mycobacterium tuberculosis* clinical isolates varies considerably between different geographical regions. The objective of the present study was the identification of *rpoB* gene mutations responsible for RIF resistance in *M. tuberculosis* isolates in Sri Lanka. Three regions of the *rpoB* gene of *M. tuberculosis*, one corresponding to a 437-bp region, including the rifampicin resistance-determining region (RRDR) and two other regions (1395 bp and 872 bp) spanning the RRDR, were polymerase chain reaction amplified, and were subjected to DNA sequencing. The two mutations found within the RRDR in the 31 RIF resistant strains isolated in this study were at codon 526 ($n=15$, 48.4%) CAC (His)→TAC (Tyr) and codon 531 ($n=3$, 9.7%) TCG (Ser)→TTG (Leu). A significant proportion ($n=15$, 48.3%) showed mutations spanning the RRDR, including two novel mutations at codon 626 ($n=13$, 41.9%) GAC (Asp)→GAG (Glu) and 184 ($n=2$, 6.4%) GAC (Asp)→GAT (Asp), a silent mutation. Two isolates revealed double mutations (codons 626+526 and 626+184). The presence of a high frequency of new mutations, and the different frequencies of the universally prevailing mutations, as reported here, emphasizes the need for expanding the geographical database of mutations for effective application of an *rpoB*-based diagnosis of multidrug resistant tuberculosis.

Introduction

One third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* (MTB), and the majority of tuberculosis (TB) cases are concentrated in developing countries.[34] Globally, 8.8 million incident cases of TB were estimated in 2009. Most of the estimated cases occurred in Asia (59%) and Africa (26%) as compared to the Eastern Mediterranean Region (7%), the European Region (5%), and the Region of the Americas (3%). Three Asian countries, namely India, China, and Indonesia, are among the five countries with the largest number of incidents of TB in 2009. India alone contributed 26% of all TB cases worldwide, while India and China together accounted for 38%.[33]

Multidrug resistant tuberculosis (MDR-TB) is defined as resistance to at least rifampicin (RIF) and isoniazid (INH). The WHO has recognized MDR-TB and XDR-TB, or extensively drug resistant tuberculosis (MDR-TB plus resistance to any fluoroquinolone, and resistance to at least 1 of the 3 injectable drugs: capreomycin, kanamycin, and amikacin) as being a major challenge for controlling TB. Around 440,000 MDR-TB cases are estimated to emerge each year, and 150,000 people die annually from MDR-TB.[32] The four countries that have the largest number of estimated cases of MDR-TB are China, India, the Russian Federation, and South Africa.[34] The WHO progress report of 2011 states that 69 countries had reported at least one case of XDR-TB by the end of 2010, and it is estimated that 25,000 cases of XDR-TB will emerge each year. In an outbreak in South Africa, 52 out of 53 XDR-TB patients died within an average of 3 weeks of being diagnosed.[32]

Although Sri Lanka is not a high-burden country, TB remains a significant public health problem. The estimated incidence rates of all forms of tuberculosis in 2009 were 66 per 100,000 population,[35] and 9643 new TB cases were notified.[15] The overall treatment success rate among smear-positive cases was 85% among the new smear-positive cases and 70% among retreatment patients in the year 2008.[35] A study conducted by the National Reference Laboratory-Sri Lanka during the period 2005–2007 revealed that 3.4% of

Originally presented as poster (abstract) entitled "Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the *rpoB* Gene Are Important Determinants of '*M tuberculosis*' (MTB) Rifampicin Resistance (RifR)" at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) held in Chicago, Illinois, 2011.
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MTB strains were resistant to RIF and 4.5% were resistant to INH, while 2.5% were multidrug resistant.[6]

RIF is the most important drug in the short-course treatment regimen due to its efficient antimicrobial action.[20] RIF interferes with initiation of DNA transcription. It is stated that approximately 90% of the world's RIF resistant isolates are also resistant to INH, making RIF resistance a useful marker of MDR-TB.[25,27] Most of RIF resistant MTB strains from different countries appear to harbor specific point mutations in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, which comprises an 81-bp region containing codons 507–533.[11,12,16,30] In addition, studies have also revealed a significant contribution to RIF resistance through mutations in the region outer to RRDR of the *rpoB* gene, suggesting that, the nature and frequency of mutations in the *rpoB* gene of RIF resistant *M. tuberculosis* clinical isolates vary considerably, between different geographical regions.[10,11,17–19] Further, it has been reported that there is discordance between results of phenotypic methods and genotypic methods that are based on RRDR mutations used for the detection of drug resistant TB.[10]

The WHO recommends performing drug susceptibility testing (DST) for at least RIF and INH in resource limited settings, especially in previously treated patients and HIV coinfected patients. An early diagnosis of drug resistant TB and the management of MDR TB patients are challenging obstacles faced by tuberculosis control programmes.[31] Presently numerous molecular-based identification methods such as INNO-LiPA Rif.TB (INNOGENETICS) and GenoType MTBDR*plus* (HainLifescience) have been commercialized for identification of RIF resistance. However, these methods are based on the detection of commonly found mutations at codon 531, 526, and 516.[34]

In Sri Lanka, RIF resistance is identified by the proportion method that requires more than 28 days for detection of drug resistance. Rapid molecular methods have yet to be established for identification of drug resistant tuberculosis. Before establishing molecular-based methods for detection of drug resistance, it is important to characterize the mutations in the *rpoB* gene in MTB isolates in a particular geographical setting. Therefore, the present study was focused on evaluating the nature and the frequency of mutations in the *rpoB* gene that are associated with resistance to RIF in *M. tuberculosis* strains isolated from Sri Lanka. The results of the present study will also contribute to expand the knowledge on the profile of mutations in the *rpoB* gene.

