LIST OF TABLES

Table No.	TITLES OF THE TABLE	Page No
2.1	**Classification of drug susceptibility testing methods**	**37**
2.2	**Comparison of Conventional Culture Methods**	**39**
2.3	**Hot spot mutations reported in *rpoB* gene for RIF resistance**	**51**
2.4	**Possible hotspot mutations occurring in *tlyA gene* from literature**	**68**
3.1	**cDNA conversion Protocol**	**78**
3.2	**Details of Primer sequence, thermal profile and Amplicon size used to standardize nRT-PCRs.**	**79**
3.3	**Clinical Specimen wise Distribution of *M. tuberculosis* isolates**	**81**
3.4	**29 MDR-TB strains included included in this study in this study**	**83**
3.5	**Second line anti-tuberculous drugs and the final concentration used i this study.**	**83**
3.6	**Clinical Specimen wise Distribution of *M. tuberculosis* isolates**	**85**
3.7	**PCR cocktail preparation**	**86**
3.8	**Primer sets used in the study to sequence the target genes with their thermal profile and expected amplicon sizes**	**87**
3.9	**Thermal Profile for EXO-SAP Treatment**	**89**
3.10	**Cycle sequencing protocol**	**90**
3.11	**Clinical specimen wise distribution of *M. tuberculosis* isolates include in this study.**	**93**
3.12	**Primer sets used in the study to sequence the target genes with their thermal profile and expected amplicon sizes**	**93**
4.1	**Results of nRT-PCRs targeting *icl_2, hspx and rRNAP1* genes on sputum samples**	**106**
4.2	**Evaluation of nRT-PCRs targeting *icl_2, hspx and rRNAP1* genes results against culture positive *M. tuberculosis* isolates by BACTEC method in 203 Sputum specimens.**	**106**
4.3	**Comparative results of 48 culture positive *M. tuberculosis* isolates against nRT-PCRs (*icl_2, hspx and rRNAP1* genes) negative clinical specimens**	**107**
5.1	**First line phenotypic drug susceptibility testing results of 326 *M. tuberculosis* by BACTEC micro MGIT method**	**113**
5.2	**clinical specimenwise distribution of 18 MDR isolates**	**114**
5.3	**Direct smear, age, sex and first line phenotypic DST results of 18 MDR-TB Patient**	**115**
5.4	**Clinical Specimenwise Distribution of 37 Polyresistant *M. tuberculosis* isolates**	**116**
5.5	**Clinical Specimenwise Distribution of 53 monoresistant *M.***	**117**

	***tuberculosis* isolates**	
6.1	**Novel mutations in Rifampicin Sensitive and Resistant *M. tuberculosis* isolates**	**127**
6.2	**Novel mutations in Isoniazid Sensitive *M. tuberculosis* isolates**	**128**
6.3	**Novel silent mutations in Isoniazid Sensitive and resistant *M. tuberculosis* isolates**	**131**
6.4	**Novel mutations in Isoniazid Sensitive and resistant *M. tuberculosis* isolates**	**131**
6.5	**Reported mutations in Ethambutol Sensitive and resistant *M. tuberculosis* isolates**	**133**
6.6	**Novel mutations in Pyrazinamide Sensitive and resistant *M. tuberculosis* isolates**	**135**
6.7	**Novel and reported mutations in Streptomycin Sensitive and resistant *M. tuberculosis* isolates**	**138**
7.1	**Energy minimization and Protein structure Quality assessment of WT and MT of Pzase.**	**152**
7.2	**Docking Studies of Pzase with PZA**	**153**
8.1	**Details of age, sex, direct smear, first line and second line drug DST results for XDR-TB strains from this study**	**161**
9.1	**Reported mutations in FQs sensitive and resistant *M. tuberculosis* isolates in Polyresistant and MDR-TB strains**	**177**
9.2	**Novel and reported mutations targeting *gyr B* region in Polyresistant and MDR-TB strains**	**180**
9.3	**Novel mutations targeting *tly A* gene in polyresistant and MDR-TB strains**	**187**
9.4	**Novel Mutations Targeting e*is Gene* in Polyresistant And MDR-TB Strains**	**189**
9.5	**Novel mutations targeting *ethA1 gene* in polyresistant strains**	**194**
9.6	**Novel mutations targeting *ethR gene* in polyresistant strains**	**197**
9.7	**Novel mutations targeting *thyA gene* in polyresistant strains**	**200**

LIST OF FIGURES

Figure No	Titles of Figures	Page No.
1.1	Cell wall of *Mycobacterium tuberculosis*	05
1.2	Acid Fast Staining (1000x)	08
1.3	Mode of transmission for *Mycobacterium tuberculosis*	09
1.4	Process of Primary tuberculosis Infection	12
1.5	Global Estimated Tuberculosis incidence rates in 2013	13
1.6	Global incidence of tuberculosis in 2013	14
1.7	Progress in global coverage of data on drug resistance from 1994 to 2013	16
2.1	Glyoxylate shunt pathway involving icl_2 gene of *M. tuberculosis*	34
2.2	*rRNAP1* gene structure of *M. tuberculosis*	35
2.3	micro MGIT culture system	41
2.4	Microscopically Observed Drug Susceptibility testing	44
2.5	Nitrate Reductase Assay	45
2.6	Colorimetric Indicator Assay	46
2.7	Phage Based Assay	47
2.8	Mode of action of Isoniazid	48
2.9	Mode of action of Rifampicin	49
2.10	Mode of action of Ethambutol	52
2.11	Mode of action of Pyrazinamide	54
2.12	Mode of action of Streptomycin	56
2.13	Ribbon representation of the structure of the *M. tuberculosis* PncA protein	63
2.14	Mechanism of action of Fluoroquinolones against *M. tuberculosis*	65
2.15	*gyrA* and *gyrB* gene structure	66
2.16	Mode of Drug Action Of Aminoglycosides	68
2.17	Mode of Drug Action Of Ethionamide	73
4.1	Agarose gel electrophoretogram showing the analytical sensitivity of indigenously designed primers targeting icl_2 gene	102
4.2	Agarose gel electrophoretogram showing the analytical sensitivity ofi ndigenously designed primers targeting *hspx* gene	102
4.3	Agarose gel electrophoretogram showing the analytical	103

Figure No	Titles of Figures	Page No.
	sensitivity of indigenously designed primers targeting *rRNAP1* gene	
4.4	**Agarose gel electrophoretogram showing the specificity of indigenously designed primers targeting *icl*$_2$ gene**	**103**
4.5	**Agarose gel electrophoretogram showing the specificity of indigenously designed primers targeting *hspx* gene**	**104**
4.6	**Agarose gel electrophoretogram showing the specificity of indigenously designed primers targeting *rRNAP1* gene**	**104**
4.7	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *icl*$_2$ gene**	**105**
4.8	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *hspx* gene**	**105**
4.9	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rRNAP1* gene**	**105**
5.1	**Pie Chart showing the distribution of resistance pattern in 18 MDR-TB strains.**	**114**
5.2	**Pie chart showing the distribution of 37 polyresistant strains in this study**	**117**
5.3	**Pie chart showing the distribution of 53 Monoresistant strains in this study**	**118**
6.1	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rpo B* gene**	**123**
6.2	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *katG I* gene**	**124**
6.3	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *katG II* gene**	**124**
6.4	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *katG III* gene**	**124**
6.5	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *katG IV* gene**	**125**
6.6	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *inhA* gene**	**125**
6.7	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *oxyR-aphC* gene**	**125**
6.8	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *emb B* gene**	**126**
6.9	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *pnc A* gene**	**126**
6.10	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rrs* gene**	**126**

Figure No	Titles of Figures	Page No.
6.11	**Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rpsl* gene**	**127**
6.12	**Agarose gel electrophotogram showing analytical sensitivity of primers targeting *gid B* gene**	**127**
6.13	**Agarose gel electrophotogram showing specificity of the primers targeting *gid B* gene**	**127**
6.14	**Agarose gel electrophoretogram showing the amplification *M. tuberculosis* isolates targeting *gidB* gene**	**128**
6.15	**Multalin results of PCR based DNA sequencing targeting *rpo B* gene**	**129**
6.16	**Multalin Results of DNA Sequence Analysis Targeting *inhA* gene for isoniazid resistance**	**131**
6.17	**Multalin Results (LTITM 623) of DNA Sequence Analysis targeting *oxyR-aphC* gene**	**132**
6.18	**Multalin Results (LTITM 714) of DNA Sequence Analysis targeting *oxyR-aphC* gene**	**133**
6.19	**Multalin Results of DNA Sequence Analysis Targeting *emb B* gene for Ethambutol resistance**	**135**
6.20	**Multalin Results of (LTITM 543) DNA Sequence Analysis Targeting *pncA* gene for Pyrazinamide resistance**	**137**
6.21	**Multalin Results of (LTITM 623) DNA Sequence Analysis Targeting *pncA* gene for Pyrazinamide resistance**	**138**
6.22	**Multalin Results (LTITIM 296) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance**	**141**
6.23	**Multalin Results (LTITIM 293) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance**	**142**
6.24	**Multalin Results (LTITIM 670) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance**	**143**
6.25	**Multalin Results (LTITIM 979) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance**	**144**
6.26	**Multalin Results (LTITIM 982) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance**	**145**
7.1	**secondary structure prediction of Wildtype of PZA using pdbsum sever**	**151**
7.2	**Secondary structure prediction of Mutant type (Ala102 Pro) of PZA using pdbsum sever.**	**152**
7.3	**Super imposition of WT and MT PZase 3D structures WT is colored in Blue and Mutant in Red. The residue at position 102 is shown stick form. enlarged view at 102nd position**	**153**

Figure No	Titles of Figures	Page No.
	(WT is colored in orange and MT in cyan)	
7.4	**Q-Mean score of WT of PZase**	**154**
7.5	**Q-Mean score of MT of PZase**	**154**
7.6	**CASTp Pocket prediction: Heme is indicated as brown sphere shape and Ala 102 Pro mutant position in Red and other pocket residues are indicated as lines with labels.**	**155**
7.7	**Docking interaction of WT PZase with PZA. PZA represented in Green colour.**	**156**
7.8	**Docking interaction of MT PZase with PZA. PZA represented in Green colour.**	**156**
7.9	**Represent Root Mean Square Fluctuation of residues in WT type of PZase**	**157**
7.10	**Represent Root Mean Square Fluctuation of residues in MT type of PZase**	**157**
9.1	**Agarose gel electrophotogram showing analytical sensitivity of the primers targeting *gyr A* gene**	**167**
9.2	**Agarose Gel Electrophotogram Showing Analytical Sensitivity Of Primers Targeting *gyr B* Gene**	**167**
9.3	**Agarose gel electrophotogram showing analytical sensitivity of primers targeting *tly A* gene**	**168**
9.4	**Agarose gel electrophotogram showing analytical sensitivity of primers targeting *eis* gene**	**168**
9.5	**Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *thyA* gene**	**168**
9.6	**Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *ethA1* gene**	**168**
9.7	**Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *ethA2* gene**	**168**
9.8	**Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *eth3* gene**	**169**
9.9	**Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *ethR* gene**	**169**
9.10	**Agarose gel electrophoretogram showing the specificity of primers targeting *gyrA* gene**	**170**
9.11	**Agarose gel electrophoretogram showing the specificity of primers targeting *gyrB* gene**	**170**
9.12	**Agarose gel electrophoretogram showing the specificity of primers targeting *tlyA* gene**	**171**
9.13	**Agarose gel electrophoretogram showing the specificity of primers targeting *thyA* gene**	**171**

Figure No	Titles of Figures	Page No.
9.14	Agarose gel electrophoretogram showing the specificity of primers targeting *ethA1* gene	171
9.15	Agarose gel electrophoretogram showing the specificity of primers targeting *ethA2* gene	172
9.16	Agarose gel electrophoretogram showing the specificity of primers targeting *eth3* gene	172
9.17	Agarose gel electrophoretogram showing the specificity of primers targeting *ethR* gene	172
9.18	Agarose gel electrophoretogram showing the specificity of primers targeting *eis* gene	173
9.19	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *gyr A* gene	173
9.20	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *gyr B* gene	173
9..21	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *tlyA* gene	173
9.22	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *eis* gene	174
9.23	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethA1* gene	175
9.24	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethA2* gene	175
9.25	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethA3* gene	175
9.26	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethR* gene	175
9.27	Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *thyA* gene	176
9.28	Multalin Results of DNA Sequence analysis targeting *gyr A* Gene	176
9.29	Multalin results of LTITM 87- resistant strain targeting *gyr B* gene	178
9.30	Multalin results of LTITM 232- resistant strain targeting *gyr B* gene	181
9.31	Multalin results of LTITM 480- resistant strain targeting *gyr B* gene	183
9.32	Multalin Results Of Dna Sequence Analysis Of MDR-TB Isolate (LTITM 17- Resistant) Targeting *tly A* Gene	184
9.33	Multalin results of DNA sequence analysis of MDR-TB isolate	185

Figure No	Titles of Figures	Page No.
	(LTITM 232- resistant) targeting *tly A* gene	
9.34	**Multalin results for LTITM 728 (MDR-TB strain)**	**186**
9.35	**Multalin results for LTITM 232 (MDR-TB strain)**	**188**
9.36	**Multalin results for LTITM 17 (MDR-TB strain)**	**190**
9.37	**Multalin results for LTITM 103 (MDR-TB strain)**	**191**
9.38	**Multalin results for LTITM 283 (Polyresistant strain)**	**192**
9.39	**Multalin results for LTITM 978 (Polyresistant strain)**	**193**
9.40	**Multalin results for LTITM 623, 238, 447 (Polyresistant strain)**	**195**
9.41	**Multalin results for LTITM 238, 447 (Polyresistant strain)**	**196**
9.42	**Multalin results for LTITM 9 (Polyresistant strain)**	**198**
9.43	**Multalin results for LTITM 238 (Polyresistant strain)**	**204**
9.44	**Multalin results for LTITM 565 (Polyresistant strain)**	**206**

LIST OF ABBREVATIONS / SYMBOLS

A	**Adenine**
AFB	**Acid-fast bacilli**
AG	**arabinogalactan**
AIDS	**Acquired Immunodeficiency Syndrome**
ATCC	**American type culture collection**
ATT	**Anti-tuberculous treatment**
BAL	**Bronchoalveolar Lavage**
C	**Cytosine**
CCD	**charge-coupled device**
CAP	**Capreomycin**
COD	**Cause of Death**
CSR	**Central serous chorioretinopathy**
CSF	**Cerebrospinal Fluid**
CCD	**Charge-Coupled Device**
CTB	**Childhood Tuberculosis**
CRI	**Colorimetric Redox Indicator**
CRT	**Cyclic reversible termination**
cDNA	**Complementary Deoxyribonucleic Acid**
CFP	**Culture Filter Protein**
CSF	**Cerebrospinal fluid**
CFU	**Colony forming units**
°C	**Degree Celsius**
Δ	**Delta**
DAP	**L-alanyl-D-*iso*-glutamnyl-*meso*-diaminopimelic acid**
DNA	**Deoxyribonucleic acid**
DEPC	**Diethyl Pyrocarmonate**
DOTS	**Directly Observed Treatment Short Course**
dsDNA	**Double stranded Deoxyribonucleic acid**
DRS	**Drug Resistance Surveillance**
DST	**Drug Susceptibility Testing**
ESAT	**Early Secretory Antigen**
ELISA	**Enzyme Linked Immunosorbent Assay**
EMB	**Ethambutol**
ERDR	**Ethambutol Resistance Determining Region**

ETH	**Ethionamide**
EDTA	**Ethylene Diamine Tetraacid**
EXO	**Exo nuclease**
EPTB	**Extra Pulmonary Tuberculosis**
XDR-TB	**Extremely Drug Resistant Tuberculosis**
F	**Female**
fg	**Femtogram**
FNAB	**Fine Needle Aspiration biopsy**
FRET	**Fluorescence Resonance Energy Transfer**
FQ	**Fluoroquinolone**
GCI-SH	**Government Chest Institue and Chest Clinic of Government Stanley Hospital**
G	**Guanine**
GC	**Growth Control**
GI	**Growth Index**
HIV	**Human Immunodefieciency Virus**
ICL	**Isocitrate lyase**
ICMR	**Indian Council of Mediacle Research**
IFN	**Interferon**
IU	**International Units**
INH	**Isoniazid**
KKCTH	**Kanchi Kamakoti CHILDS Trust Hospital**
LAM	**Lipoarabinomannan**
LTBI	**Latent Tubercle Bacilli Infection**
LRP	**Luciferase reporter phages**
LVX	**Levofloxacin**
M	**Male**
µg	**Micro gram**
µl	**Micro litre**
µM	**Micro mole**
MODS	**Microscopically Observed Direct Susceptibility Testing**
ml	**Milli litre**
mM	**Milli mole**
MIC	**Minimum Inhibitory Concentration**
MDR-TB	**Multidrug Resistant Tuberculosis**
MGIT	**Mycobacteria Growth Indicator Tube**

MAC	***Mycobacterium avium intracellularae* complex**
MA	**mycolic acid**
MT	**Mutant type**
MW	**Molecular weight**
NAM	**N-acetylmuramic acid**
NAP	**ρ-nitro-α-acetylamino-β-hydroxy-propiophenone**
NALC	**N-Acetyl L-Cysteine**
NCBI	**National centre for Biotechnology Information**
ng	**Nano gram**
nt	**Nucleotide**
NTI	**National Tuberculosis Institute**
NTP	**National Tuberculosis Programme**
nPCR	**Nested Polymerase Chain Reaction**
NTB	**Neuro Tuberculosis**
NRA	**Nitrate Reductase Assay**
OFX	**Ofloxacin**
OADC	**Oleic acid Albumin Dextrose Catalase**
OD	**Optical Density**
PAS	**Para aminosalicylic acid**
PDB	**Protein data base**
POA	**Pyrazinoic acid**
PNB	**ParaNitro Benzoic Acid**
PBS	**Phosphate Buffer Saline**
pg	**Picogram**
PG	**Peptidoglycan**
PCR	**Polymerase Chain Reaction**
PANTA	**Polymyxin, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin antibiotic mixture**
PTB	**Pulmonary Tuberculosis**
PGM	**Personal Genome machine**
PPD	**Purified Protein Derivative**
PGAAP	**Prokaryotic Genomes Automatic Annotation Pipeline**
PZA	**Pyrazinamide**
QFT-IT	**Quantiferon TB Gold In tube Test**
QRDR	**Quinolones Resistance Determining Region**
RT-PCR	**ReverseTranscriptase Polymerase Chain Reaction**

RR	**Ready reaction mix**
RNTCP	**Revised National Tuberculosis Control Programme**
RNA	**Ribonucleic Acid**
rRNA	**Ribosomal Ribonucleic Acid**
RIF	**Rifampicin**
RRDR	**Rifampicin Resistance Determining Region**
SCC	**Short Course Chemotherapy**
STN RT-PCR	**Single tube Nested ReverseTranscriptase Polymerase Chain Reaction**
SEA	**South East Asian Region**
STR	**Streptomycin**
SLD	**Second line drug**
SIRE	**Strptomycin Isoniazid Rifampicin Ethambutol**
SVM	**Support vector machine**
SBS	**Sequencing by synthesis**
T	**Tyrosine**
TCA	**Tricarboxylic acid**
TST	**Tuberculin Skin Test**
TB	**Tuberculosis**
TRC	**Tuberculosis Research Centre**
TDR-TB	**Total drug resistant tuberculosis**
UV	**Ultraviolet**
XDR-TB	**Extremely drug resistant tuberculosis**
WHO	**World Health Organization**
WGS	**Whole genome sequencing**
WT	**Wild type**
Zn	**Zinc**

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION:

Tuberculosis (TB), one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. **Tuberculosis (TB)** is the leading cause of death in the world from a bacterial infectious disease caused by *Mycobacterium tuberculosis* [Collee et al, 2002]. The disease primarily affects lungs and causes Pulmonary TB (PTB). It can also affect intestine, meninges, bones and joints, lymph glands, skin and other tissues of the body. TB has co-evolved with humans for many thousands of years, and perhaps for several million years [Ananthanarayanan et al., 2000]. The oldest known human remains showing signs of tuberculosis infection are 9,000 years old [Ryan et al, 2004]. The disease affects 1.8 billion people per year, which is equal to one-third of the entire world population [Ananthanarayanan et al, 2000]. One–third of the world's current population has been infected with *M. tuberculosis*. New infections occur at a rate of one per second [Ryan et al, 2004]. The vast majority of TB deaths are in the developing world. Left untreated, each person with active TB disease will infect on average between 10 and 15 people every year and this continues the TB transmission. The proportion of people in the general population who become sick with tuberculosis each year is stable or falling worldwide but, because of population growth, the absolute number of new cases is still increasing.