Materials and Methods

Collection of sputum samples

The sample collecting period was from March 2008 to September 2010, and only pulmonary TB cases were considered. The study population represented primary TB patients (patients that had never received treatment for TB or who had received anti-TB treatment for <1 month) as well as secondary TB patients (i.e., relapse, retreatment, treatment failure, and defaulters) (defaulter: A patient who returns to treatment, positive bacteriologically, following interruption of treatment for 2 months or more). Sputum samples were collected from secondary TB patients admitted to the Chest Hospital Welisara (the only chest hospital in Sri Lanka) for supervised treatment and from primary and secondary TB patients presenting to the Central Chest Clinic, Colombo (the location to which secondary TB patients are referred for re-evaluation and treatment from peripheral clinics). In addition, sputum from all TB suspects (cough >3 weeks) were collected from the Prison Hospital Colombo as prisoners are at a high risk of harboring drug resistant TB strains.

Isolation of M. tuberculosis *strains*

The sputum specimens were processed using the modified Petroff's method. Sodium hydroxide (4%) (Sigma)-treated sputum was centrifuged at 3000 *g* under refrigerated conditions (at 4°C). The centrifuged deposit was diluted in 1 ml of sterile distilled water for preparing the bacterial suspension for inoculation. Two slopes of the Lowenstein–Jensen (L-J) medium (Difco), one containing paranitrobenzoic acid (PNB, Sigma), were inoculated with 100 µl of the bacterial suspension. A small portion of the bacterial suspension was examined microscopically, using the Ziehl-Neelsen (ZN) stain, to determine the presence of acid fast bacilli. The inoculated media were incubated at 37°C in a 5% CO_2 incubator up to 8 weeks until growth was observed. Cultures were confirmed as MTB if they were slow growing, non-pigment producing, reduced nitrate, and did not grow in the presence of PNB. The isolates were further confirmed as MTB by detection of the insertion element *IS*6110 by polymerase chain reaction (PCR) as published previously.[14] One Isolate from each patient was used for the study. The H37Rv strain and a known RIF resistant MTB isolate confirmed by the National Tuberculosis Institute, Bangalore, India were used as quality control strains.

DST for M. tuberculosis *strains*

DST was carried out using the agar proportion method,[7] the nitrate reductase assay (NRA),[2] and the manual Mycobacteria Growth Indicator Tube (MGIT).[1] Rifampicin (Sigma) stock solution (10 mg/ml) was prepared using dimethyl sulphoxide (DMSO) (Sigma), and filter sterilized aliquots were stored at −70°C. A working solution (1 mg/ml) was prepared by diluting the stock solution with sterile distilled water. The final concentration of RIF in each drug containing the culture medium used for the DST was 1 µg/ml[7] (Data not shown).

DNA extraction

Genomic DNA was extracted from identified RIF resistant isolates using the phenol chloroform extraction method.[29] Harvested heat-killed bacteria from the L-J slopes were incubated overnight with proteinase K (Sigma). Genomic DNA was extracted using phenol–chloroform (Sigma) and precipitated with absolute ethanol (Sigma) followed by washing with 70% ethanol. The precipitated DNA was resuspended in the TE buffer (pH 8.0). The quality and concentration of the extracted DNA was tested using an agarose gel (1.5%) electrophoresis, and the DNA was stored at −20°C.

PCR amplification and DNA sequencing

Extracted DNA was used for amplification of three different segments (437 bp, 872 bp, and 1395 bp) of the *rpoB* gene (Fig. 1). The amplified segments covered around 60% of

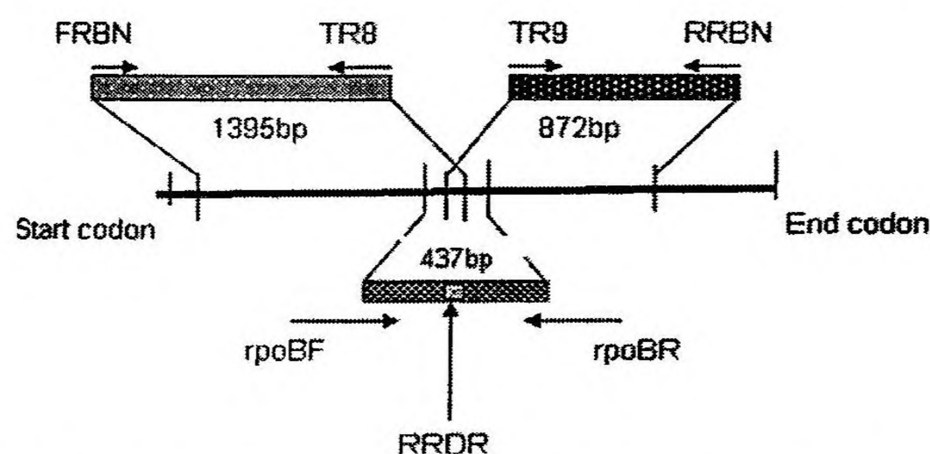

FIG. 1. Position of primers used for amplification of the three regions of the *rpoB* gene in rifampicin resistant *Mycobacterium tuberculosis* isolates.

the total length of the *rpoB* gene. Specific primers were designed using available literature[21,28] and primer-3 software. For amplification of the 437-bp region, including RRDR, rpoBF primer and rpoBR primer were used. The TR9 and RRBN primer pair was used to amplify the 872-bp region downstream of the RRDR region. The 1395-bp fragment upstream of RRDR was amplified using the FRBN and TR8 primer pair (Table 1) (all primers were purchased from Promega).