1.1.1. HISTORY OF *MYCOBACTERIUM TUBERCULOSIS:*

Phthisis is a Greek term for tuberculosis, around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times involving coughing up blood and fever, which was almost always fatal [Al-Sharrah et al, 2003]. It is transmitted from person to person via droplets from the throat and Lungs of people with the active respiratory TB disease.

M. tuberculosis was considered to be the cause of the "White Plague" of the 17th and 18th centuries [Al-Sharrah et al 2003]. *M. tuberculosis* has been present in the human population since antiquity - fragments of the spinal column from Egyptian mummies from 2400 BCE showed definite signs of tuberculosis. In 1679, Sylvius was the first to identify actual tubercles as a consistent and characteristic change in the lungs and other areas of

consumptive patients. In 1720, the English physician Benjamin Marten was the first to conjecture, in his publication, stating that tuberculosis could be caused by "wonderfully minute living creatures," which, once they had gained a foothold in the body, could generate the lesions and symptoms of the disease. The introduction of the sanatorium cure provided the first real step against tuberculosis. Hermann Brehmer, a Silesian botany student suffering from TB, was instructed by his doctor to seek out a healthier climate. He traveled to the Himalayan Mountains where he could pursue his botanical studies while trying to rid himself of the disease. He returned home cured and began to study medicine. In 1854, he presented his doctoral dissertation bearing the auspicious title, “Tuberculosis is a Curable Disease”. New advances then followed in rapid succession. In 1882, Robert Koch discovered a staining technique that enabled him to see *M. tuberculosis*.

The history of tuberculosis was changed dramatically on March 24, 1882, when Hermann Heinrich Robert Koch made his justly famous presentation to the Berlin Physiological Society. In his presentation, Koch not only presented demonstrations of the tubercle bacillus he had identified but posted his famous postulates, called the The Koch discovery was confirmed by more efficient staining models of Ehlrlich (1887) and Ziehl-Neelson (1883). Koch-Henle postulates Koch’s contributions to bacteriology were legion, and he was awarded the Noble Prize in Medicine and Physiology in 1905 for his elucidation of the etiology of tuberculosis.

The *M. tuberculosis* genome is the first major pathogen to be sequenced with 4,411,522 bp 3,924 open reading frames, and with GC content of 65.6% [Fleischmann et al., 2002]. They are complex unicellular microorganisms with a resilient cell wall structure and can suppress the host immune response by the immunomodulatory action mediated by the cell wall components.

New vaccines and drugs are needed to stem the worldwide epidemic of TB that kills two million people each year. To rationally develop new anti tubercular agents, it is essential to study *M. tuberculosis*-host interaction to learn how these bacteria circumvent host defenses and cause disease [Fleischmannet al. 2002]. In the future, some of these genes and the proteins they encode will provide new bacterial targets that can be used for creating vaccines and drugs as well as more selective diagnostic tool for the detection of *M. tuberculosis.*

1.1.2 TAXONOMY

Kingdom:	Bacteria
Phylum:	Actinobacteria
Order:	Actinomycetales
Suborder:	Corynebacterineae
Family:	Mycobacteriaceae
Genus:	*Mycobacterium*
Species:	***tuberculosis***

1.1.3. MORPHOLOGY

Mycobacteria are acid fast, slender rods, measuring around 0.2-0.6μm, weakly gram positive, non-motile, non-sporulating, and non-capsulating, and obligate aerobes [Fleischmann et al, 2002]. Under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from coccobacilli to long rods. The dimensions of the bacilli have been reported to be 1-10 μm in length (usually 3-5 μm), and 0.2-0.6 μm width.

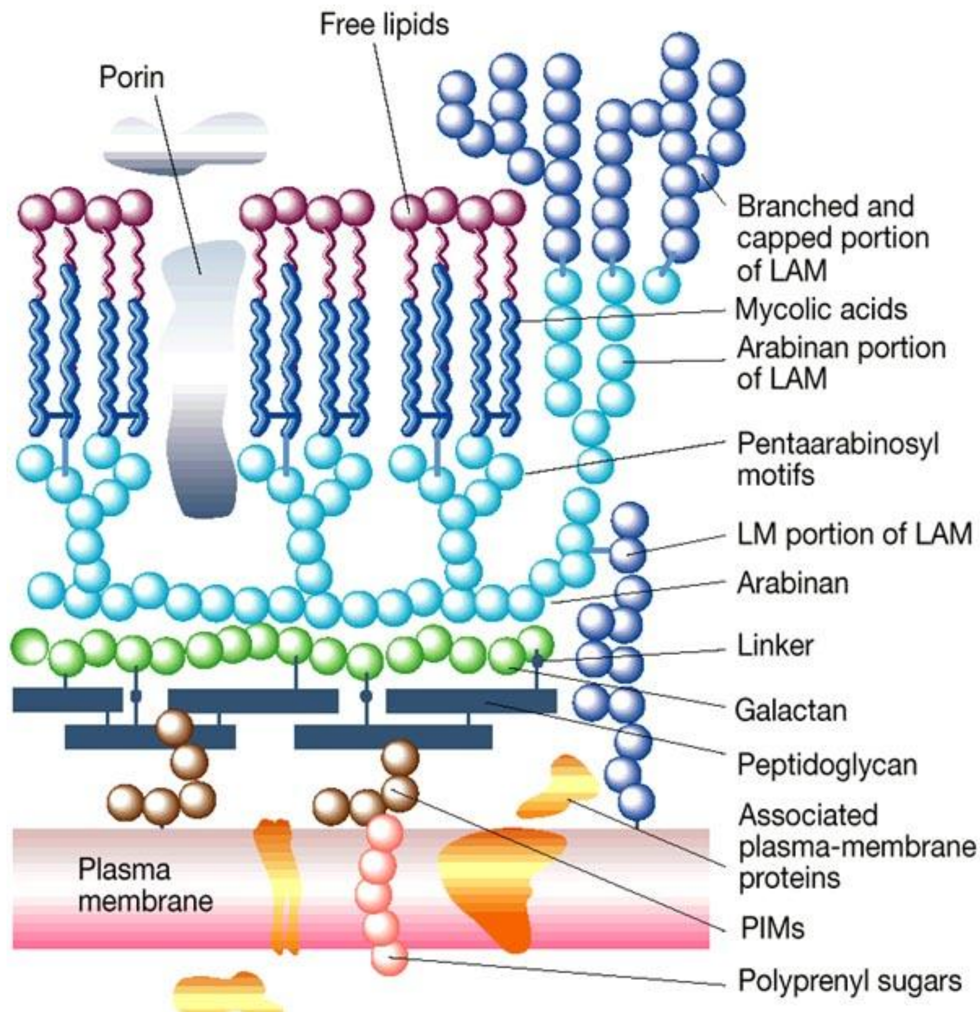
1.1.4 CELL WALL STRUCTURE

The cell wall of Mycobacterium is characterized by a unique structure which is caused by partly distinct chemical composition in comparison with the cell wall of other bacteria. These variations are thought to be advantageous in stressful conditions of osmotic shock or desiccation as well as contributing to their considerable resistance to many drugs. The Mycobacterial cell wall, in principle, consists of an inner layer and an outer layer that surround the plasma membrane. The outer compartment consists of both lipids and proteins [Trias et al, 1992]. The inner compartment consists of peptidoglycan (PG), arabinogalactan (AG) and mycolic acid (MA) covalently linked together to form a complex known as MA-

AG-PG complex that extends from plasma membrane outwards in layers, ataring with PG and ending with MAs.

The peptidoglycon is made of peptides and glycan strands. The long glycan strand typically consists of repeating N-acetylglucosamines (NAGs) linked to N-acetylmuramic acid (NAM). These strands are cross linked by peptides bound to the lactyl group on NAMs from different glycan strands [Figure 1.1]. These peptides chains normally consists of L-alanyl-D-*iso*-glutamyl-*meso*-diaminopimelic acid (DAP) from one strand linked to the terminal T-alanine residue from L-alanyl-D-*iso*-glutamyl-*meso*- DAP-D-alanine from a different strand [Trias et al, 1992]. This highly cross-linked glycan meshwork of PG that surrounds bacteria is the primary agent that maintains bacterial shape. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and unusual high copy number of cross-links. Indeed, the degree of peptidoglycon cross linking in the cell wall of *M. tuberculosis* is 70-80%, whereas that in *E.coli* is 20-30% contains lipids in high quantities [Trias et al., 1992]. The lipid content includes waxes in the form of mycolic acids having long, branched, carbon chains of 80 atoms or more which makes the cells impervious to Gram staining; acid-fast techniques are used instead.

Figure 1.1: Cell wall of *Mycobacterium tuberculosis*



LAM- Lipoarabino mannan, LM- Lipomannan, PIMs- Phosphatidylmyo-inositol Mannosides

(Source: http://www.nature.com/nature/journal/v406/n6797/full/406788a0.html)

The illustrated diagram shows the different components of cell wall of *M. tuberculosis*.

The peptidoglycan found in *M. tuberculosis* is generally similar to that found in other bacteria and consists of linear polysaccharide chains. Peptidoglycan is linked to the next polymer in the cell envelope, arabinogalactan, by a unique diglycosylphosphoryl bridge, containing rhamnose and *N*-acetyl glucosamine. Arabinogalactan is made up of a galactose backbone with arabinose branches. Mycobacteria are the only known pathogens that contain

both galactofuranose and arabinofuranose in an essential structural component of the cell envelope [Trias et al., 1992].

Mycolic acids, the single largest component of the mycobacterial cell envelope, are 3-hydroxy, 2-alkyl-branched fatty acids that contain 60 to 90 carbon atoms.they are covalently attached by ester linkages to a hexa-arabinose motif found at the terminus of the branched arabinogalactan [Trias et al., 1994]. The glycolipid lipoarabinomannan (LAM) is the final part of the complex structure is built around a core of mannose residues, to which are attached multiple, branched, mannose-capped arabinofuranosyl side chains and a phosphatidyl-inositol unit, which may be used as a linkage to the other elements of the cell envelope [Figure 1.1]. The mycobacterial cell envelope is extremely hydrophobic and forms an exceptionally strong permeability barrier rendering mycobacteria naturally resistant to a wide variety of antimicrobial agents. Primarily a pathogen of the mammalian respiratory system, infects the lungs, causing tuberculosis.

1.1.5 CLASSIFICATION OF MYCOBACTERIA

Mycobacteria genus includes obligate parasite, opportunistic pathogens and saprophytes. Koch in 1882 isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis by satisfying Koch's postulates. Tuberculosis in humans was subsequently shown to be caused by two types of the bacillus-the human and bovine types, designated *Mycobacterium tuberculosis and Mycobacterium bovis,* respectively [Ananthanarayanan et al 2000]. The term *Mycobacterium tuberculosis complex* includes, besides the human and bovine types two others mammalian types also: *Mycobacterium africanum causing* human *tuberculosis* in tropical Africa and possessing properties intermediate between human and bovine types; and *Mycobacterium microti* (vole bacillus pathogenic for voles and other small mammals but not for humans).

The second human pathogenic Mycobacterium is the lepra bacillus causing leprosy. Though this was the *Mycobacterium* first described, it is the least understood because it has not been possible to convincingly culture it in vitro so far [Ananthanarayanan et al., 2000].

The third group of *Mycobacterium* is the mixed group of isolates from diverse sources: from birds, cold blooded and warm blooded animals, from skin ulcers, and from soil, water, and

other environmental sources. They are called as atypical, anonymous, non-tuberculous, tuberculoid, opportunist and Mycobacteria other than tuberculosis (MOTT) [Tripathy et al., 1970].

Non Tuberculous Mycobacteria have been classified into four groups by Runyon (1959) based on pigment production and rate of growth:

- Group 1 – Photochromogens (*M.kansasii, M.marinum, M.simiae*)
- Group 2 – Scotochromogens (*M.scrofulaceum, M.szulgai, M.gordonae)*
- Group 3 – Nonphoto chromogens (*M.malmoensii, M.avium- intracellularae M. avim,M.intracellulare, M.xenopi, M.ulcerans)*
- Group 4- Rapid growers (*M.fortuitum, M.chelonae, M.abscessus*).

Group 1 Photochromogens: These strains form colonies that produce no pigment in the dark but when the young culture is exposed to light for 2 hour and reincubated at 37°C for 24-48 hours, a yellow orange pigment appears. They are slow growing, though growth is faster than that of tubercle bacilli [Rodrigues et al., 2007].

Group 2 Scotochromogens: These strains form pigmented colonies (yellow-orange-red) even in the dark. They are widely distributed in the environment and sometimes contaminate cultures of tubercle bacilli [Rodrigues et al., 2007].

Group 3 Nonchromogens: These strains do not form pigment even on exposure to light. Colonies may resemble those of tubercle bacilli.

Group 4 Rapid growers: This is a heterogenous group of Mycobacteria capable of rapid growth, colonies appearing within 7 days of incubation at 37°C. Within the group, photochromogenic, scotochromogenic and nonchromogenic species occur [Rodrigues et al., 2007].

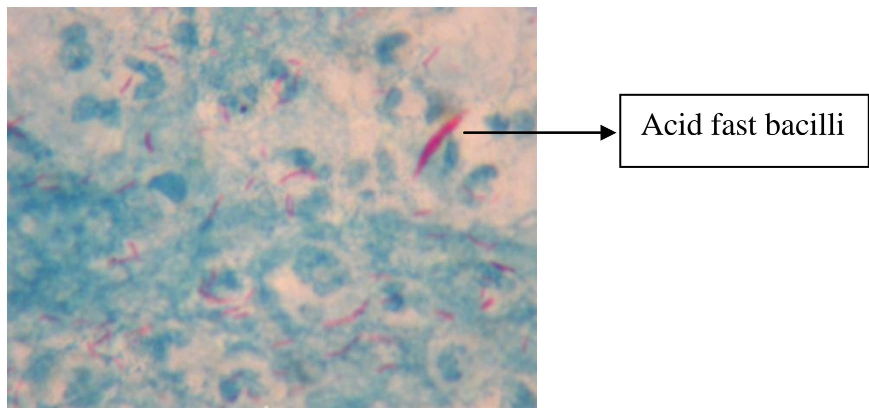
The fourth group of Mycobacterium is saprophytic Mycobacteria isolated from a number of sources. These include *M.butyricum* from butter, *M.pheli* from grass, *M.stercoris* from dung, *M.smegmatis* from smegma.

In a different class are two species of Mycobacteria, *M.ulcerans* and *M.marinum,* which are exclusively skin pathogens causing chronic ulcers and granulomatous lesions on the skin cutaeous lesion is involved because they multiply optimally at skin temperature [Tripathy et al., 1970].

1.1.6 MICROSCOPY OF *M. TUBERCULOSIS*

M. tuberculosis is characterized by caseating granulomas containing Langhans giant cells, which have a "horseshoe" pattern of nuclei. Organisms are identified by their red color on acid-fast staining [Fig 1.2] [Rodrigues et al., 2007].

Figure 1.2: Acid Fast Staining of *M. tuberculosis* (1000x)



Acid fast bacilli- pink color

Pus cells, non acid fast bacilli- blue color

1.1.7 PHYSIOLOGY

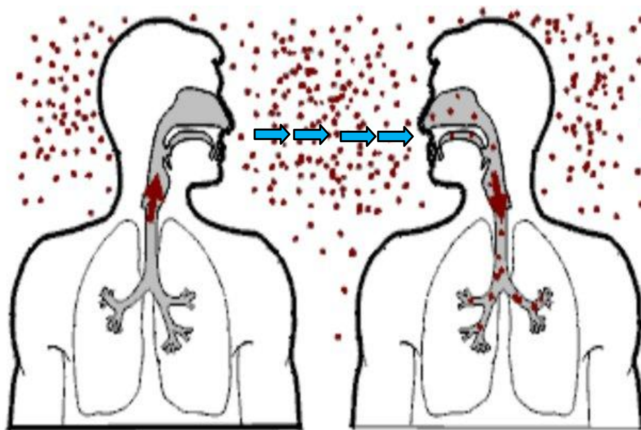
- Obligate aerobes growing most successfully in tissues with high oxygen content, such as the lungs.
- Facultative intracellular pathogens usually infecting mononuclear phagocytes (e.g. macrophages).
- It does not retain any bacteriological stain due to high lipid content in its wall, and thus is neither Gram positive nor Gram negative; hence Ziehl-Neelsen staining or acid-fast staining, is used.
- *M. tuberculosis* divides every 15-20 hours, which is extremely slow compared to other bacteria [Rodrigues et al., 2007].

- It is a small bacillus that can withstand weak disinfectants and can survive in a dry state for weeks. Its unusual cell wall, rich in lipids (e.g., mycolic acid), is likely responsible for this resistance and is a key virulence factor [Rodrigues et al., 2007].

1.1.8 MODES OF TRANSMISSION

Exposure to tubercle bacilli in airborne droplet nuclei produced by people with tuberculosis of the respiratory tract (primarily pulmonary or laryngeal) during expiratory efforts, such as coughing, singing, or sneezing [Figure 1.3]. Health care workers may be exposed during medical procedures such as bronchoscopy, autopsy or intubation. Laryngeal tuberculosis is highly contagious. Prolonged close exposure to an infectious case may lead to infection of contacts. Direct invasion through mucous membranes or breaks in the skin may occur but is extremely rare. Bovine tuberculosis occurs from exposure to tuberculous cattle, usually by ingestion of unpasteurized milk or dairy products and sometimes by airborne spread to farmers and animal handlers. Except for rare situations where there is a draining sinus, extrapulmonary tuberculosis (other than laryngeal) is generally not communicable [Gingeras et al., 1998].

Figure 1.3: Mode of transmission for *Mycobacterium tuberculosis*



Source: (http://www.ntp.mohealth.gov.eg/DOTS_Treatment.htm)

M. tuberculosis is spreaded through droplet nuclei from infected person to another person

Predisposing factors for TB infection include-

- ✓ Close contact with large populations of people

- ✓ Poor nutrition
- ✓ Intravenous drug use
- ✓ Alcoholism
- ✓ HIV infection

1.1.9 DISEASE PROCESS OF *M. TUBERCULOSIS:*

1.1.9A EVENTS IN THE INFECTIOUS PROCESS

Early Events

The mode of transmission of *M. tuberculosis* is through droplet nuclei from the infected individual. *M. tuberculosis* usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes which is found in greater numbers than macrophages in alveoli [Bermudez et al., 1996, Mehta et al., 1996]. In addition, dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages [Tascon et al., 2000] and presumably play a key role in activating T cells with specific *M. tuberculosis* antigens [Bodnar et al., 2001, Juarrero et al., 2001]. Since dendritic cells are migratory, unlike differentiated macrophages, they also may play an important role in dissemination of *M. tuberculosis* [Lipscomb et al., 2002].

The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors [Sansonetti et al., 1986]. Surfactant protein A, a glycoprotein found on alveolar surfaces, can enhance the binding and uptake of *M. tuberculosis* by upregulating mannose receptor activity [Noss et al., 2001]. On the other hand, surfactant protein D, similarly located in alveolae, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface. The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake [Neufert et al., 2001].

On entry into a host macrophage, *M. tuberculosis* initially resides in an endocytic vacuoule called the phagosome. If the normal phagosomal maturation cycle occurs, i.e., phagosome-lysosome fusion, these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Pathogenic

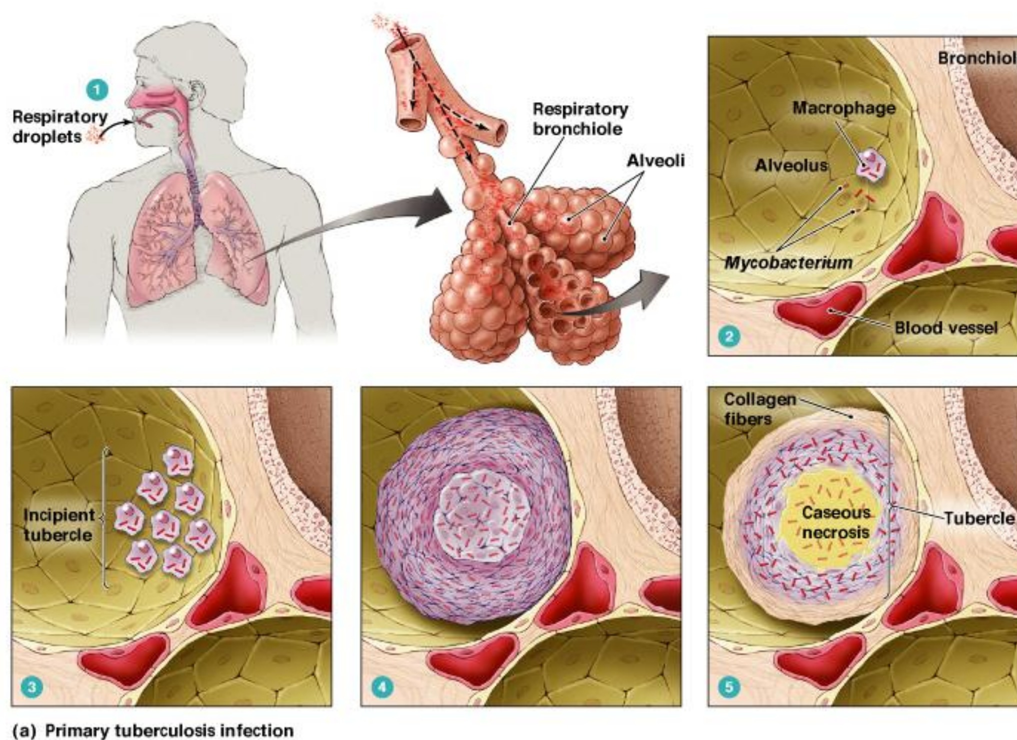
Mycobacteria also inhibit phagosome-lysosome fusion due to the exclusion of proton ATPases from the mycobacterial phagosome [Neufert et al., 2001]. It has also been postulated that a selective advantage to *M. tuberculosis* of staying in an early endosome is that there would be less host immunosurveillance by CD4 T cells. In agreement with this idea, there is a decrease in the expression of major histocompatibility complex class I (MHC-I) proteins and in the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection [Fig 1.4].

Later Events:

The infected macrophages in the lung, through their production of chemokines, attract inactivated monocytes, lymphocytes, and neutrophils [Crevel et al., 2002], none of which kill the bacteria very efficiently [Fenton et al., 1997]. Then, granulomatous focal lesions composed of macrophage derived giant cells and lymphocytes begin to form. This process is generally an effective means of containing the spread of the bacteria. As cellular immunity develops, macrophages loaded with bacilli are killed, and this results in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes [Dannenberg et al., 1994]. Although *M. tuberculosis* bacilli are postulated to be unable to multiply within this caseous tissue due to its acidic pH, the low availability of oxygen, and the presence of toxic fatty acids, some organisms may remain dormant but alive for decades.

The strength of the host cellular immune response determines whether an infection is arrested here or progresses to the next stages. This enclosed infection is referred to as latent or persistent TB and can persist throughout a person's life in an asymptomatic and nontransmissible state. In persons with efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by an unknown process and then serves as a rich medium in which the now revived bacteria can replicate in an uncontrolled manner [Hickman et al., 2002]. At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB).

Figure 1.4: Process of Primary tuberculosis Infection



Source: (http://www.nature.com/nrmicro/journal/v8/n11/fig_tab/nrmicro2437_F1.html)
The illustrated figure shows the pathogenitic mechanism of *M. tuberculosis*

1.1.10 PATTERNS OF INFECTION

There are three major patterns of disease with Tuberculosis

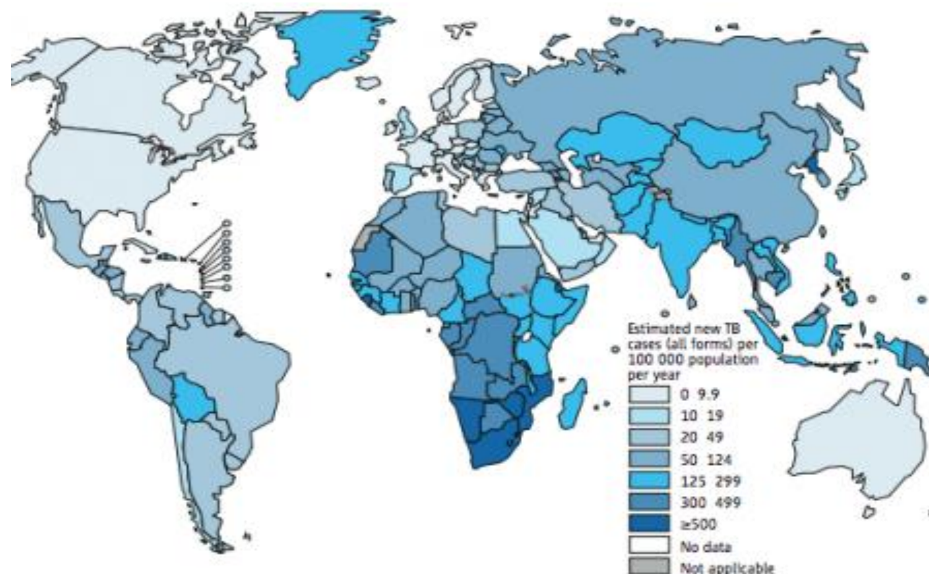
- **Primary tuberculosis**: seen as an initial infection, usually in children. The initial focus of infection is a small subpleural granuloma accompanied by granulomatous hilar lymph node infection. Together, these make up the Ghon complex. In nearly all cases, these granulomas resolve and there is no further spread of the infection.
- **Secondary tuberculosis**: seen mostly in adults as a reactivation of previous infection (or reinfection), particularly when health status declines. The granulomatous inflammation is much more florid and widespread. Typically, the upper lung lobes are most affected, and cavitation can occur.
- **Disseminated tuberculosis**: The spread of the disease within the body may result if infected macrophages moving through the blood and lymph transport the bacteria to

other sites. Once infected, symptoms of disseminated tuberculosis correspond to the locations infected.

1.1.11 GLOBAL TUBERCULOSIS BURDEN:

In the year 2013, it is estimated that globally there were 8.6 million incident TB cases equivalent to 122 cases per 100 000 population [WHO., 2013]. Most of the estimated number of cases in 2013 occurred in **Asia (58%)** and the African Region (27%);2 smaller proportions of cases occurred in the Eastern Mediterranean Region (8%), the European Region (4%) and the Region of the Americas (3%) [WHO., 2013]. The 27 High burden countries that have been given highest priority at the global level since 2000 accounted for 81% of all estimated incident cases worldwide. The five countries with the largest number of incident cases in 2013 were **India (2.0 million– 2.4 million),** China (0.9 million–1.1 million), South Africa (0.4 million–0.6 million), Indonesia (0.4 million–0.5 million) and Pakistan (0.3 million–0.5 million) [Figure 1.5]. India and China alone accounted for 26% and 12% of global cases, respectively. There were an estimated 12 million prevalent cases of TB and 1.3 million TB deaths in 2012. Approximately 75% of total TB deaths occurred in the African and South-East Asia Regions in 2012. India and South Africa accounted for about one-third of global TB deaths [WHO., 2013].

Figure 1.5: Global Estimated Tuberculosis incidence rates in 2013



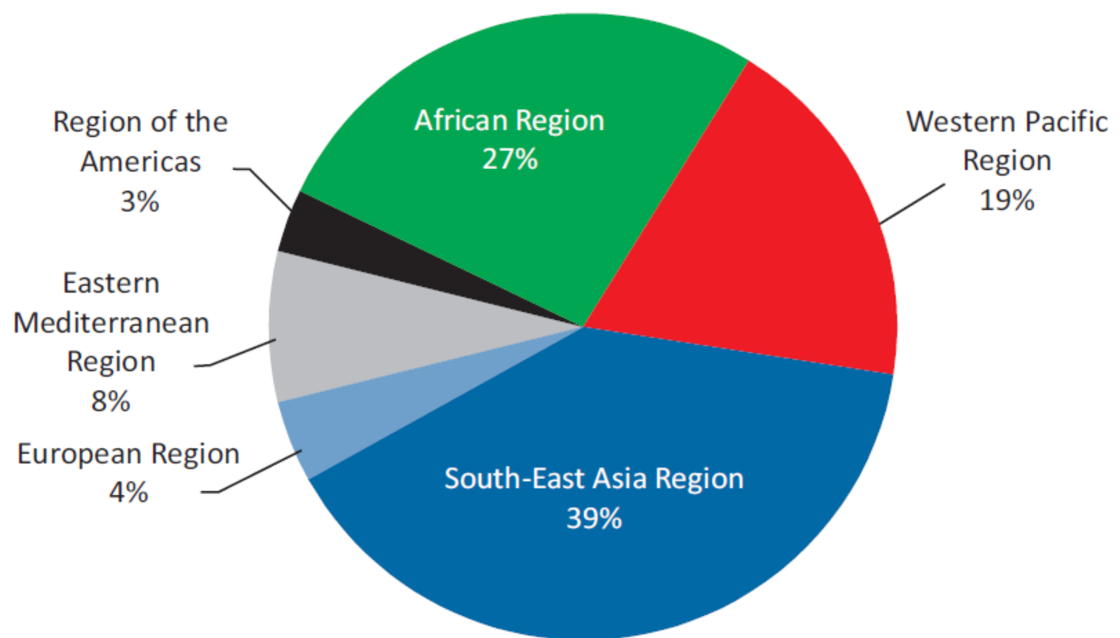
Source: (Global tuberculosis report 2013, WHO, Geneva 2013 [WHO /HTM/ TB/ 2013. 11])

Globally, 3.6% new TB cases and 20.2% of previously treated cases are estimated to have Multi drug resistant tuberculosis (MDR-TB). Eastern European and especially central Asian countries continue to have the highest levels of MDR-TB. Extensively drug-resistant TB (XDR-TB) had been reported by 92 countries globally by the end of 2013 [WHO., 2013].

1.1.12 TUBERCULOSIS BURDEN IN INDIA:

The World Health Organization South-East Asia (SEA) Region, "The regional report – 2013", indicate that there were 5 million prevalent and about 3.5 million incident cases of tuberculosis in 2012, which carries about 40% of the global burden of the disease [Figure 1.6]. India stands first among the 27 TB high burden countries in the world [WHO., 2013].

Figure 1.6: Global Incidence of tuberculosis in the year 2013



Source: (Global tuberculosis report 2013, WHO, Geneva 2013[WHO/HTM/TB/2013.11])

India ranks first among the high burden countries and contributed one-fourth of estimated global incident TB cases in 2012. In 2012, out of the estimated global annual incidence of 8.6 million TB cases, 2.0 million– 2.4 million were estimated to have occurred in India. It is

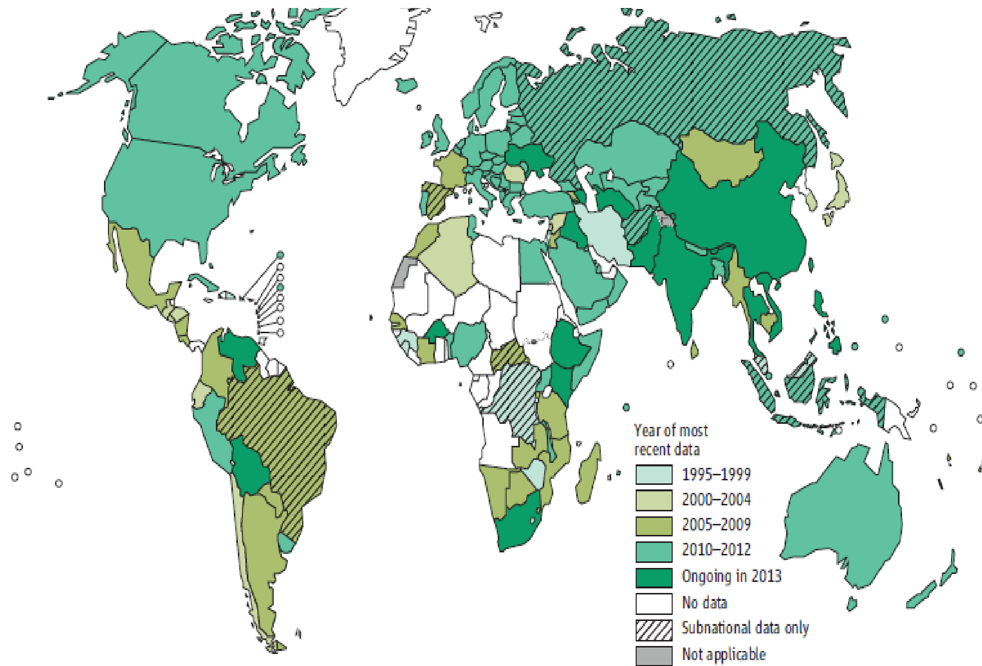
estimated that about 40% of Indian population is infected with TB bacillus. The prevalence of TB has been estimated at 2.8 million TB cases for the year 2012. WHO estimated TB mortality in India as 270,000 (23/100,000 population) in 2012.

According to the latest report released by the World Health Organisation (WHO), there are about 63,000 notified cases of MDR-TB in India. The MDR-TB prevalence rate in India is estimated to be 2.2% among the new cases and 15% among treated cases. Prevalence of XDR-tuberculosis cases is notably high in Eastern Europe, sub-Saharan Africa and **Asia** [WHO, 2013]. The prevalence of XDR-TB among all MDRTB patients was 6.6% overall worldwide, 6.5% in industrialized countries and 13.6% in Russia and Eastern Europe, **1.5% in Asia**, 0.6% in Africa and Middle East, and 15.4% in Republic of Korea.

1.1.13 FIRST, SECOND AND THIRD LINE DRUGS FOR DRUG RESISTANT TUBERCULOSIS:

Combinations of drugs are used for treating tuberculosis. The first line anti-tuberculosis drugs are Rifampicin, Isoniazid, Ethambutol, Streptomycin and Pyrazinamide. The second line anti-tuberculosis drugs (less toxic and effective) are aminoglycosides, polypeptides, fluoroquinolones, Ethioamides, cycloserine and p-aminosalicyclic acid. The third line of anti-tuberculosis drugs (efficacy not proven) are rifambutin, macrolides, linezolid, and thioacetazone [Frieden TR et al, 2002]. This downward trend ended and the numbers of new cases started increasing in the mid-1980s. The major causes of this were increased drug resistance to *M. tuberculosis* and the emergence of AIDS, with its destruction of the cell-mediated immune response in co-infected persons [Figure 1.7].

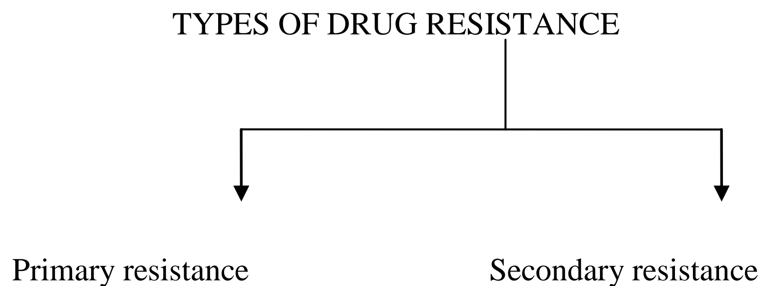
Figure 1.7: Progress in global coverage of data on drug resistance from 1994 to 2013



Source: (Global tuberculosis report 2013, WHO, Geneva 2013[WHO/HTM/TB/2013.11])

1.1.13.1 TYPES OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*:

Drug resistance in TB may be broadly classified as primary and acquired resistance.



- Primary drug resistance is defined as drug resistance in a patient who has not received any anti-tubercular treatment in the past [Frieden et al., 2002].

❖ The resistance that develops in a patient who has received prior chemotherapy is defined as acquired drug resistance.

Epidemics of drug-resistant strains of TB are generated by three independent but interacting processes:

(i) Transmission of drug resistant strains to uninfected individuals (transmitted resistance)

(ii) conversion of wild-type pan sensitive cases to drug-resistant cases during treatment (acquired resistance)

(iii) The progressive acquisition, by drug-resistant strains, of resistance to more drugs during repeated treatment episodes (amplified resistance).

1.1.13.2 FACTORS RELATED TO THE DEVELOPMENT OF DRUG RESISTANCE:

The emergence of drug resistance in TB patients is a result of a deficient or deteriorating TB control programme [WHO, 2013].

The Factors include:

(iv) inadequate or inefficient administration of effective treatment

(v) poor case holding

(vi) use of sub-standard drugs

(vii) inadequate or irregular drug supply

(viii) ignorance of healthcare workers in the treatment and control of TB

(ix) interruption of chemotherapy due to side effects

(x) non-adherence of patients to the prescribed regimens

(xi) availability of anti-TB drugs without prescription

(xii) illiteracy

(xiii) low socio-economic status of patients

(xiv) massive bacillary load

(xv) laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates

(xvi) lack of the use of uniform laboratory methodology and quality control measures.

1.1.14 MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB):

Multidrug-resistant tuberculosis (MDR-TB) is defined as tuberculosis that is resistant to the two most important first line antituberculosis drugs, Isoniazid and Rifampicin with or without resistance to other first line drugs [WHO, 2013]. The acquisition of resistance by the bacterium is a random event, and in a given mycobacterial population, 1in 10^6 bacteria mutates to develop Isoniazid resistance, while 1 in 10^8 mutates to develop Rifampicin resistance. MDR-TB has emerged as a significant global health concern [Surendra et al., 2011, WHO, 2013]. There are alarming reports of increasing drug resistance from various parts of the globe which potentially threaten to disrupt the gains achieved in tuberculosis (TB) control over the last decade.

Currently tuberculosis is treated with an initial intensive 2-month regimen comprising first line anti-tuberculosis drugs —Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), and Ethambutol (EMB) or Streptomycin (STR). The next 4 months, only RIF and INH are administered to eliminate any persisting tubercle bacilli. INH and RIF, the two most potent antituberculous drugs, kill more than 99% of tubercule bacilli within 2 months of initiation of therapy [Vareldzis et al., 1994]. Along with these two drugs, PZA, with a high sterilizing effect, appears to act on semidormant bacilli not affected by any other antitubercular drugs [Vareldzis et al., 1994]. Using these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. Therefore, the emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates, especially among HIV-infected persons [Hopewell et al., 2006].
The probability of resistance for INH, STR and EMB is 10^6 and for RIF and PZA is 10^8. Consequently, the probability of a mutation is directly proportional to the bacterial load. Because the mutations conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities; thus the probability of MDR is multiplicative [Sharma et al., 2006]. When the patient is exposed to a second course of drug therapy with yet another drug, mutants resistant to the new drug are selected, and the patient may eventually have bacilli resistant to two or more drugs. Serial selection of drug resistance, thus, is the predominant mechanism for the development of MDR strains.

1.1.15 EXTENSIVELY DRUG RESISTANT TUBERCULOSIS (XDR-TB):

Extensively drug-resistant tuberculosis (XDR-TB) is a form of TB caused by *Mycobacterium tuberculosis* that are resistant to Isoniazid and Rifampicin (i.e. MDR-TB) as well as any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin) [WHO., 2013]**.** MDR TB cases threaten the effectiveness of chemotherapy for both treatment and control of TB and require the use of second-line drugs that are more expensive, toxic, and less effective than first-line anti-TB drugs. In 2000, the Stop TB Partnership's Green Light Committee created to increase access to second line drugs (SLDs) worldwide while ensuring their proper use to prevent increased drug resistance. While assisting MDR TB treatment programs worldwide, and ensuring the proper use of SLDs in resource limited countries the committee encountered reports of multiple cases of TB with resistance to virtually all SLDs [WHO., 2013] .This led to the emergence of new terminology in relation to drug-resistant TB, i.e., extensively drug-resistant TB (XDR TB). XDR-TB can be developed when the second-line drugs are misused or mismanaged and therefore results in ineffective treatment. Because XDR-TB is resistant to first- and second-line drugs, treatment options are seriously limited. It is therefore vital that TB control is managed properly.

Extensively drug-resistant tuberculosis (XDR-TB) is a form of TB caused by *Mycobacterium tuberculosis* that are resistant to Isoniazid and Rifampicin (i.e. MDR-TB) as well as any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin)**.**

The basis of tuberculosis drug resistance is the selection of bacterial mutants with innate resistance to chemotherapy. Acquired resistance (Conversion of wildtype pan-susceptible strains to drug resistant strains during treatment) and amplified (Increasing development of resistance in drug-resistant strains because of inappropriate chemotherapy) drug resistance is the primary means by which tuberculosis drug-resistant strains have been generated. However, the key determinant that has led to the exponential rise in XDR tuberculosis cases is likely to have been transmitted (Transmission of drug-resistant cases) resistance.