A 50 µl PCR mixture containing 50 mM KCl, 10 mM Tris (pH 8.0),1.5 mM $MgCl_2$, 200 µM of deoxynucleotides triphosphates (dNTPs) (Promega), 1 U *Taq* polymerase (GenScript), 20 pmol of each primer (Promega), and 2.5 µl of genomic DNA (10 ng) was used for each PCR reaction. The thermo-cycling parameters used for amplification of the three segments of the *rpoB* gene are shown in Table 1. The amplified PCR products were electrophoresed on ethidium bromide stained agarose gels (1.5%) and visualized under a UV illuminator.

Each resistant strain was PCR amplified in duplicate and both amplified products were custom DNA sequenced (Macrogen DNA sequencing service in Korea). A DNA sequence analysis was done using NCBI and Sea View software (version 4.2.12). All resistant isolates were also subjected to *IS6110* restriction fragment length polymorphism typing using the *Pvu*II restriction enzyme.[29]

Results

Three hundred and seventy-three (373) MTB strains were isolated from sputum samples during the period March 2008 to September 2010. The majority of patients were from the Western Province (Colombo, Gampaha, and Kalutara districts) of Sri Lanka, which has a high prevalence of TB.[15] Thirty one RIF resistant isolates were identified among the 373 MTB strains by one or more phenotypic method (APM, NRA, and manual MGIT). Thirteen RIF resistant isolates were from primary cases of TB and the remaining 18 isolates from patients with secondary TB.

DNA sequencing of amplified fragments of the *rpoB* gene revealed that mutations in the RRDR region accounted for resistance in 18 (54%) isolates and were confined to two codons, 526 (n = 15, 48.4%) and 531 (n = 3, 9.7%). The remaining mutations were detected in the regions outer to RRDR and occurred at codons 626 (n = 13, 41.9%) and 184 (n = 2, 6.4%). Twenty nine isolates showed single-point mutations at codon 526 (n = 14, 45.2%), 626 (n = 11, 35.5%), 531 (n = 3, 9.7%), and 184 (n = 1, 3.2%). Dual mutations (at codon 526 + 626 and at codon 184 + 626) were detected in 2 strains (Table 2).

The mutation observed at codon 526 (CAC → TAC) and codon 531 (TCG → TTG) was a C-to-T transition mutation, while the novel mutation at codon 626 (GAC → GAG) was a C-to-G transversion mutation and at codon 184 (GAC → GAT), a transition mutation. All point mutations in this study revealed only a single mutation pattern, and there were no multiple patterns of base changes at a single codon.

TABLE 1. PRIMER SEQUENCES AND THERMO-CYCLING PARAMETERS USED FOR THE AMPLIFICATION OF RIFAMPICIN RESISTANCE-DETERMINING REGION AND 5′ AND 3′ REGIONS OUTER TO RIFAMPICIN RESISTANCE-DETERMINING REGION OF THE *RPOB* GENE IN RIFAMPICIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

Forward primer (5′-3′)	*Reverse primer (5′-3′)*	*Length of the fragment*	*Thermo-cycling parameters*
rpoBF TGGTCCGCTTGCACGAGGGTCAGA	rpoBR CTCAGGGGTTTCGATCGGGCACAT	437 bp	94°C-10min–denaturing 94°C-1min, 57°C-1min, 72°C-1 min } 40 cycle 72°C-10 min–extension
TR9 TCGCCGCGATCAAGGAGT	RRBN GCGCCATCTCGCCGTCGTCAGTACAG	872 bp	94°C-10min–denaturing 94°C-1min, 62°C-1min, 72°C-1 min } 40 cycle 72°C-10 min–extension
FRBN GCAAAACAGCCGCTAGTCCTAGTCCGA	TR8 TGCACGTCGCGGACCTCCA	1395 bp	94°C-10min–denaturing 94°C-1min, 60°C-1min, 72°C-1 min } 40 cycle 72°C-10 min–extension

Table 2. Frequencies and Types of Mutations in the *rpoB* Gene of *Mycobacterium tuberculosis* Isolates from Sri Lanka

Mutated codon	*Specific mutation*	*Primary/secondary TB (No.)*	*Percentage (%)*
526[a]	CAC (His)→TAC (Tyr)	Secondary-10 Primary -4	45.2
626[b,c]	GAC(Asp)→GAG (Glu)	Secondary-7 Primary -4	35.5
531[a]	TCG (Ser)→TTG (Leu)	Secondary-0 Primary-3	9.7
184[b,c]	GAC(Asp)→GAT(Asp)	Secondary-0 Primary-1	3.2
626,184	GAC→GAG, GAC→GAT	Secondary-0 Primary-1	3.2
526, 626	CAC→TAC, GAC→GAG	Secondary-1 Primary-0	3.2
Total			100

The mutated codons with corresponding amino acids are indicated.
[a]Codons within RRDR.
[b]Codons outer to RRDR.
[c]novel mutations.
RRDR, rifampicin resistance-determining region; TB, tuberculosis.

NCBI Accession numbers for DNA sequences: HQ377336-HQ377338, HQ377340- HQ377345, HQ377351, and JQ314433-JQ314444.