1.1.16 MOLECULAR DIAGNOSTIC METHODS FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS*

During the last decade, major advances in understanding the genetic structure of Mycobacteria have been made. Based on this newer knowledge about the specific gene sequences, several gene probes/gene amplification systems for tuberculosis have been developed [Katoch et al., 1997]. Various gene amplification based methods have been applied for the rapid detection and identification (or differentiation) of mycobacterial species. Gene amplification - restriction assays targeting several genes have been developed in recent years [Shankar et al., 1997, Gunisha et al., 2001, Siddiq et al., 2002]. These molecular tools and methods can be used for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also molecular detection of drug resistanc

A number of molecular assays [such as PCR-based DNA sequencing, PCR-Single Strand Conformational Polymorphism (PCR-SSCP), Polymerase Chain Reaction- heteroduplex formation, Line probe assay or LiPA (solid phase hybridization assay), Micro array, Whole genome sequencing] have been designed to detect the presence of *M. tuberculosis*. The potential advantages of molecular assays are the ability to

(1) Design assays that are highly sensitive and specific

(2) Manufacture some assays in large quantities, allowing for decreased cost and ease of standardization in field use

(3) Yield rapid results

(4) Be used more widely, because they require less training and infrastructure than do conventional mycobacterial cultures and anti-Mycobacterial susceptibility testing.

1.1.16.1 DIRECT DETECTION OF MYCOBACTERIA FROM CLINICAL SPECIMENS

Many Mycobacterial species, including *M. tuberculosis*, grow extremely slowly in the laboratory and require 3–8 weeks of incubation on solid medium or at least 2 weeks in a liquid culture system (BACTEC). This slow growth often leads to a delay in TB diagnosis. Nucleic acid amplification (NAA) methods allow for detection of mycobacterial DNA or RNA directly from the specimens before the culture results are available.

The Food and Drug Administration (FDA) has approved two NAA tests for direct detection of *M. tuberculosis* from clinical specimens. These are the Enhanced *Mycobacterium tuberculosis* Direct Test (E-MTD; Gen- Probe, San Diego, CA) and the Amplicor *Mycobacterium tuberculosis* Test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ).

1.1.16.1.2 Enhanced *M. tuberculosis* Direct Test (E-MTD- Gen- probe, San Diego, CA):

The E-MTD test is based on the transcription-mediated amplification system developed by Kwoh et al [Kwoh et al., 1989]. In this assay, rRNA is released from the target cells by sonication, and a promoter-primer binds to the 16S rRNA target. Reverse transcriptase is then used to copy rRNA to a cDNA-RNA hybrid. The initial RNA strand is degraded, and a second primer binds to the cDNA and is extended, leading to the formation of double-stranded cDNA, which is then transcribed by DNA-directed RNA polymerase to produce more rRNA molecules. The new transcripts serve as templates for reverse transcription and further amplification. The RNA amplicons are detected with an acridinium ester-labeled DNA probe in a solution hybridization assay. Importantly, the amplification procedure is isothermal and the reaction is performed in a single tube, which helps to reduce carryover contamination. After standard decontamination of the clinical specimen, the E-MTD test can be completed in 3.5 h [Kwoh et al., 1989].

The E-MTD test has been reported to perform well with both AFB smear-positive and smear-negative specimens. The overall sensitivity (compared with culture) for respiratory specimens was 90.9 –95.2%, the specificity was 98.8–100%, the positive predictive value was 83.3–100%, and the negative predictive value was 98.4–99.6%. The E-MTD test is FDA-approved for detection of *M. tuberculosis* in both AFB smear-positive and smear-negative specimens.

Scarparo et al [Scarparo et al., 2000] compared the performance of the E-MTD and the Cobas Amplicor tests with 486 respiratory and non respiratory specimens obtained from 323 patients. No significant differences were observed between the results of the assays. However, it was noted that although the turnaround time is shorter for the E-MTD test, the Amplicor test can be fully automated and has an internal control for monitoring amplification inhibitors.

In conclusion, the currently available NAA tests can enhance diagnostic speed, but they do not replace AFB smear or culture. Because the tests can only detect *M. tuberculosis*, cultures

are still needed for identification of nontuberculous mycobacteria and for drug susceptibility testing. Because the tests cannot distinguish between live and dead organisms, they cannot be used to monitor TB therapy. Clinicians should interpret the NAA test results based on the clinical situation, and the tests should usually be performed at the request of the clinician.

1.1.16.1.2 AMPLICOR *M. tuberculosis* test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ)

This technique detects the presence of the Mycobacterial 16S ribosomal RNA (rRNA) gene by PCR amplification followed by an ELISA reaction. The clinical specimen is processed with the standard N-acetyl-L-cysteine-NaOH decontamination method, followed by amplification of the mycobacterial 16S rRNA gene. After amplification, the amplicons are denatured to form single strands and added to a microtiter plate containing a bound, *M. tuberculosis* complex-specific oligonucleotide probe. An avidin-horseradish peroxidase conjugate then binds to the bound, biotin-labeled amplicons. The conjugate then reacts with peroxide and 3, 39, 5, 59-tetramethylbenzidine in dimethylformamide to form a red color complex. The results are measured with a photometer [Bergman et al., 1996]. False-positive results produced by carryover contamination are prevented by the incorporation of dUTP coupled with uracil-*N*-glycosylase restriction. An automated version of this test is available in Europe (Cobas Amplicor) [Bergman et al., 1996].

Soini et al found that the overall sensitivity of the Amplicor test (compared with culture) for respiratory specimens is 79.4 –91.9%, the specificity is 99.6 –99.8%, the positive predictive value is 92.6 –96.6%, and the negative predictive value is 98.6– 98.7%. However, the sensitivity for smear negative specimens is somewhat lower, 40.0–73.1% [Bergman JS et al, 1996, Stauffer F et al, 1995]. Therefore, the Amplicor test has been approved by the FDA only for direct detection of *M. tuberculosis* in AFB smear-positive respiratory specimens. Stauffer et al [Stauffer et al., 1995] reported that the sensitivity of the Amplicor test was similar to that of culture (58% vs 56%) for detecting *M. tuberculosis* from respiratory specimens when the clinical case definition of TB was used as the reference standard. However, Tevere et al [Tevere et al., 1996] reported that although the Amplicor test had excellent specificity (100%), it was less sensitive than culture (42% vs 73%) for diagnosis of minimal active pulmonary TB (patients suspected of having TB but without spontaneous sputum or with AFB-negative smears).

An automated version of the test, the COBAS Amplicor MTB test together with the COBAS Amplicor analyzer (Roche Diagnostics, Switzerland) allows automation of the amplification and detection steps in one system. More recently, the qualitative **COBAS TaqMan MTB** test has also been introduced using real-time PCR and hybridization and performed in the COBAS TaqMan 48 analyzer running up to 48 samples simultaneously in 2.5 hours.

OTHER MOLECULAR METHODS:

1.1.16.1.3 BD PROBETEC MTB TEST (Becton Dickinson, Sparks, MD, USA):

This was first introduced several years ago as a semiautomated system for the rapid diagnosis of tuberculosis. It is based on the strand-displacement amplification technique that uses enzymatic replication of target sequences in *IS6110* and the 16S rRNA gene. The amplified products are then detected with a luminometer. The method was evaluated in studies [Bergmann et al., 1998] with respiratory samples with a reported sensitivity of 100% in smear-positive specimens and 92–100% in smear-negative samples; the overall specificity was 96–99% in the same studies. The major drawback was that the sample preparation required at least 2 hours.

An improved version of this system, the **BDProbe Tec ET**, which includes an internal amplification control to detect the presence of inhibitors, has been more recently evaluated in respiratory and nonrespiratory specimens in a clinical setting. As with the other NAA tests described above, higher sensitivity and specificity has been found in respiratory smear-positive samples. The BDProbe Tec ET system is not yet approved by the US FDA.

1.1.16.1.4 Simplified Isothermal Amplification Techniques:

A recent development based on isothermal amplification is the loop-mediated isothermal amplification (LAMP) assay, which is based on autocycling strand displacement DNA synthesis using the large fragment of Bst DNA polymerase. The main characteristic of LAMP is its ability to synthesize large amounts of DNA. Pyrophosphate, which is produced as a byproduct, yields a white precipitate of magnesium pyrophosphate that can be detected visually in the reaction vial. The presence or absence of this precipitate allows the detection of DNA amplification [Boehme et al., 2007]. Furthermore, the increase in the turbidity of the reaction mixture correlates with the amount of DNA synthesized, allowing real-time monitoring of the LAMP reaction by real-time measurement of the turbidity. Using a slightly modified version of this methodology, adding SYBR Green I to the reaction for easy

detection of colour,were able to detect the *M. tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulare* directly from sputum specimens and in culture isolates grown in the Mycobacterium growth indicator tube (Becton Dickinson) or Ogawa's medium. The whole procedure was carried out in a single tube with the isothermal reaction held at 63°C. With the exception of a water bath or heating block, no other laboratory equipment was necessary.

When compared with the Amplicor test, the LAMP assay showed comparable performance using a very small volume of DNA and a period of 60 mins incubation. The sensitivity in smear- and culture-positive sputum samples was 97.7%, while in smear-negative, culture-positive specimens it was only 48.8%. The specificity in culture-negative samples was 99% [Boehme et al., 2007].

The simplicity of the assay and the lack of requirement for major equipment render the LAMP assay a promising candidate as a rapid molecular test for the detection of *M. tuberculosis*. Further studies, however, are needed to assess the accuracy and ease of implementation of this technique in resource-limited settings [Boehme et al., 2007]. The major conclusion was that the accuracy of these NAATs with respiratory samples was highly variable, with sensitivity giving lower values than specificity. For these reasons, they probably still do not have enough clinical relevance and could not be recommended to replace the conventional tests for diagnosing pulmonary tuberculosis.

1.1.16.2 IDENTIFICATION OF MYCOBACTERIAL SPECIES FROM CULTURE

Mycobacterial isolates have traditionally been identified to the species level based on their reactions in a series of phenotypic and biochemical tests. However, the biochemical reactions of isolates of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained. Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using molecular methods for species identification.

1.1.16.2.1 Nucleic Acid Probes:

Commercial DNA probes (AccuProbe; Gen-Probe Inc.) have been available for the identification of clinically important mycobacterial species, including *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*. The tests are based on species-specific DNA probes that hybridize with rRNA released from bacteria. The probes are labeled with acridinium ester, and results are measured with a luminometer. For

culture-positive specimens, the turnaround time for the method is approximately 2 hours. The method is very easy to perform, and no special instrumentation is needed. The probes have been evaluated extensively in clinical practice and are rapid, sensitive, and specific [Boehme et al., 2007].

The probes can also be combined with the BACTEC or other liquid based culture systems to further decrease the time needed for species identification [Goto et al., 1991]. However, probes are not available for all pathogenic mycobacterial species, and those isolates must be identified by other methods.

1.1.16.2.2 PCR-based DNA Sequencing:

PCR-based DNA sequencing has become the gold standard for identification of Mycobacterial species. The method consists of PCR amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons. The organism is identified by comparison of the nucleotide sequence with reference sequences. The target most commonly used is the gene coding for the 16S rRNA. This gene is present in all bacterial species and contains both conserved and variable regions, making it an ideal target for taxonomic purposes. The 16S rRNA gene has been sequenced from a large number of mycobacterial species, and the identification method based on this gene has been evaluated extensively in diagnostic laboratories. Sequencing of two hypervariable regions of the 16S rRNA gene allows for identification of the majority of mycobacterial species. The genes coding for the 32-kDa protein (*85B* gene) [Soini et al., 1994], the 65-kDa heat shock protein (*hspx* gene), and the 16S-23S rRNA internal transcribed spacer (*ITS* gene) [Kapur et al., 1995] contain enough sequence diversity to distinguish all clinically important mycobacteria except for the members of the *M. tuberculosis* complex.

This method also allows for direct detection of mycobacterial species that cannot be grown in conventional laboratory culture media, and several previously unrecognized species have been identified [Metzker et al., 2005].

1.1.16.2.3 DNA microarray:

High-density oligonucleotide arrays (DNA microarrays) offer the possibility of rapid examination of large amounts of DNA sequences with a single hybridization step. This approach has recently been applied to simultaneous species identification and detection of mutations that confer Rifampin resistance in *M. tuberculosis*. This technique is based on hybridization of fluorescently labeled PCR amplicons generated from bacterial colonies to a

DNA array containing nucleotide probes. The bound amplicons emit a fluorescent signal that is detected with a scanner. The turnaround time for this method when performed on culture positive specimens was only 4 hours.

1.1.16.3 Identification of Antibiotic Resistance-Associated Mutations

Drug-resistant *M. tuberculosis* isolates are a serious threat to TB control because only a few effective drugs are available for treatment of this disease. *M. tuberculosis* acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to multidrug-resistant TB. Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate.

After the primary culture results are available, conventional drug susceptibility testing performed on solid medium takes; 2–4 weeks. When the BACTEC (radiometric, micro MGIT) drug susceptibility testing system is used, several weeks are needed to obtain results. Advances in molecular biology have made it possible to investigate the genetic mechanisms of drug resistance in *M. tuberculosis* and to develop methods for rapid detection of mutations associated with resistance.

1.1.16.3.1PCR-based DNA sequencing:

PCR-based sequencing is the main technique used to elucidate the genetic mechanisms of drug resistance in *M. tuberculosis*. It is the most direct and reliable method for studying mutations and allows for detection of both reported and novel mutations.

1.16.3.2 Line-Probe Assay:

The Line Probe assay (LiPA; Inno-Genetics N.V., Zwijndrecht, Belgium) has been developed for rapid detection of RIF resistance in *M. tuberculosis*. The test is based on the reverse hybridization method, and it consists of PCR amplification of a segment of the *rpoB* gene and denaturation and hybridization of the biotinylated PCR amplicons to capture probes bound to a nitrocellulose strip. The bound amplicons are then detected with alkaline phosphatase-conjugated streptavidin and BCIP/NBT chromogen, producing a color reaction [Morgan et al., 2005].

The LiPA test strip contains five probes for wild-type *rpoB* sequences and four probes for specific *rpoB* mutations, in addition to a conjugate control and *M. tuberculosis* control probes. The interpretation of the banding pattern on the strip allows for identification of *M. tuberculosis* complex and detection of *rpoB* mutations. The test can be performed on *M.*

tuberculosis cultures or directly from clinical specimens. The turnaround time for the test is 48 hours [Morgan et al., 2005].

Overall concordance of the LiPA test with phenotypic susceptibility testing and direct sequencing, when performed from cultures, has been reported to be good, varying from 92.2% to 99.0% [Morgan et al., 2005]. Although the LiPA test only detects 4 of the 35 distinct *rpoB* mutations, 75% of the RIF-resistant clinical isolates carry 1 of the 4 mutation [De Beenhouwer et al., 1995], making the LiPA test a useful method for rapid detection of RIF resistance. However, the test cannot be used for detection of rare mutations.

1.1.16.3.3 Real-Time PCR:

Real-time PCR technology has also been proposed for the rapid detection of drug resistance in *M. tuberculosis*. Realtime PCR is based on hybridization of amplified nucleic acids with fluorescent-labelled probes spanning DNA regions of interest (*16S rRNA, IS6110*) and monitored inside thermal cyclers. The fluorescent signal increases in direct proportion to the amount of amplified product inside the reaction vial. For the detection of drug resistance different probes have been used, especially rifampicin or isoniazid, such as TaqMan probes, fluorescence resonance energy transfer probes, molecular beacons and biprobes [Michos et al., 2006] .

The main advantage of real-time PCR is the speed in giving results, 1.5–2.0 hours after DNA extraction, and the lower risk of contamination because both reaction and detection occur in a single tube. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Realtime PCR has been evaluated in several studies for detection of *M. tuberculosis* and resistance to antibiotics [Michos et al., 2006].

1.1.16.3.4 DNA microarray:

DNA microarray technology described for mycobacterial species identification can also be used for rapid detection of mutations that are associated with resistance to TB drugs. Gingeras et al [Michos et al., 2006] studied 44 RIF-resistant *M. tuberculosis* isolates with a DNA array containing sequences from the *rpoB* gene. A total of 40 isolates had a previously recognized mutation, one new mutation was detected, and no *rpoB* mutations were found in the remaining 3 isolates by either the DNA array or sequencing. In a study performed by Troesch et al [Troesch et al., 1999], 15 RIF-resistant and 1 sensitive *M. tuberculosis* isolate were analyzed with a similar DNA array. All 15 resistant isolates had mutations in the *rpoB*

gene, and the mutations were correctly detected with the DNA array. The results were completely concordant with DNA sequencing results [Troesch et al., 1999]. A DNA microarray for simultaneous detection of various drug resistance determinants is currently being developed. It contains sequences from the *katG*, *inhA*, *rpoB*, *rpsl*, and *gyrA* (associated with fluoroquinolone resistance) genes [Troesch et al., 1999]. This approach has the potential of becoming the most effective and rapid method for detection of drug resistance mutations in *M. tuberculosis.*

1.1.16.3.5 Molecular Beacons

Molecular beacons are hairpin-shaped probes able to detect the presence of specific nucleic acids. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A quenching moiety is attached to the end of one arm and a fluorescent moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem hybrid and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence that can be detected. This is monitored in real-time, where the fluorescence increases every cycle in proportion to the amplification of the hybridizing target, which is not detected in cases when the target is not complementary to the beacon [Troesch et al., 1999].

Beacons are highly sensitive and specific: a single mismatch in the target sequence diminishes the beacon-target hybrid stability, allowing the detection of point mutations. Beacon assays are performed in sealed wells preventing amplicon contamination, they are easily implemented, automated and can be used in high throughput analysis. In the case of RIF, a set of 5 beacons has been designed to cover the rpoB core region in a single reaction, with excellent results [Troesch et al., 1999]. The assay is sensitive enough to detect 2 bacilli, offers results in 3 hours from sputum collection, and identifies the *M. tuberculosis* species proving its strong potential in clinical settings. A different set of molecular beacons has been

designed to screen for mutations in the regions with higher frequency of mutations associated with drug resistance for INH [katG (position 315), the promoter region of inhA, the oxyR-ahpC intergenic region and positions 66, 269, 312 and 413 of kasA] [Troesch et al., 1999]. Results indicate that the assay is highly specific, but further research is required to identify the full set of mutations responsible for INH R.

1.1.16.3.6 Single-Strand Conformation Polymorphism (SSCP)

This technique determines the presence of mutations in specific DNA regions by their migration patterns in polyacrylamide gels. The target region of a gene is amplified by PCR and the product is denatured into two single-stranded molecules and subjected to non denaturing polyacrylamide gel electrophoresis. Under non-denaturing conditions, the single-stranded DNA (ssDNA) molecule has a secondary structure determined by the nucleotide sequence, buffer conditions, and temperature. Mutant ssDNAs migrate to different positions than the wild type control. SSCP has been used to detect point mutations in *rpoB*, with promising results [Scarpellini et al., 1997]. However, there are reports of the presence of silent mutations (mutations that do not change the amino-acid sequence) in this region not resulting in RIF resistance leading to false positives by SSCP [Scarpellini et al., 1997]. This technique has risk of amplicon contamination due to the extensive post-PCR manipulation required. This technique is not practical for other antibiotics because they require screening larger DNA regions and more than one region per antibiotic.

1.1.16.3.7 FRET probes

Fluorescence resonance energy transfer (FRET) probes, also known as light cycler probes, are highly specific probes able to detect the presence of mutations in real time PCR reactions. The detection system of light cycler probes is based on FRET with two different specific oligonucleotides. Hybridization probe 1 is labeled with fluorescein, and hybridization probe 2 is labeled with the fluorophore Light Cycler Red 640. Both probes can hybridize in a head-to-tail arrangement, bringing the two fluorescent dyes into close proximity. A transfer of energy between the two probes results in emission of red fluorescent light. The level of fluorescence is proportional to the amount of DNA generated during the PCR process. In case the template is mutant, the probe-target stability will decrease and the

probe will remain in solution hindering FRET, resulting in a flat signal. FRET probe assays for the rapid detection of isoniazid and Rifampicin resistance were developed and tested with clinical isolates. The technique yields concordant results with sequencing data in less than 2 hours [Torres et al., 2003]. This technique has the advantage that the region covered is larger than the region screened by molecular beacons, but because it entails the use of two fluorophore-labeled probes, the cost is doubled.