Discussion

It is widely reported that MTB expresses a high degree of geographic diversity in mutations to the *rpoB* gene.[9,13,22,26,36,37] Mutations of codon 531, 526, and 516 have been reported as the most frequent mutations in the mutation hotspot region of the *rpoB* gene of MTB worldwide.[3,23,36] In the present study, the majority of mutations occurred at codon 526, and the mutation frequency (48.4%) observed was slightly higher than frequencies reported by China (40%),[36] Japan (35%),[16] and Korea (38%).[9] However, the frequency of occurrence of the mutation at codon 526 in India, the closest neighbor to Sri Lanka, is much higher (93%) as reported by Sharma *et al.* (2003).

Although the majority of published reports have shown CAC to GAC (transversion mutation) as the most prevalent base change at codon 526 (29.7% among Italian isolates[18] and 12% in Greece isolates[12]), our study revealed that the base change was CAC to TAC (transition mutation) in all isolates, similar to reports from India.[24]

The frequency of mutation at codon 531 (9.7%) was comparatively low in MTB isolates from Sri Lanka in relation to mutation frequencies reported by other countries, including India (47%),[10] Australia (52%),[37] Vietnam (31%),[4] and Poland (41%).[23] However, the base change observed at codon 531 was a TCG to TTG (transition mutation) as commonly reported by several Asian countries (Japan,[16] China,[36] Korea,[9] and India[11]) and other countries such as Greece[12] and Italy.[18] Previous studies have reported that a mutation at codon 531 is frequently associated with the codon 526 mutation in certain regions of the world.[22] However, this phenomenon was not observed in our study.

The mutation at codon 626 ($n=13$, 41.9%) GAC (Asp)→GAG (Glu) was the second major mutation found in the present study. Codon 626 is located downstream of RRDR and mutation in this codon has not been previously reported. This novel mutation was present in 13 isolates, and these strains showed different DNA fingerprinting patterns with 1–15 copies of the *IS6110*. The absence of identical banding patterns demonstrates the presence of epidemiologically nonrelated strains. Thus, it is plausible that *M. tuberculosis* strains with mutation at codon 626 represent epidemiologically different strains.

Two isolates showed a silent mutation at codon 184 GAC (Asp)→GAT (Asp) that is located upstream of RRDR. In one isolate, the mutation at codon 184 was associated with a mutation at codon 626. Several mutations in the upstream region of RRDR also have been reported from India.[10]

Mutations at codon 516 have been reported as prominent in several geographical areas such as India, 13%[10] and Iran, 10%[5] and therefore, commercially available RIF resistance diagnosis kits have been optimized to detect mutation at codon 516 among other mutation in the RRDR. However, no mutations were observed at codon 516 in the present study.

Several publications have stated that ~4% of phenotypically resistant isolates did not show any mutations within the RRDR.[4,8] Yue *et al.* (2003) have reported that 10% of phenotypically resistant MTB strains did not show any mutations at RRDR. According to Lingala *et al.* (2010), 21% of phenotypically RIF resistant isolates did not show any mutations when a 255-bp region of RRDR and 350-bp region of non-RRDR of the *rpoB* gene were sequenced. This may be due to mutations occurring in the region outer to RRDR, as was observed in 41.9% of RIF resistant isolates of MTB in the present study. A single discordant result was observed between phenotypic and genotypic results in the present study (phenotypically resistant, but no mutation), although the isolate showed a silent mutation at codon 184. This isolate was confirmed as resistant to RIF by APM and MGIT. It is possible that an additional point mutation is present in this isolate at a codon in the *rpoB* gene not investigated in the present study.

Current molecular diagnostic methods such as INNO-LiPARif are designed to detect common *rpoB* mutations at codon 531, 526, and 516. Therefore, they may not be

applicable universally across various settings, considering the geographical diversity of the mutations.[10,24]

Although the mutations in our strains are limited to a few types, the occurrence of a high percentage of mutations outside the RRDR presents important implications for the use of currently available rapid molecular methods, which target mutations in the RRDR region. Molecular tests used for rapid detection of drug resistance need to be customized to enable detection of novel mutations in different geographical areas. Therefore, identification of drug resistant mutations and their relevant frequencies is an important first step before introducing molecular DST in different geographical settings.

In conclusion, two novel point mutations outer to the RRDR and two universally prevailing mutations in the RRDR of the *rpoB* gene were observed in Sri Lankan RIF resistant MTB isolates. The new mutations and different frequencies of universally prevailing mutations that are reported here emphasize the need for expanding the geographical database of the mutations for effective application of the *rpoB*-based diagnosis of MDR-TB in public health settings. It may be necessary to customize the *rpoB*-based molecular diagnostic methods so as to make them suitable to detect locally prevalent mutations.

Acknowledgments

The authors thank Dr. Inoka Corea, Sirithilak Gamage, Srirani Hendalage, S.M.P Senevirathne, and Gihani Perera and the laboratory assistant staff (Department of Microbiology, Faculty of Medicine, the University of Colombo) for providing assistance. Funding: The study was supported by the National Science Foundation of Sri Lanka.

Ethical Approval

The Ethics Review Committee of the Faculty of Medicine, the University of Colombo, Sri Lanka, approved the study (ERC number is EC/06/062).

Disclosure Statement

All authors report no conflicts of interest relevant to this article.