Molecular methods offer many advantages over conventional methods in the identification of mycobacterial species. The results are obtained rapidly, are reliable and reproducible, and even mixed or contaminated cultures can be analyzed. The probes are already widely used in clinical laboratories for the identification of the most common mycobacterial species. Because automatic DNA sequencers and the programs used for analyzing sequence data have become technically simpler, the PCR-based sequencing method is now being used in many mycobacterial reference laboratories as the routine method for species assignment. The DNA microarray method holds great promise for the future because it is easy to perform, it can be readily automated, and it allows for identification of a large number of mycobacterial species in one reaction.

The currently available molecular methods may aid in rapid detection of mutations associated with drug resistance, but the test results must always be confirmed by phenotypic methods.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Nested Reverse transcriptase PCRs (nRT-PCRs) targeting mRNA of *icl*$_2$, *hspx* and *rRNAP1* genes to detect viable *M. tuberculosis* directly from clinical specimens

Reverse transcriptase polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR). In RT-PCR, however, RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional PCR [Freeman et al.,1999]. Recently, detection of actively multiplying *M. tuberculosis* directly from Clinical specimens targeting mRNA of various genes has been studied by many study groups. Among these, the important drug targets reported were *85B*, *icl*$_2$*, hspx and rRNAP1* genes.

The 3 steps of conventional PCR are denaturation, annealing, and primer extension. Initially, DNA is taken from the clinical specimen, as well as certain sequence-specific oligonucleotide primers, thermostable DNA polymerase, nucleotides, and10X buffer. The temperature of these is increased to 90-95°C in order to separate (denature) the two strands of target DNA. In the second step, the temperature decreases (45-60°C), depending on the primers, to permit annealing (strengthening) of the target DNA primers. Finally, nucleotides complementary to the target DNA are added extending each primer by the thermostable DNA polymerase [Freeman et al., 1996]. The target DNA segment is amplified in the range of 10^5 - to 10^6 -fold by repeating this cycle no less than 30-40 times. The amplified segment may be observed using electrophoretic gel or can be identified by Southern blot analysis, using specific DNA probes for that segment.

The initial diagnosis of *M. tuberculosis* often is based on clinical data, definitive diagnosis usually involves the isolation and identification of the *M. tuberculosis* in the laboratory [Katoch et al., 1997]. The usual laboratory procedure for clinical specimens involves decontamination and digestion of the specimen, microscopic examination for the presence of acid-fast bacilli (AFB), isolation by BACTEC culture, and identification and drug susceptibility testing of the isolated organism. Because of the slow growth rate of *M. tuberculosis*, isolation, identification, and drug susceptibility testing can take several weeks or longer. The rise of multidrug-resistant (MDR) and extensively drug resistant (XDR) TB has drawn attention to the need for rapid diagnosis of tuberculosis.

The potential advantages of molecular assays are the ability to design assays that are highly sensitive and specific, manufacture some assays in large quantities, allowing for decreased cost and ease of standardization in field use, yield rapid results and be used more widely, because they require less training and infrastructure than do conventional mycobacterial cultures and anti-Mycobacterial susceptibility testing [McFadden et al., 1990].A number of molecular assays have been designed to detect the presence of *M. tuberculosis* [Katoch et al., 1997, McFadden et al., 1990].

Many study groups reported on RT-PCR targeting mRNA of *M. tuberculosis*. The most widely used gene targets were *85B* gene, followed by *icl*$_2$*, hspx and rRNAP1* genes [Li et al., 2010, Muñoz-Elías EJ et al, 2005, Yuan et al., 1998]. Thus nRT-PCR targeting mRNA of *icl*$_2$*, hspx and rRNAP1* genes could serve as better diagnostic tool for early detection of actively multiplying *M. tuberculosis* from the clinical specimens which leads to early initiation of anti-tuberculous treatment.

2.1.1 *85B* GENE:

85B gene is one of the 85 antigen complex of mycobacteria and constitutes up to 41% of the total mycobacterial protein in log-phase culture supernatants [Salata et al., 1991]. It is reasonable to expect similarly high levels of 85B mRNA expression. The 85B antigen complex is present in all mycobacteria, and there is considerable evidence that the complex contains both species-specific and shared epitopes [Salata et al., 1991].

In the previous study done in our laboratory (L&T Microbiology research centre, Sankara nethralya) by Gayathri et al on standardization and application of Reverse transcriptase PCR targeting *85B* gene was done on direct clinical specimens [Gayathri et al., 2011]. RT-PCR targeting *85B* gene was standardized and applied on the 801 new cases for the detection of viable *M. tuberculosis*. Despite the high specificity and sensitivity of RT-PCR targeting 85B gene, out of the 354 culture positive specimens 49 (13.8%) were negative by RT-PCR targeting 85B gene (unpublished data). The inability of the assay to detect mRNA despite continued growth of *M. tuberculosis* in culture raises concern that the threshold of detection for this marker is too high. So there is an urgent need to target few more genes to increase the sensitivity of the RT-PCR to detect viable *M. tuberculosis.* There are three new target genes available in the recent literature for the detection of viable *M. tuberculosis*

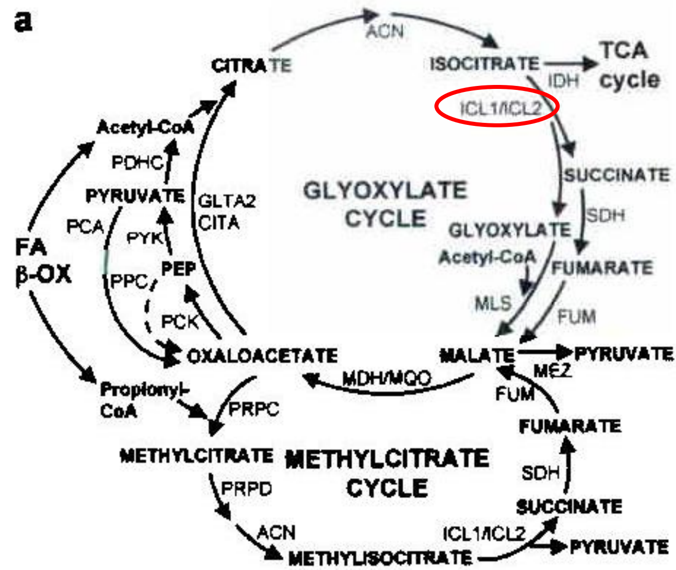
namely *hspX* (encoding alpha-crystalline homologue protein), *icl*$_2$ (encoding isocitrate lyase) and *rrnA-P1* (non-coding ribosomal promoter region) [Li et al., 2010].Thus, in this study optimization of nRT-PCRs targeting *icl*$_2$, *hspx* and *rRNAP1* genes were done to detect viable *M. tuberculosis* directly from clinical specimens.

2.1.2 *ICL*$_2$ GENE:

M. tuberculosis mainly growing on acetate or fatty acids as the sole carbon source employ the glyoxylate bypass for the biosynthesis of cellular material. The key enzymes of this by pass are isocitrate lyase and malate synthase. The former cleaves isocitrate to succinate and glyoxylate, and the latter condenses glyoxylate with acetyl coenzyme A (acetyl-CoA) to yield malate [McKinney et al., 2000]. The glyoxylate bypass circumvents the loss of two carbon dioxides of the tricarboxylic acid cycle (TCA cycle), thereby permitting net incorporation of carbon into cellular structures during growth on acetate. In addition, even during operation of the TCA cycle, many fatty acids are partially metabolized to acetyl-CoA, thus requiring the presence of iso citrate lyase [McKinney et al., 2000].

icl$_2$ gene encoding for isocitrate lyase (ICL) was previously demonstrated to play a pivotal role in the intracellular metabolism of actively multiplying *M. tuberculosis*. ICL is one of the key enzymes of the glyoxylate metabolism shunt [McKinney et al., 2000]. During the growth on C2 substrates, such as fatty acids or acetate, most microorganisms employ the glyoxylate shunt as the main metabolic route for the biosynthesis of cellular materials and survive inside the host macrophages. The role of isocitrate lyase (ICL) in the glyoxylate cycle and its necessity for persistence [Figure 2.1] and virulence of *M. tuberculosis* has been well described [Muñoz-Elías et al., 2005, Li et al., 2010]. Recent reports states that the role for isocitrate lyase (ICL) for *M. tuberculosis* survival within macrophage was suggested by the finding that disruption of the *icl*$_2$ gene inhibited the persistence of MTB in macrophage and in mice [McKinney et al., 2000]. Hence, *icl*$_2$ production and activity are markedly upregulated in intracellular infections with *M. tuberculosis.*

Figure 2.1: Glyoxylate shunt pathway involving *icl*$_2$ gene of *M. tuberculosis*.



Source : [Munoz-Elías et al., 2005]

The highlighted icl1/icl2 shown in the pathway is where the *icl*$_2$ gene involved in the Glyoxylate pathway

2.1.3 *HSPX* GENE*:*

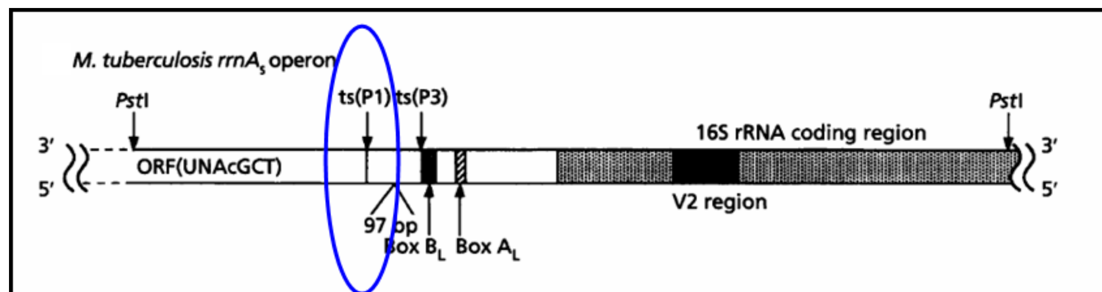
Hspx gene encodes alpha-crystalline homologue protein. It is a 16-kDa heat shock protein requires for mycobacterial persistence within the macrophage [Li et al., 2010]. This protein, originally defined by using the monoclonal antibody TB68 [Jackett et al., 1988], is an immunodominant antigen that is recognized by the majority of patients with active tuberculosis. Many reports states that *hspx* gene is upregulated in macrophages during initial stages of tuberculosis. Recent reported highlighted the high levels of *hspX* mRNA in log-phase cultures of *M. tuberculosis* [Hu et al., 2006, Yuan et al., 1998]. Experimental evidence has also indicated that *hspx* gene is level is elevated during growth of *M. tuberculosis* in cultured macrophages.

The identification by Lee et al [Lee et al., 1992] of hspX protein as the major membrane protein of *M. tuberculosis* suggests that hspX protein does not function purely cytoplasmically. More recently, proteome analysis has identified HspX protein in both whole-cell lysates and culture filtrates of *M. tuberculosis* [Rosenkrands et al., 2000].

2.1.4 *RRNAP1* GENE:

rRNAP1 gene is a non coding ribosomal promoter region mainly involve in the transcription of *M. tuberculosis.* rrnA-P1, one of five rrn promoters in Mycobacteria, has been described as a novel target to detect *M. tuberculosis* [Kempsell et al., 1992]. *M. tuberculosis* have single **rRNA *(rrn)*** operon. The single ***rrn*** operons of the slow-growers studied are sufficiently closely related in both their primary and secondary structures to be regarded as members of a family, described as ***rmA,*** operons which is located within a gene, which terminates 427 bp upstream of the 16S rRNA coding region. This gene encodes a protein that is significantly similar to an enzyme involved in bacterial cell wall synthesis suggesting that the production of the enzyme and transcription of the rRNA are linked [Li et al, 2010]. Analysis of *M. tuberculosis* RNA revealed two products corresponding to transcripts directed by promoters homologous with PI and P3 (Figure 2.2). Among these promoters, rRNAP1 promoter is considered to be expressed in high level during active tuberculosis infection.

Figure 2.2: *rRNAP1* gene structure of *M. tuberculosis*.



This diagrammatic picture shows the specific location of promoter region P1 in rrnA operon

2.2 First line and second line drug susceptibility testing by phenotypic drug susceptibility testing using "micro MGIT BACTEC method"

2.2.1 Drug-Susceptibility Testing (DST) Methods

Drug susceptibility of *M. tuberculosis* can be determined either by observation of growth or metabolic inhibition in a medium containing anti-tuberculosis drug, or by detection, at the molecular level, of mutations in the genes related to drug action [WHO/CDC/TB/2000.278,

Kent et al., 1985]. From a technical standpoint, drug susceptibility is determined on the basis of growth (or metabolic) inhibition induced by the drug by means of:

a) macroscopic observation of growth in drug-free and drug-containing media [WHO/CDC/TB/2000.278, Kent et al., 1985]
b) detection or measurement of the metabolic activity or products
c) lysis with mycobacteriophage and
d) detection of genetic mutations using molecular techniques

There are basically two types of detecting drug resistance- Direct and Indirect method [Morlock et al., 2000].

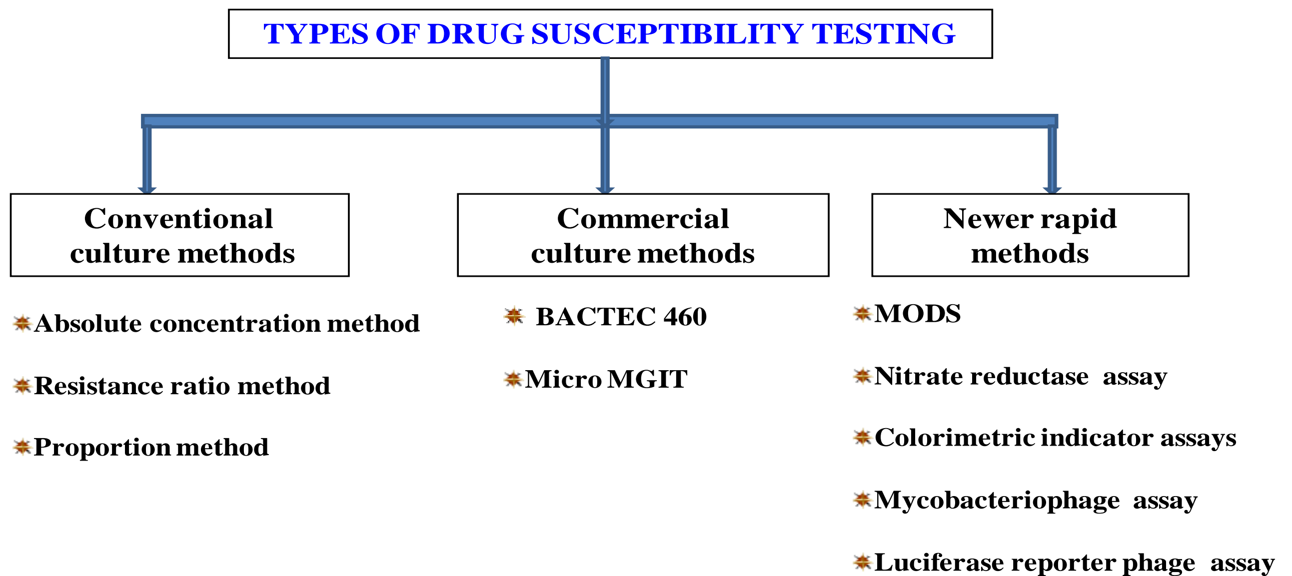
2.2.1A DIRECT METHODS:

The drug susceptibility testing can be performed based on mycobacterial cultivation on solid media, either egg or agar-based [Ramachandran et al., 2002]. In the direct test a set of drug-containing and drug-free media is inoculated directly with concentrated specimen. The advantage of the direct method over indirect method is that the results are available quickly and represent the patient's original bacterial population better.

2.2.1B INDIRECT METHODS:

In the indirect test, the pure culture is inoculated in drug containing and drug free slopes either in egg-based Lowenstein-Jenson medium or agar based 7H11 medium [Tripathy et al., 1970]. The phenotypically sensitive strains will not grow on the drug containing media and the phenotypically resistant strain will grow on the drug containing media

Table 2.1: Classification of drug susceptibility testing methods



2.2.2 CONVENTIONAL CULTURE METHOD:

2.2.2A Absolute concentration methods:

This method uses a standardized inoculum grown on drug-free media and media containing various concentrations of the drugs to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth; ie. Minimal inhibitory concentration (MIC) [Morlock et al., 2000].

2.2.2B Resistance ratio method:

It compares the growth of unknown strains of tubercle bacilli with that of standard laboratory strain (*M. tuberculosis* H37Rv). Parallel sets of media containing two-fold dilutions of the drug are inoculated with standard strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test strain in the same set [Ramachandran et al., 2002]. This test is also greatly affected by the inoculum size as well as the visibility of the strains. In addition, any variation in the susceptibility of the standard strain also affects the resistance ratio of the test strain.

2.2.2C Proportion method:

This method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted on to both control (drug-free) and drug-containing media; at least one dilution should yield isolated countable (50-100) colonies [Morlock et al., 2000]. When these numbers are adjusted by multiplying by the dilution of the inoculum used, the total number of viable colonies on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population used. The proportion method is currently the method of choice for estimating drug resistance and this principle is being applied to the following rapid testing methods [Morlock et al., 2000]:

i. BACTEC TB 460

ii. MGIT 960

iii. MB/BacT systematic, and

iv. ESP II system

Table 2.2: Comparison of Conventional Culture Methods

Criterion	Absolute concentration method	Resistant ratio method	Proportion method
Principle	uses a standardized inoculum grown on drug-free media and media containing graded concentrations of the drugs to be tested	compares the growth of test strains of tubercle bacilli with that of a std. strain –*M.tuberculosis* (H37Rv).	precise estimation of the proportion of mutants resistant to a given drug
Resistant pattern	Lowest concentration of the drug that inhibits growth i.e., Minimal inhibitory concentration (MIC).	Ratio of the MIC of the test strain to the MIC for the standard strain in the same set	Proportion of bacilli resistant to a given drug -expressed as percentage
Interpretation	INH -MIC $\geq$ 5-Resistant RIF -MIC $\geq$128-Resistant EMB-MIC $\geq$ 8 -Resistant	Sensitive - RR of 2 or less Resistant -RR of 8 or more	>1%proportion-Resistant <1% proportion-Sensitive
Comments	greatly affected by inoculum size and by the viability of the organisms	greatly affected by inoculum size and by the viability of the organisms	Currently the method of choice

MIC- Minimal inhibitory concentration, INH- Isoniazid, RIF-Rifampicin, RR-resistance ratio

2.2.3 COMMERCIAL CULTURE METHODS:

2.2.3A BACTEC TB 460 SYSTEM

The basic principle of the BACTEC radiometric susceptibility assay for Mycobacteria is similar to the one utilized in the primary isolation procedure. When Mycobacteria grow in 7H12 medium containing ^{14}C-labeled substrate, they utilize the substrate and $^{14}CO_2$ is produced. The amount of $^{14}CO_2$ detected reflects the rate and amount of growth occurring in the vial, and is expressed in terms of the “Growth Index” (GI) [Rodrigues et al., 2007]. When an antituberculous drug is added to the medium, suppression of growth occurs if the test organisms are susceptible. This suppression can be detected by either a decline or a

very small increase of the daily GI as compared to the control. However, if the organisms are resistant, little or no suppression occurs.

To determine the 1% proportion of resistance, the bacterial inoculum used in the control vial is one-hundred fold less than that used for the drug-containing vial. The drug and control vials are tested daily after inoculation. The rate of increase in the GI, or the amount of change over that of the previous day, called delta (Δ) GI, is compared between the control vial and the vials containing drugs. If the daily GI increase in the drug vial is equal to or greater than that in the control vial, the test organisms are considered resistant to the drug. For a susceptible population, the daily GI increase for the control would be higher than that of the drug vial. For example, if 1% of the mycobacterial population is resistant to isoniazid (INH), then 99% of the organisms would be inhibited by INH and only 1% will grow in the drug vial. The growth rate in the drug vial would be similar to the growth rate in the control vial in which the original bacterial inoculum was only 1/100 of that in the drug vial [Rodrigues et al., 2007].

2.2.3B MANUAL MICRO MGIT BACTEC CULTURE SYSTEM [Pfyffer et al, 1997]**:**

Principle:

Among the novel growth-based strategies for antimicrobial susceptibility testing (AST) is the Mycobacteria Growth Indicator Tube (MGIT) (Figure 2.3). The MGIT tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be observed using a 365 nm UV transilluminator or longwave UV light (Woods lamp) [Pfyffer et al., 1997]. Growth can also be detected by the presence of a non-homogeneous turbidity or small grains or flakes in the culture medium. The medium components are substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by tubercle bacilli and plays an important role in the metabolism of mycobacteria. Albumin acts as a protective agent by binding free fatty acids, which may be toxic to

Mycobacterium species, thereby enhancing their recovery. Dextrose is an energy source. Catalase destroys toxic peroxides that may be present in the medium .

Figure 2.3 micro MGIT (BACTEC)

The picture showing the micro MGIT BACTEC reader

2.2.3C BACTEC MGIT 960 SYSTEM

The BACTEC MGIT 960 SIRE Kit is a 4 – 13 day qualitative test. The test is based on growth of the *Mycobacterium tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control). The BACTEC MGIT 960 instrument continuously monitors tubes for increased fluorescence [Tortoli et al., 1999]. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube is used by the instrument to determine susceptibility results.

The BACTEC MGIT 960 instrument automatically interprets these results and reports a susceptible or resistant result. The BACTEC MGIT 960 susceptibility test does not interpret the degree of susceptibility of the isolate being tested. Results are reported as either S, susceptible, or R, resistant, for the drug and concentration tested. The BACTEC MGIT 960 SIRE test was developed with critical concentrations for streptomycin, Isoniazid, Rifampin and Ethambutol that are slightly lower than the critical concentrations used in the method of proportion in order to avoid false susceptibility [Tortoli et al., 1999]. Testing of the higher concentrations, as recommended, will enhance the ability to detect isolates with low-level resistance.

2.2.3D MB/BacT SYSTEM

MB/BacT is a nonradiometric antimicrobial susceptibility system for testing MTC isolates cultured from clinical samples. It was developed to provide susceptibility results for STR, INH, RIF, and EMB since 1997, but recently, critical concentrations (CCs) of the drugs listed above were modified and a new acidified vial for standardized PZA testing was introduced. The system consists of a bottle that contains a colorimetric sensor embedded in its bottom. As microorganisms grow and produce carbon dioxide, the sensor changes from dark green to yellow. The change is continuously monitored by a detection unit and promptly reported by the instrument [Benjamin et al., 1998]. Primary culture bottle growing *M. tuberculosis* is used to inoculate drug-containing bottles and a drug-free control. Drug susceptibility testing sets are entered into the instrument and continuously monitored until a positive or negative result is obtained. An organism is determined to be susceptible when the antibiotic-containing bottle remains negative or shows a positive detection time greater than drug-free control. In contrast, when the antibiotic containing bottle becomes positive or has a positive time to detection shorter than the drug-free control, the tested organism is determined to be resistant [Benjamin et al., 1998].

2.2.3E ESPII SYSTEM

The ESP Culture System II is a fully automated method originally developed for blood cultures and subsequently adapted to the detection of Mycobacteria in body fluids. The ESP technology is based on the continuous monitoring of pressure changes due to the consumption or production of gas resulting from metabolic activity of microorganisms growing in a liquid medium. Mycobacterial metabolism, characterized by consumption of oxygen, makes these organisms detectable by the reduction of pressure in the headspace of sealed culture bottles. *Mycobacterium avium-intracellularae* complex (MAC) strains were detected faster with the ESP system. For each isolate tested, six ESP II bottles were inoculated: one drug-free growth control (GC), one each with rifampin, ethambutol, and streptomycin, and two with isoniazid. Briefly, 1.0 ml of ESP II Myco growth supplement containing 7.5% (wt/vol) bovine serum albumin, 3.0% (wt/vol) dextrose, 0.0009% (vol/vol) oleic acid, 72 U of catalase per ml, and 1.275% (wt/vol) sodium chloride was added to each ESP II bottle [Tholcken et al., 1997]. To appropriately labeled ESP II bottles, 1.0 ml of each drug solution was added according to the manufacturer's protocol, giving final

concentrations of 0.1 and 0.4 µg/ml for isoniazid, 1.0 µg/ml for rifampin, and 8.0 µg/ml for both ethambutol and streptomycin. These values were selected by AccuMed based on results of experiments they had previously conducted to determine what final drug concentrations in the ESP II system correlated with the critical concentrations used with the method of proportion [Tholcken et al., 1997]. The GC bottle received 1.0 ml of distilled water. Each ESP II bottle was then inoculated with 0.5 ml of a 1:10 dilution (in sterile saline) of the McFarland-equivalent inoculum or with 0.5 ml of a 1:10 dilution of broth from the seed bottle. A connector was placed on each bottle, and the bottles were loaded into the ESP II instrument. To test for sterility, a blood agar plate was inoculated with 0.5 ml of the inoculum and incubated at 37°C for 48 h.

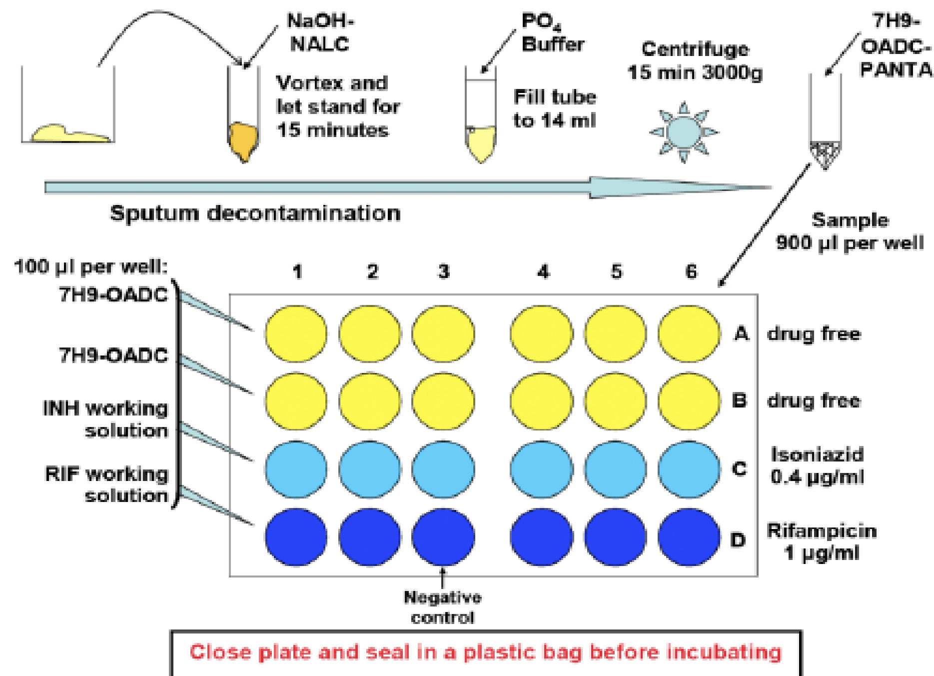
An isolate was considered susceptible to a drug if there was no growth in the drug-containing bottle or if the time to detection of growth in the drug-containing bottle was greater than the time to detection of growth in the GC bottle (rounded to the nearest whole number) plus 3 days. An isolate was considered resistant to a drug if the time to detection of growth in the drug-containing bottle was less than the time to detection of growth in the GC bottle (rounded to the nearest whole number) plus 3 days. To confirm that the positive signal was due to growth of resistant MTBC and not to bacterial contamination, a blood agar plate was inoculated with 0.5 ml of broth from each positive ESP II bottle, incubated at 37°C, and examined for bacterial colonies at 48 h. According to the manufacturer's protocol, if the GC is not positive by day 10, the run must be considered invalid, and testing of all drugs must be repeated [Tholcken et al., 1997].

2.2.4 RAPID CULTURE METHODS:

2.2.4A MICROSCOPICALLY OBSERVED DRUG SUSCEPTIBILITY (MODS):

The MODS assay is a microcolony method, based on direct inoculation of patient specimens to drug-free and drug-containing liquid media (incubated at 37°C for 3 weeks) followed by microscopic examination of early culture growth (Figure 2.4). The media used is Middlebrook 7H9 broth with OADC and PANTA in 24 wells tissue culture plate. Growth of *M. tuberculosis* is identified by typical cord formation under an inverted light microscope [Angeby et al., 2002].

Figure 2.4 Microscopically Observed Drug Susceptibility



OADC-PANTA- Oleic acid, Albumin, Dextrose, Catalase- Polymyxin, Amphotericin, Nalidixic acid, Trimethoprim, Azirocillin

INH- Isoniazid, RIF- Rifampicin, PO_4 buffer- Phosphate buffer

Interpretation:

- Sensitive-Growth of *M.tuberculosis* only in drug free wells
- Resistant-Growth of *M.tuberculosis* in drug free media and in drug containing wells

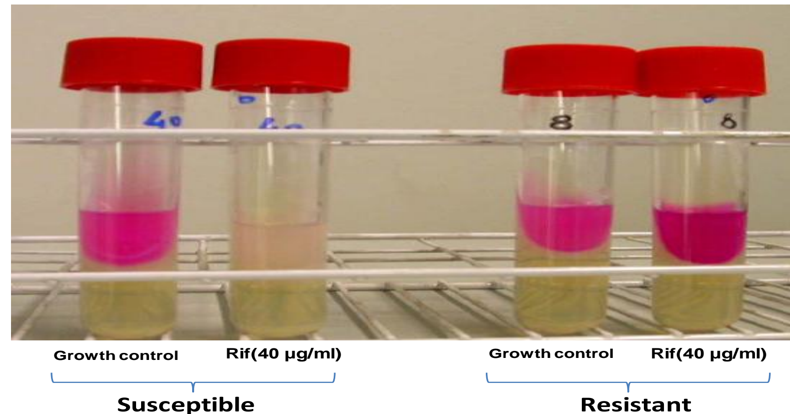
Advantages:

- *M. tuberculosis* grows faster in liquid medium
- Typical chord formation observerd before visualization of colonies on solid agar
- Provides low cost, safe ,early detection of resistant strains

2.2.4B NITRATE REDUCTASE ASSAY (NRA):

The NRA is a solid culture technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding a specific reagent (Griess reagent) to conventional Löwenstein-Jensen (LJ) medium into which 1 mg/ml of potassium nitrate (KNO3) has been incorporated [Yajko et al., 1995]. The reduction of nitrate is detected by a coloured reaction. Resistance testing is done by inoculating drug-free and drug-containing media (Figure 2.5). Detection the coloured reaction on the drug-free medium alone indicates a positive culture and drug susceptibility; growth in both drug-free and drug-containing media indicates resistance. The NRA test can be used as a direct or indirect test.

Figure 2.5 Nitrate Reductase Assay



Interpretation:

Red colour- drug-free medium alone -susceptible

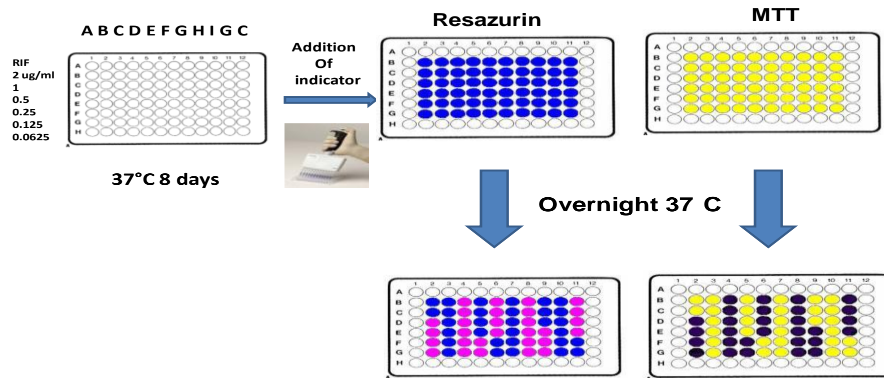
Red colour- both drug-free and drug-containing media -resistance.

2.2.4C COLORIMETRIC INDICATOR ASSAY (CRA):

CRI methods are indirect methods based on the reduction of a colored indicator added to liquid culture medium in a microtiter plate after *M. tuberculosis* has been exposed in vitro to different antibiotics and different drug concentrations [Heydenrych et al., 2002]. Resistance is detected by a change in color of the indicator, which is proportional to the number of viable Mycobacteria in the medium. Among the different growth indicators used are the tetrazolium salts XTT (2,3-bis(2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide and MTT(3(4,5-dimethylethyl- thiazol-2-yl)-2,5-

diphenyltetrazoliumbromide), and the redox-indicators Alamar blue and resazurin [Heydenrych et al., 2002] (Figure 2.6).

Figure 2.6: Colorimetric Indicator Assay



Interpretation:

Minimal inhibitory concentration (MIC)-lowest drug concentration that prevent the change of color

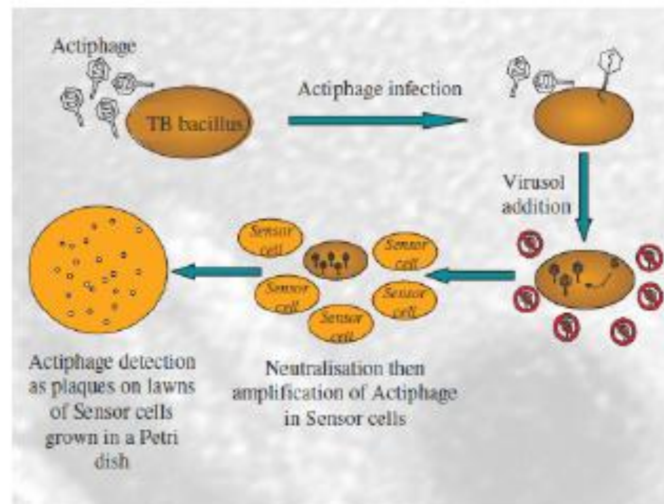
2.2.4D PHAGE BASED ASSAY:

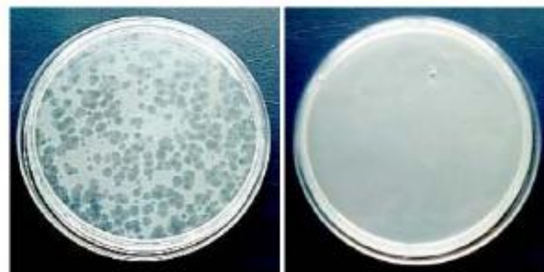
Phage-based assays utilize bacteriophages to infect and detect the presence of viable *M. tuberculosis* in clinical specimens and culture isolates. Two main approaches have been developed [Heydenrych et al., 2002]:

- Amplification of phages after their infection of *M. tuberculosis*, followed by detection of progeny phages using sensor bacteria and measuring plaque formation, and
- Detection of light produced by luciferase reporter phages (LRP) after their infection of live *M. tuberculosis*. When these assays detect *M. tuberculosis* in drug-free specimens, but fail to detect *M. tuberculosis* in drug-containing specimens, the strains are classified as drug susceptible. Several investigators have evaluated indigenously amplification assays using D29 phages and their results have been included in the systematic reviews.
- Currently, there is one commercial phage-based test on the market, ie. the FASTPlaque™ assay (Biotec Laboratories Limited, Ipswich, Suffolk, UK). This assay is based on infection of *M. tuberculosis* present in the specimen by specific mycobacteriophages that,

after amplification and release, are allowed to infect a lawn of non-pathogenic organisms within an agar plate, resulting in plaque formation on the surface of the agar [Heydenrych et al., 2002]. Appearance of plaques is indicative of *M. tuberculosis* growth. The first generation test for detection of rifampin resistance, the FASTPlaque – RIF™ or FASTPlaque – MDRi™, was used only with *M. tuberculosis* isolates from cultures, ie. in indirect testing. This has now been replaced by the FASTPlaque – Response™ which can be used as a direct test on patient specimens as well as an indirect test on *M. tuberculosis* isolates [Albert et al., 2004].

Figure 2.7: Phage Based Assay





Plaques – viable MTB cells present

No plaques – no viable MTB cells present

Interpretation:

- Resistant -plaque formation in both drug free and drug containing plate. sensitive-plaque formation only in drug free plate not in drug containing plate

2.3 PCR based DNA sequencing technique targeting drug resistance genes of First line anti-tuberculosis drugs (Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide).

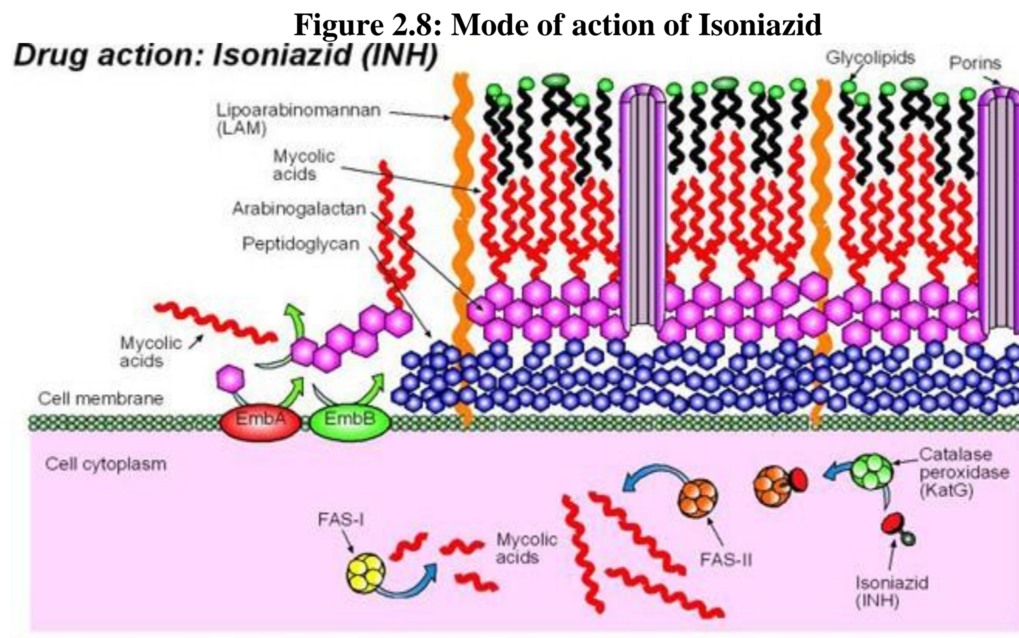
2.3.1 Isoniazid:

Isoniazid was first introduced for the treatment of *M. tuberculosis* in 1952 and because of its relative efficacy and low toxicity it has become one of the mainstays of first-line therapy for tuberculosis [Banerjee et al., 1994]. Although it is also the most intensively studied of anti mycobacterial agents, the molecular mechanisms of its bactericidal activity are still not well understood.

2.3.1A Mode of action of Isoniazid (Figure 2.8):

Isoniazid is a prodrug that requires activation by the mycobacterial catalase peroxidase KatG after it enters the cell by passive diffusion. The activated isoniazid targets two principal enzymes that are involved in the elongation cycle of the fatty acid molecules, an enoyl-acyl carrier protein reductase (inhA) and a beta ketoacyl-acyl carrier protein synthase, resulting in the inhibition of synthesis of the mycolic acids necessary for the mycobacterial cell wall [Banerjee et al., 1994].

Figure 2.8: Mode of action of Isoniazid



Source: **(http://immunopaedia.org.za/index.php?id=261)**

The first line antibiotic drug INH interfers with cell wall biosynthesis in *M. tuberculosis*. INH is a prodrug and is coverted to an enzyme form by catalase peroxidise. Activated INH inhibits the action of enoly-acly carrier protein reductase and interrupts mycolic acid synthesis.

2.3.1B Genes encoding for Isoniazid drug resistance:

Drug resistance mutations in the *katG* gene results in loss of the ability of the catalase to activate the prodrug of Isoniazid. Mutations in the *inhA* gene or its promoters may alter the activated isoniazid binding site or increase InhA production resulting in INH resistance [Banerjee et al., 1994]. Although in *katG* gene insertions, deletions, and frameshift mutations do occasionally happen and induce complete loss of the functional gene product and correspondingly high rates of Isoniazid resistance, most mutations identified in clinical isolates are single-point mutations that result in intermediate resistance.

While *katG* gene mutations may confer high-level isoniazid resistance, *inhA* mutation may cause low-level isoniazid resistance to INH. Although Isoniazid mutations most frequently occur in the *katG* and *inhA* genes, they also occur in other enzymes coding genes such as *ndh, ahpC*, and *furA*. Between 31–97% of INH resistance has been attributed to katG mutations (at codon 315), with higher frequencies occurring in TB-endemic countries [Banerjee et al., 1994]. In a recent study by Dalla Costa et al [Costa et al., 2004], of 224 INH-resistant *M. tuberculosis* isolates from Argentina, Brazil, and Peru the frequency of *inhA* mutations was 11%. Eighty-six percent had either a katG or inhA mutation associated with INH resistance.