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Adikaram *et al. BMC Infectious Diseases* 2012, **12**:326
http://www.biomedcentral.com/1471-2334/12/326

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

The manual mycobacteria growth indicator tube and the nitrate reductase assay for the rapid detection of rifampicin resistance of *M. Tuberculosis* in low resource settings

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Abstract

Background: Tuberculosis (TB) is a disease of poverty that contributes significantly to ill-health in developing countries. Drug resistant TB is a major challenge to disease control. Early diagnosis and rapid determination of drug sensitivity is of paramount importance in eradication of TB. Although automated liquid culture based methods are available for rapid detection of drug resistance, the high cost of these tests prevent them from being used routinely in low resource settings. This study compares two phenotypic methods, the manual Mycobacteria Growth Indicator Tube (MGIT) and the Nitrate Reductase Assay (NRA) in liquid medium, with the agar proportion method (APM), the gold standard for susceptibility testing of *Mycobacterium tuberculosis*.

Methodology: Fourteen day old *M. tuberculosis* strains (n=373) grown on solid media were used for drug susceptibility testing by APM, NRA and the manual MGIT method. Rifampicin free and rifampicin incorporated (final concentration, 1 μg/ml) media were inoculated with the recommended concentrations of mycobacterial suspensions and incubated at 37°C in 5% CO_2. In the APM, the proportion of colonies in the drug containing medium was determined. In the NRA, the colour change in the medium was compared with a standard colour series after day 6 and day 12 of incubation. Growth in the MGIT was detected using the manual MGIT reader from day 2 onwards. The 2 methods were compared with the gold standard, APM to determine sensitivity and specificity and agreement between the methods was calculated using kappa statistics.

Results: Thirty one (31) rifampicin resistant isolates were identified. When compared with the APM, the sensitivity of detection of rifampicin resistance was 85% for the NRA and 93% for the manual MGIT and the specificity was 99% and 100% respectively. Both assays, NRA (κ=0.86) and manual MGIT method (κ= 0.94) were in excellent agreement with the APM. The mean turnaround time for manual MGIT method and NRA were 08 days and 10 days respectively.

Conclusion: The NRA in liquid medium and manual MGIT are useful alternatives to APM for drug susceptibility testing of *M. tuberculosis* in low resource settings.

Keywords: Drug resistant tuberculosis, Determination of drug sensitivity, Manual MGIT, NRA in liquid medium, Agar proportion method

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Background

The population of Sri Lanka is about 20 million and it is considered a low TB prevalence country in the Asian region. In Sri Lanka, the estimated incidence of all forms of tuberculosis in 2009 was 66 per 100,000 population. In 2009, 9643 new TB cases were notified and 5186 among them being sputum smear positive TB cases. The notification rate of TB was slightly increased when compared to the year 2008 [1]. The drug resistant rate in Sri Lanka is also low. It is around 0.2% among new TB patients and 18%–21% among re-treatment cases. The HIV co-infection rate among TB patients is currently estimated to be less than 0.1% [2].

Tuberculosis control activities in Sri Lanka operate through the National Programme for Tuberculosis Control & Chest Diseases (NPTCCD) which is a decentralized unit under the Ministry of Health. There are 26 chest clinics in the 25 administrative districts of Sri Lanka and the internationally recommended Directly Observed Treatment Short course (DOTS) strategy is used for treatment of TB patients throughout the island [1].

Multi Drug Resistant Tuberculosis (MDR-TB) is defined as resistance to isoniazid and rifampicin, the two most effective drugs among the currently used anti TB drugs [3]. Rifampicin is the important drug especially in the short-course treatment regimen. Significantly, rifampicin resistant isolates are also resistant to isoniazid, making rifampicin resistance a useful marker of MDR-TB [4,5]. Early and accurate diagnosis of MDR TB is very important for prevention and control of the disease. Currently several rapid and automated liquid culture methods such as the BACTEC 460 radiometric system and the MGIT 960 system for diagnosis of MDR-TB [6,7] have been commercialized [3]. However, these methods are beyond the reach of laboratories in most developing countries including Sri Lanka, due to high cost and the need for complex infrastructure facilities [8-11].

In Sri Lanka, drug susceptibility testing (DST) is carried out using the conventional proportion method on Lowenstein-Jensen (L-J) medium which requires a minimum of 28 days. This significantly delays the detection of drug resistance and appropriate treatment. Establishing a rapid culture based method for identification of rifampicin resistance is essential for control and prevention of MDR-TB. Therefore the objective of this study was to evaluate the suitability of the manual Mycobacteria Growth Indicator Tube and the nitrate reductase assay for the rapid detection of rifampicin resistance in a low resource setting.

Methods

M. tuberculosis strains

Three hundred and seventy three (373) isolates of *M. tuberculosis* cultured from suspected TB patients during the period March 2008 to September 2010 were used for the study. The reference strain, *M. tuberculosis* H37Rv and a known rifampicin resistant isolate confirmed by the National Tuberculosis Institute, Bangalore were used as quality control strains.

Preparation of rifampicin solution

Rifampicin stock solution (10 mg/ml) was prepared by dissolving 10 mg of rifampicin powder (Sigma, USA) in 1 ml dimethyl sulphoxide (Sigma, USA). Filter sterilized aliquots of stock rifampicin solutions were kept at −70 °C until use. A working solution (1mg/ml) was prepared by diluting the stock solution with sterile double distilled water and used only once [12].

Isolation of *M. tuberculosis* from clinical specimens

Sputum specimens were processed using the modified Petroff's method and concentrated by centrifugation at 3500 g in a refrigerated (4°C) centrifuge for 15 minutes [13]. Sediment was diluted in 1 ml sterile distilled water. A small portion of the suspension was stained with Ziehl-Neelsen (ZN) stain and examined microscopically for the detection of acid fast bacilli [14]. Two slopes of L-J (Difco, US) (one containing paranitrobenzoic acid to detect *Mycobacterium* other than tuberculosis (MOTT) species) were inoculated with 100 μl of above bacterial suspension. The inoculated culture media were incubated at 37°C in 5% CO_2 until growth was observed or discarded as negative after 8 weeks. Culture isolates were confirmed as *M. tuberculosis* if they were slow growing, non-pigment producing, reduced nitrate and did not grow in the presence of paranitrobenzoic acid. Further, species confirmation was carried out by PCR amplification of heat killed bacterial DNA [15] using primers derived from *IS* 6110 insertion element of *Mycobacterium* genome, pt18 (5'-GAA CCG TGA GGG CAT CGA GG-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3') [16] (1st base -Singapore).

Drug susceptibility testing

Agar Proportion method

Agar proportion method (APM) remains the gold standard for culture based drug susceptibility testing for *M. tuberculosis*. The proportion method was carried out on Middlebrook 7H10 agar (Difco, US) plates as per CLSI guidelines [12]. Fourteen day old, fresh *M. tuberculosis* cultures grown on L-J medium were suspended in 7H9 broth medium to achieve a turbidity of McFarland No.1 standard. This suspension was further diluted (two fold and four fold) and used for inoculating rifampicin incorporated (1 μg/ml) agar plates and rifampicin free control agar plates. Colonies on each plate were counted on the 28th and 42nd day of inoculation, and the proportion of

organisms growing in the presence of rifampicin was calculated using the following equation [12].

$$\frac{\text{No. of colonies on drug containing medium}}{\text{No. of colonies on drug fee control medium}} \times 100$$

(Strains that showed ≥ 1% were considered as resistance to rifampicin)

Manual Mycobacterium Growth Indicator Tube (MGIT)

The MGIT (BD diagnostics, US) contains 4 ml of modified Middlebrook 7H9 broth with a fluorescence-quenching-based oxygen sensor embedded on the bottom of the tube. The level of fluorescence that the tube emits corresponds to the amount of oxygen consumed by organisms, which in turn is proportional to the number of bacteria present [17,18]. The rifampicin containing tubes (final rifampicin concentration of 1 μg/ml) were inoculated with 500 μl of a 1:5 dilution of a McFarland No: 0.5 bacterial suspension and rifampicin free control tubes were inoculated with 500 μl of a 1:500 dilution of McFarland No: 0.5 bacterial suspension as per manufacturer's guidelines. The emission of fluorescence was measured using the manual MGIT reader from day 2 onwards. If the drug free control tube gave positive reading and the drug containing tube did not show a positive reading up to 15 days of inoculation the test strain was read as sensitive to rifampicin. The test was repeated when the drug free control tube failed to give a reading in the positive range within 13 days of inoculation. The presence of *Mycobacterium* in the test and control tubes was confirmed microscopically by ZN stain.

Nitrate Reductase Assay

The nitrate reductase assay is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite by the nitrate reductase enzyme and in the present study, NRA in liquid medium was evaluated as a drug susceptibility test method [19]. Middlebrook 7H9 broth medium with 0.1% sodium nitrate was used as the medium. The final concentration of rifampicin in the medium was 1 μg/ml. Each drug containing medium was inoculated with 100 μl of McFarland No: 1 bacterial suspension prepared from 14 day old *M. tuberculosis* strains grown on L-J medium. The control medium was inoculated with 100 μl of a 1:10 dilution of the same bacterial suspension [20]. Two sets of the drug free and drug containing media were inoculated per isolate. After 6 days, the first set was examined for a colour change by sequentially adding of Griess reagent (10 μl of 50% HCI, 20 μl of 0.2% sulfanilamide and 20 μl of 0.1% N-napthtylethylene-diamine) to the culture medium. Readings were obtained visually by comparing with the prescribed colour standards (Figure 1) [14]. Readings between +5 to +3 of the standard colour series (Figure 1) were considered positive. The drug free control medium was tested after 6 days and if it was negative, the second set was tested at day 12. If the drug free control gave a colour change within the positive range of the standard color series and the drug containing medium did not yield a colour change even after 12 days of incubation the isolate was considered sensitive to rifampicin (Figure 2). In order to prevent false negative results (if the produced nitrite have further reduced to nitric oxide), a small amount of powdered zinc (Sigma, USA) was added to each negative tube [21] and tested for generated nitrite in the medium by sequentially adding of HCI, sulfanilamide and N-napthtylethylene-diamine as described above. Development of a dark pink colour indicated the absence of the *M. tuberculosis* in the test medium. Bacterial contamination may cause false positive results. Therefore, to ensure that the media were free of contaminants the inoculated NRA broths were streaked on blood agar plates to detect any non-specific bacterial growth before testing for colour changing.