Subsequent studies of the role of ahpC in isoniazid resistance have yielded inconsistent results. Others have suggested that ahpC may have a more direct role in isoniazid resistance. Telenti et al [Telenti et al., 1995] proposed that AhpC may be a primary contributor to resistance, noting that a significant percentage of isoniazid-resistant clinical isolates had ahpCpromoter mutations even in the absence of katG mutations. Zhang et al [Leung et al., 2006, Rinder et al., 1998] hypothesised that excess AhpC may act independently from KatG to block activation of isoniazid.

2.3.2 Rifampicin:

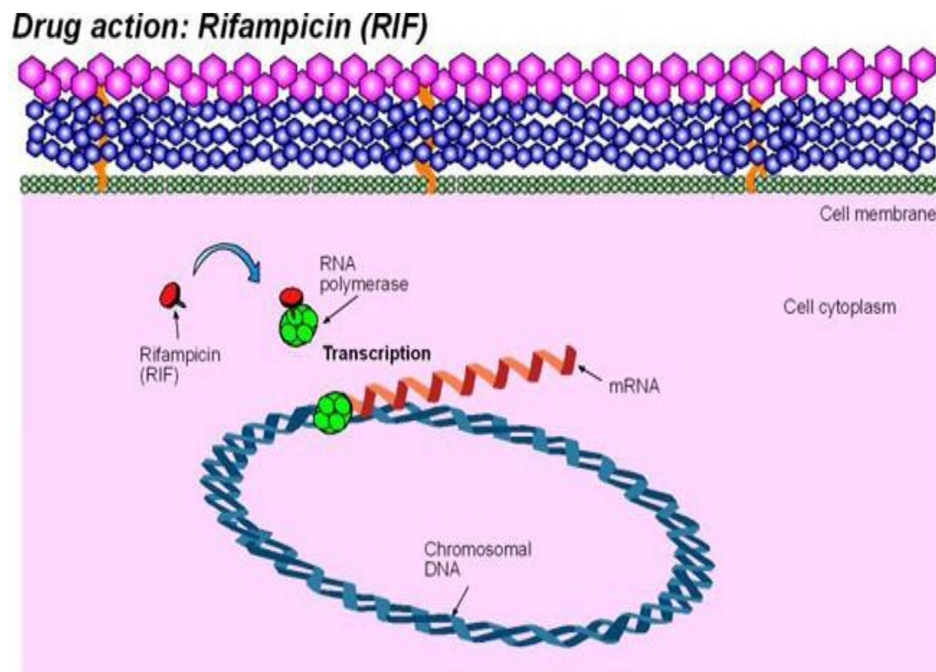
Rifampicin is another widely used first-line anti-tuberculous drug to which resistance has become increasingly common in the past decade. Rifampicin acts by binding to bacterial RNA polymerase and disrupting mRNA synthesis. Clinical resistance to this agent results almost exclusively from mutations in the *rpoB* gene that encodes the beta subunit of the bacterial RNA polymerase Rifampin inhibits transcription and thus protein synthesis by

targeting one of the four subunits, the β subunit, of the mycobacterial DNA-dependent RNA polymerase which is coded by the *rpoB* gene [Rattan et al., 1999]. Certain mutations in the *rpoB* gene reduce the binding affinity of rifampin for the RNA polymerase, resulting in drug resistance.

2.3.2A Mode of action of Rifampicin (Figure 2.9):

RNA polymerase is a complex oligomer composed of four different subunits (α, β, β', and σ encoded by rpo A, rpo B, rpo C and rpo D, respectively). The subunits are assembled in two major forms: a core enzyme (α_2, β, β') and a holoenzyme (α_2, β, β'plus a σ subunit). The core enzyme can perform RNA polymerization but requires σ subunit to initiate site-specific transcription at promoter sites. RIF interferes with transcription and elongation of RNA by binding to the β subunit of RNA polymerase and there by interruption of transcription process.

Figure 2.9: Mode of action of Rifampicin



Source: (http://immunopaedia.org.za/index.php?id=261)

The first line antibiotic drug RIF interfers with RNA transcription in *M. tuberculosis*. RIF binds to the β-subunit of the DNA dependent RNA polymerase enzyme complex and inhibits transcription of mRNA and thereby interrupting the protein synthesis

2.3.2B Gene coding for Rifampicin drug resistance:

The genetic basis for Rifampicin resistance in approximately 95 % of the cases is due to mutations in an 81-bp rifampicin resistance determining region (RRDR) of the *rpoB* gene corresponding to 507-533 codons that code for the β subunit of RNA polymerase of *M. tuberculosis*. The types of mutations include single-nucleotide changes and deletions and insertions. High-level resistance to rifampin occurs at a rate of 10^{-8} in *M. tuberculosis in vitro.* Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been shown to be strongly associated with RIF resistant phenotypes in multiple study populations.

Alterations at codon 531of *rpoB* gene have been identified as being the most common alteration in RIF-resistant *M. tuberculosis*. Most commonly affected amino acid lies at codon 531of the *rpoB* gene that results in replacement of wild type ser531 with Leu, Ala or Trp [Hirano et al., 1999]. The next most common mutations were the amino acid substitutions Asp516Val or Asp516Gly, His526Tyr, His526Leu, or His526Arg. Rare mutations occurred at codons Ser509Arg, Leu511Val, Asn518Thr, Ser522Gln, Lys527Asn, Arg528Pro, and Arg528His, Gln510His [Hirano et al., 1999].

Mutations in codons 516 and 521 conferred low-level resistance (MIC, <40 µg/ml) to rifampin, whereas mutations in codons 510, 526, 527, 528, and 531 were seen to confer high levels of resistance (MICs, ≥64 µg/ml) [Table 2.3]. Amino acids 526 to 531 appear to be very important in drug target interactions, and mutations in them result in MICs in the range of 64 µg/ml and above [Zhang et al., 2006]. Rifampin resistance is considered a major surrogate marker for MDR TB, since greater than 90% of isolates resistant to rifampin are also resistant to isoniazid.

Table 2. 3: Hot spot mutations reported in rpoB gene for RIF resistance

S.No	Mutations at Codons For RIF resistance	Resistance level	MIC
1	531, 510, 526, 527, 528, 516, 509, 511	High level resistance	≥64 µg/ml
2	516 and 521	Low level resistance	<40 µg/ml

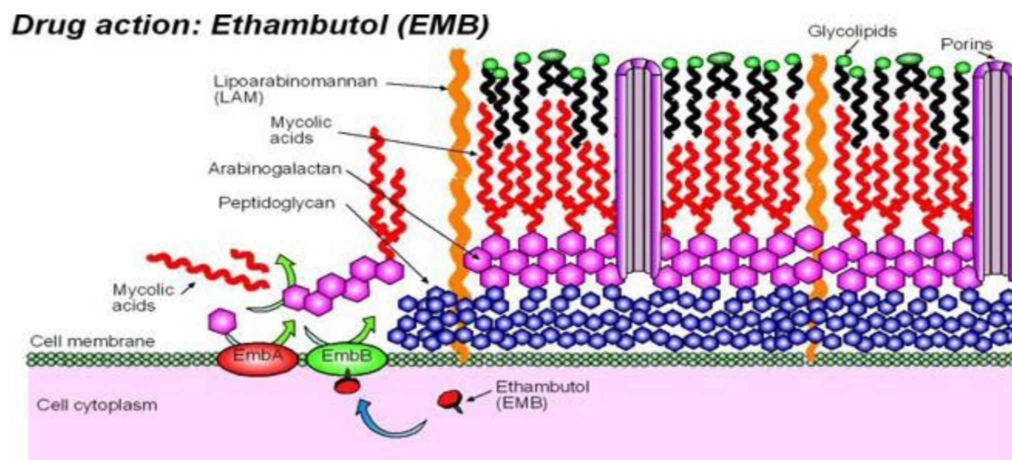
2.3.4 Ethambutol :

Ethambutol (EMB) is a chemical synthetic molecule. Its chemical name is dextro-2,2_-(ethylenediimino)di-1-butanol and its molecular formula is $C_{10}H_{24}N_2O_2$

2.3.4A Mode of Action of Ethambutol (Figure 2.10):

EMB is an important first-line tuberculosis drug. The minimum inhibitory concentration for susceptible strains is 1 to 8 μg/ml, depending on the culture medium used. EMB targets the mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalacton(AG) and lipoarabinomannan (LAM) biosynthesis. It specifically inhibits polymerization of cell-wall arabinan, thereby leading to accumulation of b-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA) leading to accumulation of mycolic acid, which results in cell death [Wolucka et al., 1994].

Figure 2.10: Mode of action of Ethambutol



Source: (http://immunopaedia.org.za/index.php?id=261)

The first line antibiotic drug EMB interfers with the cell wall biosynthesis in *M. tuberculosis*.EMB inhibits the action of arabinosyl transferase involved in synthesis of arabinogalactan

2.3.4B Gene coding for Ethambutol drug resistance:

The *emb* genes are conserved among different mycobacteria. The arabinosyl transferases are encoded by *emb* genes belonging to the *emb* operon and have been identified in *M. tuberculosis.* The Emb proteins form a family of large (>1100 residues) trans membrane

proteins with a cytoplasmic N-terminal domain, 13–15 trans membrane segments and a large extra cytoplasmic C-terminal domain. Emb proteins are considered to be good drug targets, since the current antitubercular drug, ethambutol, and causes cessation of the synthesis of arabinan polymers (AG and LAM). In *M. tuberculosis,* they belong to an operon comprising three genes, *embC, embA* and e*mbB that* are required for the biosynthesis of the arabinan components of AG and LAM [Sreevatsan et al., 1997]. It appears that both EmbA and EmbB are dedicated to the biosynthesis of the arabinan portion of AG 32, whereas EmbC is involved in LAM biosynthesis. It was recently identified that the *embCAB* gene cluster from *M. tuberculosis* showed that EMB resistance could result from overproduction of the Emb protein, structural mutation of the EmbB protein, or both.

Alterations at codon 306 of *embB* have been identified as being the most common alteration in EMB-resistant *M. tuberculosis* clinical isolates. The association between *embB*306 mutations and EMB resistance in clinical *M. tuberculosis* isolates is so strong that it has been proposed as a marker for EMB resistance in diagnostic tests [Sreevatsan et al., 1997]. Mutations leading to replacement of amino acid residues are found to be present in EMB-resistant organisms cultured from humans. Most commonly affected amino acid lies at codon 306 of the *embB* gene that results in replacement of wild type Met306 with Ile, Leu or Val. Most studies show that 65% clinical isolates harbour mutation at 306 amino acid position, forming ethambutol resistance determining region (ERDR) [Safi et al., 2010]. The second most frequent mutation encountered was at codon Asp299Glu followed by Asn296Lys.The other rare mutations occurred at codons Leu239Pro, Asp240His, Asp311His, Leu304Trp and Arg257Trp. Ramaswamy *et al* [Ramaswamy et al., 2000] identified up to seven nucleotide polymorphisms not altering the amino acid i.e., at codon 259 (GCA →GCC) and codon 303 (ATC →ATT) and they also suggest targeting amino acids 300 to 500 of the *embB* gene for mutation studies [Perdigã et al., 2009]. Identification of additional mutations occurring in EMB-resistant organisms will be useful in further understanding of the mechanisms of resistance to this primary antituberculosis agent.

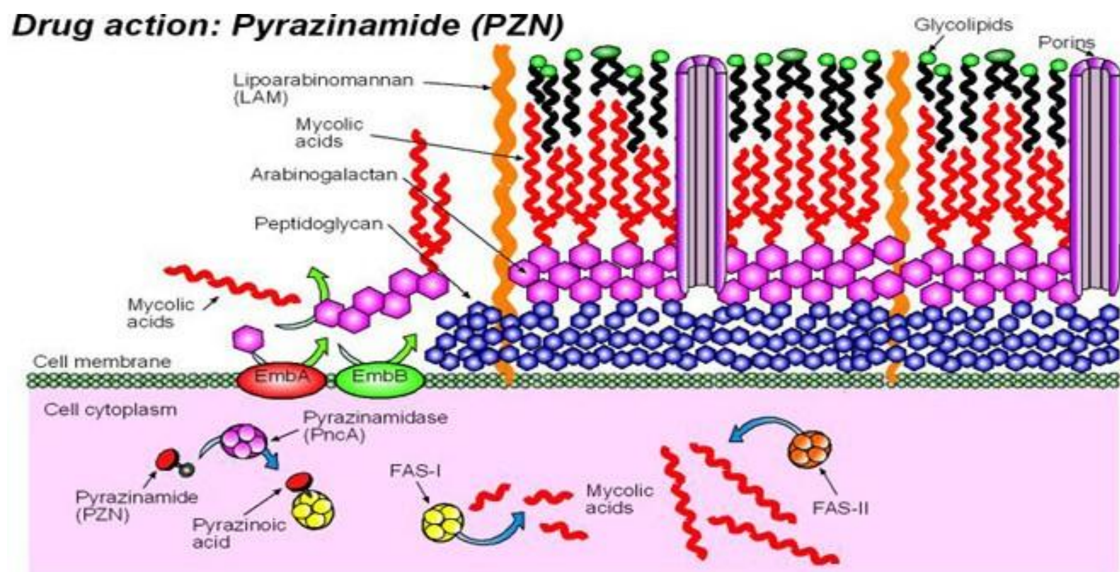
2.3.5 Pyrazinamide:

Pyrazinamide (PZA) is the first-line drug used in the treatment of tuberculosis along with Isoniazid and Rifampicin and it inhibits semi-dormant Mycobacteria only at low pH *invitro*.

2.3.5A Mode of action of Pyrazinamide (Figure 2.11):

Pyrazinamide is a prodrug that must be metabolised by mycobacterial pyrazinamidase to pyrazinoic acid, which interferes with fatty acid synthesis. Most mutations that provide pyrazinamide resistance in clinical tuberculosis isolates are seen in *pncA* gene region and impair pyrazinamidase-mediated activation of the prodrug although other mutations may interfere with drug uptake or efflux.

Figure 2.11: Mode of action of Pyrazinamide



Source: (http://immunopaedia.org.za/index.php?id=261)

The first line antibiotic drug PZA interfers with cell wall biosynthesis in *M. tuberculosis*. PZA is a pro drug and is coverted into pyrazonic acid by nicotinamidase peroxidase and interepts the fattyacid synthesis.

2.3.5B Gene coding for Pyrazinamide resistance:

The *pncA* gene encodes pyraziniamidase (PZase), and mutations in *pncA* gene are associated with resistance to PZA [Dalme et al., 1936, Yeager et al.,1952] or loss of PZase activity. PZA acts by targeting the fatty acid synthase/synthetase enzyme, and is

responsible for the killing of persistent tubercle bacilli in the initial intensive phase of chemotherapy. It is a prodrug that is converted to its active form namely, pyrazinoic acid (POA) by the catalytic action of PZase enzyme, encoded by the *pncA* gene in *M. tuberculosis.* Interestingly, PZA is active only at low pH since acidic environment favours accumulation of POA in the cytoplasm due to an ineffective efflux pump, thereby leading to improper efflux out of the amidase from the cell to the exterior [Yeager RL et al.,1952, Morlock et al.,2000].

A large number of mutations have been described, but no mutational hotspots have been identified so far [Scorpio et al., 1997]. This can be explained by the fact that mutations occur along the entire length of the *pncA* gene [Somoskovi et al., 2001]. But studies have shown that the mutations that confer the PZA resistance occur mainly in the putative promoter region of the *pncA* gene.

Scorpio and Zhang in 1996 [Scorpio et al., 1996] had identified the PZase gene (*pncA*) from *M. tuberculosis* and had shown that *pncA* gene mutations are a major mechanism of PZA resistance [Scorpio et al., 1996]. The identified *pncA* mutations are largely missense mutations causing amino acid substitutions, and in some cases nucleotide insertions or deletions and nonsense mutations in the *pncA* structural gene or in the putative promoter region of *pncA* [Sreevatsan et al., 1997]. The uniqueness in the mutations of *pncA* gene is its diversity and scattering along the whole gene though there does appear to be some degree of clustering at three regions of *pncA* protein (3 to 17, 61 to 85, and 132 to 142).

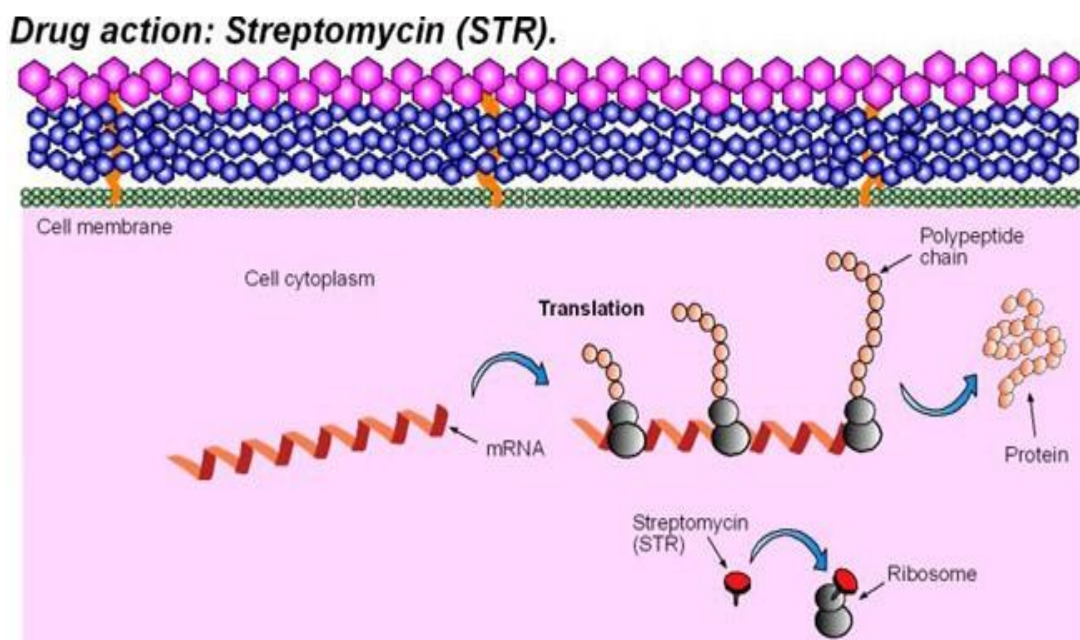
2.3.6 Streptomycin:

STR, an aminocyclitol glycoside, is an alternative first line anti-TB drug recommended by the WHO. STR is therefore used in the re-treatment of TB cases together with the four drug regimen that includes INH, RIF, PZA and EMB [Brzostek et al, 2004].

2.3.6A Mode of action of Streptomycin (Figure 2.12):

Streptomycin is a protein synthesis inhibitor. It binds to 16S rRNA of the smaller ribosomal subunit, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit leading to codon misreading, eventual inhibition of protein synthesis and ultimately death of *M.tuberculosis* [Brzostek et al, 2004].

Figure 2.12: Mode of action of Streptomycin



Source: (http://immunopaedia.org.za/index.php?id=261)

The first line antibiotic drug STR interfers with translation of mRNA transcripts in *M. tuberculosis* and inhibits the protein synthesis

2.3.6B Gene coding for Streptomycin drug resistance:

Mutations associated with streptomycin resistance have been identified in the genes encoding 16S rRNA (rrs) [Gale EF et al., 1981]protein S12 (rpsL) [Douglass et al., 1993, Finken et al., 1993] comprises the highly conserved pseudoknot structure formed by 16S rRNA [Cooksey et al., 1996]. Amino acid substitutions in *rpsl* affect the higher-order structure of 16S rRNA [Gale EF et al., 1981] and confer streptomycin resistance. Alterations in the 16S rRNA structure disrupt interactions between 16S rRNA and streptomycin, a process that results in resistance [Okamoto et al., 2007]. Mutations in rpsL and rrs are the major mechanism of STR resistance [Okamoto et al., 2007, Spies et al., 2008], accounting for respectively about 50% and 20% of STR-resistant strains [Okamoto et al., 2007, Spies et al., 2008]. The most common mutation in *rpsl* gene is a substitution in codon 43 from lysine to arginine, causing high-level resistance to STR. Mutation in codon 88 is also common. Mutations of the *rrs* gene occur in the loops of the 16S rRNA and are clustered in two regions around nucleotides 530 and 915 [Okamoto et al., 2007, Spies et al., 2008]. However, about 20–30% of STR-resistant strains with a low level of resistance

(MIC < 32 μg/ml) do not have mutations in *rpsl or rrs* genes, which indicates other mechanism(s) of resistance. A mutation in gidB, encoding a conserved 7-methylguanosine (m (7) G) methyltransferase specific for 16S rRNA, has been found to cause low-level SM resistance in 33% of resistant *M. tuberculosis* isolates [Spies et al., 2008]. A subsequent study showed that while Leu16Arg change is a polymorphism not involved in STR resistance, other mutations in *gidB* gene appear to be involved in low-level STR resistance. In addition, some low-level STR resistance seems to be caused by increased efflux as efflux pump inhibitors caused increased sensitivity to STR, although the exact mechanism remains to be identified.