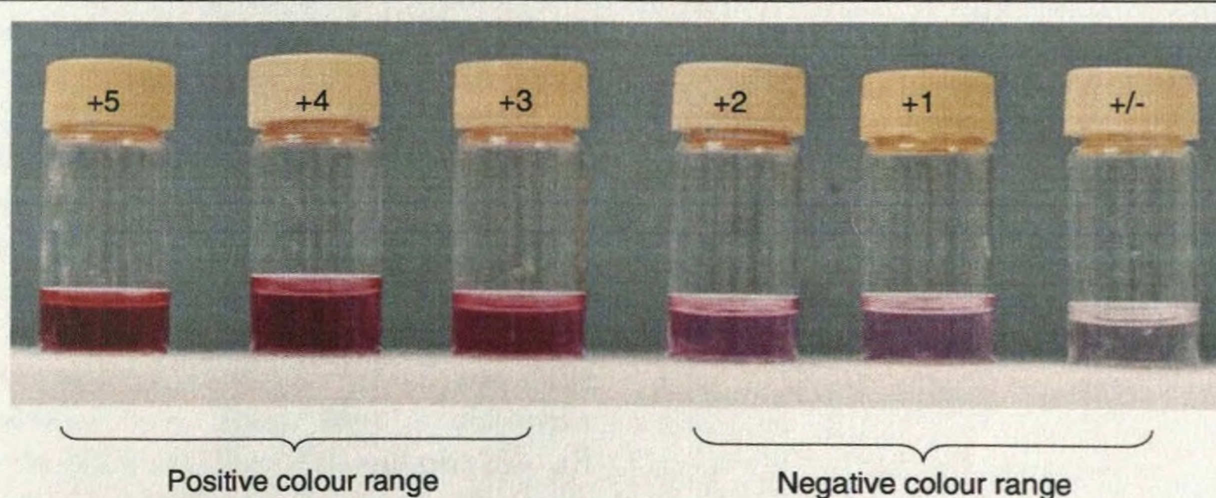

Figure 1 Standard colour series for nitrate reductase assay (WHO, laboratory services in tuberculosis control culture part iii). Colour range from +5 to +3 was considered as positive.

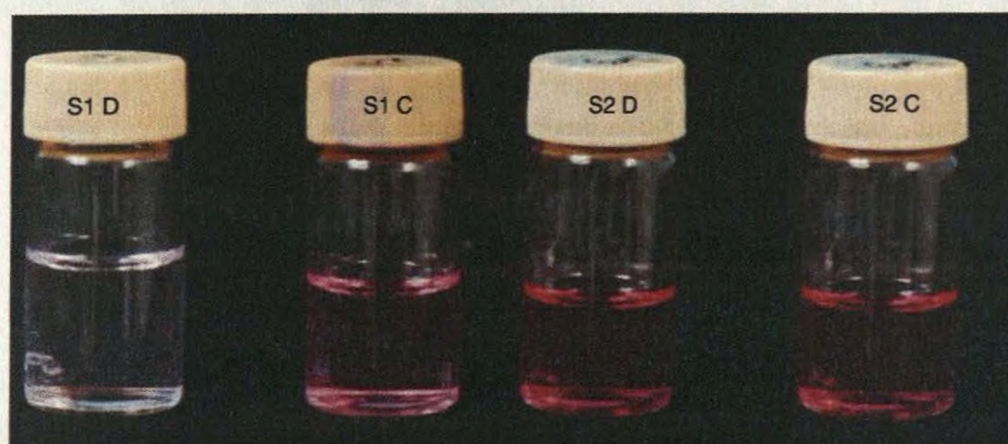

Figure 2 An example of a test result of an isolate tested using NRA. C-Rifampicin free control, D-Rifampicin containing medium, S1-Rifampicin susceptible strain of *M. tuberculosis*, S2- Rifampicin resistant strain of *M. tuberculosis*.

DNA sequencing

The *rpoB* gene mutations, of the rifampicin resistant isolates, detected by any one of the phenotypic methods, were identified by DNA sequencing (Macrogen –Korea).

Data analysis

The suitability of the manual MGIT and the NRA in comparison with the APM was evaluated in terms of sensitivity (the ability to detect true drug resistance), specificity (the ability to detect true drug susceptibility), positive likelihood ratio and negative likelihood ratio. A positive likelihood ratio above 10 or a negative likelihood ratio below 0.1 was considered to indicate excellent test-performance, and ratios above 5 and below 0.2 were considered to indicate adequate performance. The agreement between the NRA results or the manual MGIT results, and the APM were estimated by kappa statistics. The kappa value(k), a measure of test reliability, was interpreted as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥0.81, excellent [22]. The consumable costs per test were calculated in determining the cost for each test method.

Results

Thirty one rifampicin resistant isolates (resistant by one of the 3 methods) were identified among the 373 *M. tuberculosis* strains isolated during the study period March 2008 to September 2010. All 31 phenotypically resistant strains showed point mutations in the *rpoB* gene responsible for coding for rifampicin resistance (data not shown – see Additional file 1).

Twenty seven strains out of 31 were detected as rifampicin resistant by the APM, the currently used gold standard for conventional methods. MGIT and NRA identified 26 isolates each. There were 3 discordant results between the NRA and the APM and one discordant result between the MGIT and the APM. Three isolates that were detected as rifampicin resistant by NRA were susceptible by the manual MGIT and APM. Furthermore, one isolate that was rifampicin resistant by the manual MGIT was susceptible by APM and NRA (Table 1). Repeat testing of these 4 isolates provided the same results.

The sensitivity and specificity of the NRA in 7H9 broth medium when compared with the APM were 85% and 99% respectively (Table 2). Thus, there was a very good agreement between NRA and APM for detection of rifampicin resistance (κ= 0.86). An excellent agreement was also observed between the manual MGIT and APM (κ= 0.94) (Table 3) with 93% sensitivity and 100% specificity. The average turnaround time for MGIT and NRA in liquid medium was 08 days (mean) and 10 days (mean) respectively.

Discussion

Rapid and accurate detection of drug resistance is a prerequisite for initiating effective anti-TB treatment. In Sri Lanka, presently, DST for *M. tuberculosis* is carried out

Table 1 Pattern of Individual strains (n=31) showing resistance to rifampicin with the DST methods used in the study

Strain no (Lab No.)	DST method/s that confirmed rifampicin resistance
C4, C6, C7, C8, C9, C10, C20, C73, C83, C86, C88, C115, M60, M127, M15, C27, C22 C23,C163, C254 C150, C135, C110 (n=23)	APM, MGIT and NRA
M9, M33 (n=2)	APM and MGIT
PCR 88,PCR 57 (n=2)	Only APM
C120 (n=1)	Only MGIT
M46 , C25, M22 (n=3)	Only NRA

Table 2 Sensitivity, specificity, positive and negative likelihood ratio for NRA compared to APM (n=373)

APM	NRA		Sensitivity %	Specificity %	Likelihood ratio	
	No.of resistant isolates	No.of susceptible isolates			Positive	negative
Resistant (27)	23	4	85		98	0.15
Susceptible (346)	3	343		99		

only at the central reference laboratory, Welisara using solid based DST method. Liquid culture based or molecular based DST methods for detection of drug resistance are not yet available. Establishment of a more rapid DST method would positively impact the management of a patient harbouring a drug resistant strain. As rifampicin resistance is considered a surrogate maker of MDR TB, WHO recommends performing DST at least for rifampicin, especially in low resource settings [3].

In contrast to solid medium based DST methods, NRA and MGIT use an indicator to detect growth in the liquid medium, eliminating the need for visualization of growth as colonies. Therefore, NRA in liquid medium and MGIT methods are an attractive alternative to conventional methods. In this study, a good agreement was observed between APM and NRA in liquid medium or manual MGIT in the detection of rifampicin resistance. Similar results for detection of rifampicin resistance by manual MGIT and NRA have been reported previously [18,19,23,24].

The consumable cost per test for APM and NRA is around US$ 4.00 and US$ 3.00 respectively. Comparatively, the manual MGIT is more expensive (~US$ 7.00). However, both the NRA and the manual MGIT methods can be initiated with low technical expertise and initial cost. Additionally both methods are more rapid than the APM as the results of susceptibility testing will be available in less than 2 weeks.

In the evaluation of the manual MGIT for identification of rifampicin resistance, an in-house preparation of rifampicin solution was used instead of the commercially available rifampicin drug preparation kit (BD diagnostics, US). The appropriate volume of rifampicin solution was added to obtain a final concentration of 1 μg/ml of drug in the 4 ml broth medium. The excellent agreement between the MGIT and APM detecting rifampicin resistance confirms the suitability of using in-house preparation of drugs instead of commercially available drug kits that increases the cost of the test. The manual MGIT reader is a reliable and suitable instrument for use in low resource countries as no housing is required and the results can be read by placing the tube in the reading slot (See additional file 2). The time spent to take a reading is about 30 seconds. The cost of a MGIT reader is around US$ 3000 and it is a once only investment. Alternatively, in the absence of a manual MGIT reader a simple ultra violet (UV) lamp (365 nm) may be used to detect growth [25].

In the NRA, a standard colour series [14] was used to interpret test results. In the case of intermediate results, the test should be repeated for accurate interpretation. In our series, 2 of the 373 tests required repeat testing. The intermediate results in NRA may occur due to low inoculum in the medium. Contamination of the test medium can also lead to erroneous results as several other bacteria can reduce nitrate to nitrite. Therefore, it is important to ensure that there is no bacterial contamination, prior to reporting results of the NRA. Performing a purity test by sub culturing a blood agar plate with a loopful of test medium will prevent reporting of false positive test results due to contaminating bacterial flora.

Conclusion

In conclusion, both the NRA in liquid medium and the manual MGIT agreed well with the APM in determination of rifampicin resistance. Introduction of these methods for low resource settings will make the determination of rifampicin resistance faster and cost effective. As the need for sophisticated instruments and high technical skills is minimal, the initial test establishment cost will be low. Therefore, the NRA in liquid medium and the manual MGIT are suitable alternatives to APM that can be used to determine rifampicin resistance especially in low resource settings.

Table 3 Sensitivity, specificity, positive and negative likelihood ratio for the MGIT method compared to APM (n=373)

APM	MGIT		Sensitivity %	Specificity %	Likelihood ratio	
	No.of resistant isolates	No.of susceptible isolates			Positive	negative
Resistant (27)	25	2	93		167	0.04
Susceptible (346)	1	345		100		

Ethics approval

The Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka approved the study (ERC Number is EC/06/062). The samples collected for the purpose of this research were from patients attending the chest clinic for diagnosis and treatment of tuberculosis and their informed consent was obtained prior to sample collection.

Additional files

Additional file 1: Phenotypic method/s detecting rifampicin resistance and the distribution of *rpoB* gene mutations of 31 isolates.

Additional file 2: Manual MGIT reader.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CPA, AJP and WSSW designed the study; CPA carried out the laboratory work and analyzed the data; CPA, AJP and WSSW interpreted the data. CPA drafted the manuscript. AJP and WSSW supervised the work of CPA and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank E.M. Corea, Gihani Perera, Sirithilak Gamage, S.M.P. Senevirathne, Shirani Hendalage, and the laboratory assistant staff (Department of Microbiology, Faculty of medicine, University of Colombo) for providing technical and secretarial assistance.

Funding

The study was supported by the National Science Foundation of Sri Lanka (Grant No: RG/ 2007/ BT/03).

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Received: 3 February 2012 Accepted: 25 November 2012
Published: 27 November 2012

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doi:10.1186/1471-2334-12-326
Cite this article as: Adikaram et al.: The manual mycobacteria growth indicator tube and the nitrate reductase assay for the rapid detection of rifampicin resistance of M. Tuberculosis in low resource settings. *BMC Infectious Diseases* 2012 12:326.

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