2.3.7 REPORTS OF MDR-TB FROM VARIOUS PARTS OF INDIA:

The Indian Council of Medical Research (ICMR) [ICMR, 1968] undertook drug resistance studies during 1965-67 in 9 urban areas of the country. The first study was on patients who had denied any history of previous treatment, while in the second study, patients with and without previous chemotherapy were included. The results showed that in the first study resistance to INH ranged from 11-20%, to STR from 8-20% and to both drugs from 4-11%. The second study showed resistance to INH to range from 15-69%, to streptomycin from 12-63% and to both drugs from 5-58%. Further, the level of drug resistance was proportional to the duration of previous treatment.

A decade later, a study at the Government Chest Institute and Chest Clinic of Government Stanley Hospital (GCI-SH), Chennai [Chandrasekaran et al., 1992] reported similar results to those of earlier ICMR surveys [ICMR, 1968], indicating that the prevalence of initial drug resistance had not risen during the span of ten years.

During the 1980s, though the levels of initial drug resistance to INH and STR in 3 reports [Krishnaswamy et al., 1976, Chandrasekaran et al., 1992, Narang et al., 1992] were similar to those in the earlier studies, RIF resistance was observed in all the centres studied except Gujarat [Narang et al., 1992]. The level of MDR-TB in all the centres (except Wardha) was observed to be less than 5%. The reason for the emergence of RIF resistance during this period may be the introduction of short course chemotherapy (SCC) regimens containing RIF. In the early 1990s, a retrospective study done at New Delhi showed a

high level of initial drug resistance to INH (18.5%) and a low level (5.7%) of resistance to RIF [Narang et al., 1992].

C.N. Paramasivan et al [Paramasivan et al., 2002] from Tuberculosis Research Centre (ICMR), Chennai determined the levels of drug resistance in new and previously treated cases of pulmonary tuberculosis in the composite districts of North Arcot (Tamil Nadu State) and Raichur (Karnataka State) in South India. They found that in North Arcot district, resistance to any drug tested was 27.7% in new cases; INH resistance in 23.4%, RIF resistance in 2.8% and Multidrug resistance (resistance to both INH and RIF: MDR) in 2.8%. In previously treated cases, resistance to any drug was observed in 81.2% of *M. tuberculosis* isolates and any resistance to INH, RIF and both INH and RIF in 81%, 69% and 69%, respectively. In Raichur district, resistance to any drug was observed in 21.9% of new cases; any resistance to INH, RIF and INH-RIF (MDR) was found in 18.7%, 2.5% and 2.5%, respectively. All previously treated patients were resistant to INH and RIF (100%).

Almeida et al [Almeida et al., 2003] compared the incidence of multidrug resistance in 150 consecutive *M. tuberculosis* isolates obtained from a rural center (in Sakawar, India) and an urban tertiary care center (in Mumbai, India). The study highlights an alarmingly high percentage of multidrug-resistant *M. tuberculosis* isolates in Mumbai (51%) as compared with that at the rural center (2%).

Aleyamma Thomas et al [Aleyamma Thomas et al., 2007] from Tuberculosis Research Centre studied on management of multi drug resistance tuberculosis Thiruvallur district, south India (1999-2003). They found that, of 66 MDR-TB patients (46 from the rural and 20 from the Non government organization) started on treatment form the study population 20 (30%) were resistant to one or more second line drugs including a case of “XDR-TB”. Successful treatment outcome was observed only in 37% of cases and treatment failure in 27%.

A study was conducted by Udwadia ZF et al from the Hinduja Hospital and Research Center in Mumbai with 1354 patients. 724 were culture positive and 45% were MDR-TB. 11% of the MDR-TB were turned to be XDR-TB [Udwadia et al., 2007].

Sharma et al [Sharma et al., 2011] from All India Institute of Medical Sciences, New Delhi conducted a study on “Prevalence of multidrug-resistant tuberculosis among Category II

pulmonary tuberculosis patients". Of 196 category II sputum positive pulmonary TB patients in New Delhi were analysed and found 40 (20.4%) were MDR-TB. Out of 40 MDR-TB patients, 4 patients showed resistance to STR in addition to RIF and INH.

Hanif et al estimated the prevalence of MDR-TB among previously treated tuberculosis (TB) cases at the State TB Demonstration Centre, Delhi. They found that the MDRTB rate was 47.1% and they concluded by saying that high rate of MDR was observed among treatment failures compared to relapses and defaulters [Sharma et al., 2011].

Singla et al [Singla et al., 2009] from Lala Ram Sarup Institute of Tuberculosis and Respiratory Diseases, New Delhi studied on constraints and issues of a pilot DOTS-Plus experience in an urban setting in India. Of 126 MDR patients enrolled in their study, 61% were cured, 19% died, 18% defaulted and 3% failed treatment and there was an average delay of 5 months in the diagnosis of MDR-TB. They came to a conclusion that the DOTS-Plus programme in resource-poor settings may provide reasonable results; however, it may confront significant operational difficulties in the timely diagnosis and early initiation of treatment.

Joseph et al [Joseph et al., 2011] from National Institute of Research in Tuberculosis, Chennai has studied the outcome of standardized treatment for patients with MDR-TB and found that the standardized regimen recommended by RNTCP for the treatment of MDR-TB cases in India appears to be effective in terms of high culture conversion, high cure (66%) and low death (8%) in this setting.

A recent study from Hinduja hospital, Mumbai done by Udwadia, F et al [Udwadia et al., 2011] reported four cases of "total drug resistant tuberculosis"(TDR-TB) . According to this report, these patients have shown resistance to all the first line TB drugs (isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin) and to seven second line anti-TB drugs (ofloxacin, moxifloxacin, kanamycin, amikacin, capreomycin, para-aminosalcyclic acid and ethionamide).

Maurya et al [Maurya et al., 2013] reported on the prevalence of MDR-TB (July 2007-Dec 2010), first-line drug resistance patterns and its changing trends in Northern India from July 2007 to December 2010. They found the overall prevalence rate of MDR-TB to be 38.8%, increasing from 36.4% in 2007 to 40.8% in 2010 and the prevalence of MDR-TB in new and previously treated cases was 29.1% and 43.3% ($P < 0.05$; CI 95%). The increasing

trend of MDR-TB was more likely in pulmonary TB when compared with extra-pulmonary TB ($P < 0.05$; CI 95%).

Behera et al [Behera et al., 2013] carried out various surveys in India by well-qualified and accredited laboratories and reported that MDR-TB in new cases is about 2.1% (1.5-2.7) and is about 15% (13-16) in treated cases.

In the global scenario, about 9% of all MDR cases are XDR-TB. Although, the exact extent of XDR-TB in India is not known, the Gujarat survey carried out by the Central TB Division through National Institute for Research in Tuberculosis (NIRT), Chennai showed that of 1,571 isolates from new patients, 1,236 (78.7%) were susceptible to all first-line drugs, 173 (11%) had only INH resistance and MDR-TB were only 37 (2.4%) with 95% confidence interval (CI) 1.6-3.1). Of 1,047 isolates from previously treated patients, 564 (54%) were susceptible to all first-line drugs, 387 (37%) had only INH resistance, and 182 (17.4%, 95% CI 15.0-19.7%) were MDR-TB.

2.4 Insilico Analysis of Novel Mutation Ala102Pro Targeting *pncA* Gene of *Mycobacterium tuberculosis* using Bioinformatics tools.

A large number of mutations have been described, but no mutational hotspots have been identified so far [Scorpio et al., 1997]. This can be explained by the fact that mutations occur along the entire length of the *pncA* gene [Somoskovi et al., 2001]. But studies have shown that the mutations that confer the PZA resistance occur mainly in the putative promoter region of the *pncA* gene.

Scorpio and Zhang in 1996 [Scorpio et al., 1996] had identified the PZase gene (*pncA*) from *M. tuberculosis* and had shown that *pncA* gene mutations are a major mechanism of PZA resistance [Scorpio et al., 1996]. The identified *pncA* mutations are largely missense mutations causing amino acid substitutions, and in some cases nucleotide insertions or deletions and nonsense mutations in the *pncA* structural gene or in the putative promoter region of *pncA* [Sreevatsan et al., 1997]. The uniqueness in the mutations of *pncA* gene is its diversity and scattering along the whole gene though there does appear to be some degree of clustering at three regions of *pncA* protein (3 to 17, 61 to 85, and 132 to 142). These regions are likely to contain catalytic sites for the Pzase enzyme [Lemaitre et al., 1999]. The catalytic residues comprise the active site (D8, K96, A134 and C138) and the

metal-binding site (D49, H51 and H71) [Lemaitre et al., 2001, Ramaswamy et al., 1998]. Cys-138, Ala-134, Thr-135, Trp-68, and Asp-8 in the *M. tuberculosis* PZase could be key residues for hydrolysis of PZA [Lemaitre et al., 1999]. At the protein level, these regions were found to be well conserved among the amino acid sequences of *pncA* proteins from different bacterial species.

However, a critical point in this approach is to correctly interpret at the level of the structure/ function relationships of *PncA* the effects of the mutations detected by the pncA sequencing, first because of the high degree of diversity of the mutations that can be found in PncA, and secondly, because a significant proportion of PZA-susceptible clinical isolates display amino acid substitutions in PncA that have no significant effect on the pyrazinamidase activity of the enzyme.

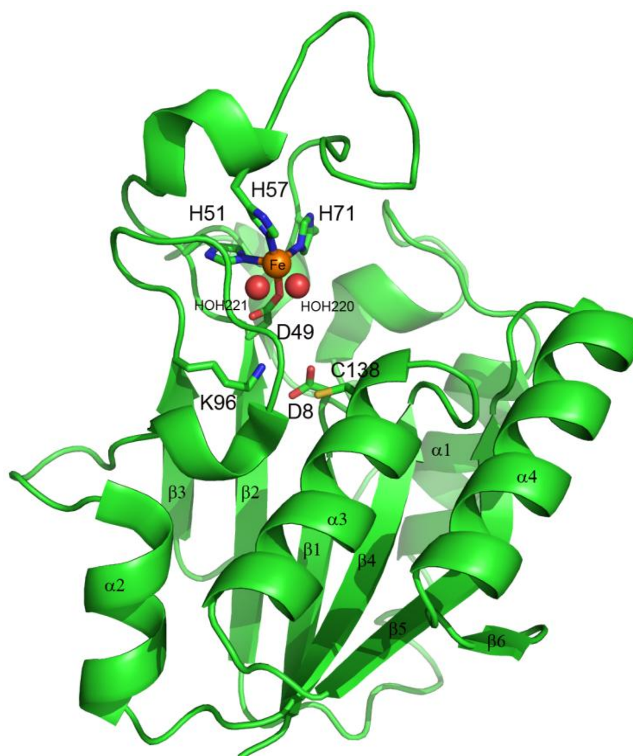
2.4.1 Crystal structure of pncA protein:

Different models of the three-dimensional structure of PncA of *M. tuberculosis* were established with this purpose [Lemaitre et al., 2001]. The first to be reported was based on the structure of the N-carbamoylsarcosine amidohydrolase (CSHase), an enzyme harbouring, like PncA, the isochorismatase domain (PF00857) [Lemaitre et al., 2001]. Using this model, they found that the level of activity of PncA mutants correlated with the location of the mutations with respect to the active site residue Cys138 involved in the nucleophilic attack of PZA. Du and colleagues established the crystal structure of the pyrazinamidase from Pyrococcus horikoshii (PhPncA) [Du et al., 2001]. The enzyme was found to have an active cysteine residue at position 133 in the *P. Horikoshii* numbering system (Cys138 in MtPncA), as well as a Zn2+ ion coordinated by 2 His residues, His54 and His71 (His51 and His71 in MtPncA) and one aspartate residue Asp52 (Asp49 in MtPncA). Formation of the acylenzyme was suggested to involve the thiolate form of Cys133 activated by another aspartate, Asp10 (Asp8 in MtPncA) acting as general base, and stabilized by Lys94 (Lys96 in MtPncA). A cis-peptide bond was identified between Val128- Ala129 in PhPncA (Ile133-Ala134 in MtPncA) which contributes to the formation of an oxyanion hole between the main chain NH of Cys133 on one hand and the one of Ala129 on the other Cys138 and Ala134 in MtPncA, respectively).

In PhPncA, the function of the Zn^{2+} ion was suggested to be catalytic through activation of a water molecule for hydrolysis of the thioester bond formed between the catalytic cysteine residue and the substrate in the acylenzyme intermediate. In a more recent publication, the crystal structure of *Acinetobacter baumanii* PncA (AbPncA) in complex with nicotinic acid has been established [Du X et al., 2001]. The enzyme was found to be a divalent cation-dependent enzyme with Fe^{2+} /Zn^{2+} (ratio 1:1) hold on by 3 coordinating residues (Asp54, His56 and His89). Nicotinic acid was found to be directly tethered to the Zn^{2+} ion , its carboxylate group being hydrogen-bounded to the main-chain amides of cis-Ala155 and Cys159 forming the oxyanion hole in AbPncA (corresponding to Ala134 and Cys138 in MtPncA) [Figure 2.13].

The crystal structure of the *M. tuberculosis pncA* protein has been determined, showing significant differences in the substrate binding cavity when compared to the pyrazinamidases from *Pyrococcus horikoshii* and *Acinetobacter baumanii*. In *M. tuberculosis*, this region was found to hold a Fe^{2+} ion coordinated by one aspartate and three histidines, the most crucial structural elements in this loop appears to be the specific positioning of residue His57 which is directly involved in the coordination of the Fe^{2+} ion. The overall architecture of the pyrazinamidase of *M. tuberculosis* is similar to that reported for the other pyrazinamidases of *A. baumanii* and *P. horikoshii* [Petrella et al., 2011].

Figure 2.13: Ribbon representation of the structure of the *M. tuberculosis* PncA protein.



Source:

(http://openi.nlm.nih.gov/detailedresult.php?img=3025910_pone.0015785.g001&req=4

The secondary structures, the iron binding site (Asp49, His51, His57, His71) and the catalytic traid (Cys138, Asp8, Lys96) are annotated. The iron ion is represented by the orange sphere, the two water molecules by red spheres

In the *pncA* model, the putative catalytic centre would be located in a pocket formed by one α-helix (αE) and four ß-strands (ß1, ß2, ß3 and ß4) consisting of ß1 (Asp-8 and Phe-13), ß2 (Asp-49), ß3 (Lys-96), ß4 (Ala-134 and Thr-135) and αE (Cys-138). In this pocket, the conserved active cysteine residue Cys-138 is located close to the conserved residues: Asp-8, Trp-68, Lys-96, Ser-104, Ala-134, and Thr-135 [Figure 2.13]. In the *pncA* model, the side chains of the two residues Asp-8 and Lys-96 is found to point towards Cys-138 of the active-site. The modification of the amino acid residues Asp-8, Lys-96 and Ser-104 in the mutants D8G, K96T and S104R resulted in enzymes showing specific activities drastically impaired (%0.004 unit mg), thus suggesting that these residues are essential for the *pncA* activity. The amino acids found at positions 8, 13, 61, 69, 96, 103, 104 and 146 are

functionally and or structurally important in *pncA* [Lemaitre et al., 2001]. Hence, in this study we attempt to utilize this empirical structural data to analyze to the impact of Ala102Pro mutation on PZA resistance.

2.5 **PCR based DNA sequencing technique for drug resistance genes coding resistance to Second line anti-tuberculosis drugs (Fluoroquinolones, Aminoglycosides, Para-amino salicylic acid, Ethionamide).**

2.5.1 Fluoroquinolones:

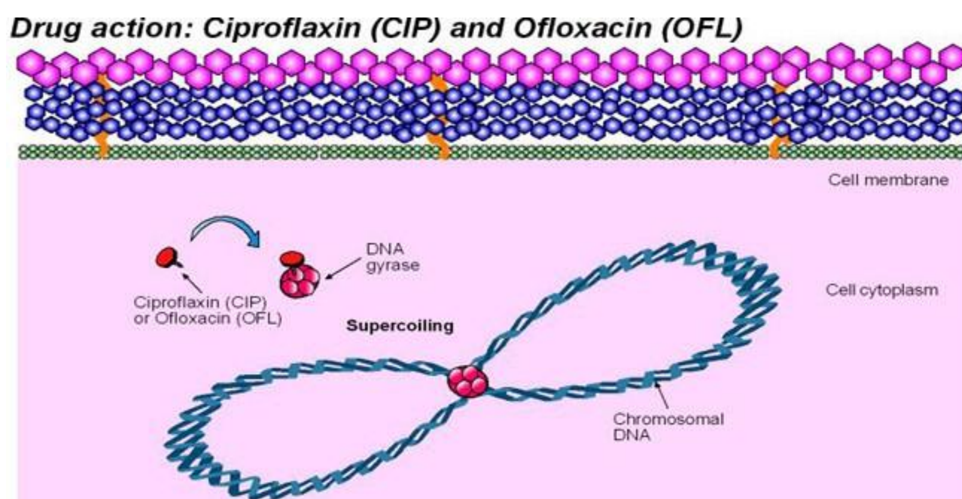
Fluoroquinolones (FQs), a class of synthetic antimicrobial agents, interact and inhibits DNA gyrase and topoisomerase IV and causes bacterial cell death. C-8-methoxy fluoroquinolones (Moxifloxacin, Norfloxacin, Levofloxacin) were introduced onto the market having been developed for use against a wide variety of pathogens. These agents exhibited excellent activity against *M. tuberculosis* [Wang et al., 2006]. They specifically bind to the enzyme-DNA complex and thereby stabilizing the covalent enzyme tyrosyl-DNA phosphate ester (a transient intermediate in the topoisomerase reaction). Hydrolysis of this linkage leads to the accumulation of double-stranded DNA fragments, which probably accounts for the bactericidal activity of FQs treatment. Fluoroquinolones also have excellent in vitro and in vivo activity against *M. tuberculosis* and have proven to be among the most effective second- line antimicrobial drugs used for the treatment of individuals infected with MDR-TB and patients experiencing severe adverse effects due to first-line drugs [Fluoroquinolones have a broad-spectrum of activity that covers many important bacterial pathogens [Wang et al., 2006]. FQs include Ciprofloxacin, Ofloxacin, Levofloxacin, and Moxifloxacin. Adverse effects are relatively infrequent (0.5–10% of patients) and include gastrointestinal intolerance, rashes, dizziness, and headache.

2.5.1A Mode of action of Fluoroquinolones (Figure 2.14):

DNA gyrase and topoisomerase is a ATP-dependent enzymes act by a transient double-stranded DNA break and cooperate to facilitate DNA replication and other key DNA transactions [Mitchison et al., 1985]. DNA gyrase is unique in catalyzing the negative supercoiling of DNA and is essential for efficient DNA replication, transcription, and recombination, whereas topoisomerase IV has a specialized role in chromosome segregation.

DNA gyrase is a tetrameric A2B2 protein. The A subunit (90to100kDa) carries the breakage-reunion active site, whereas the B subunit (70 to 90 kDa) promotes ATP hydrolysis, needed for energy transduction. *M. tuberculosis* genes encoding DNA gyrase were identified from the genome analysis as a gyrB-gyrA contig in which *gyrA* gene and *gyrB* gene encode the A and B subunits, respectively [Ginsburg et al.,2003]. It appears that DNA gyrase is the sole topoisomerase target for FQs in *M. tuberculosis*. Clearly, analysis of FQs interactions with DNA gyrase will be important in understanding and optimizing the antimycobacterial properties of this class of drugs.

Figure 2.14: Mechanism of action of Fluoroquinolones against *M. tuberculosis*



Source: (http://immunopaedia.org.za/index.php?id=261)

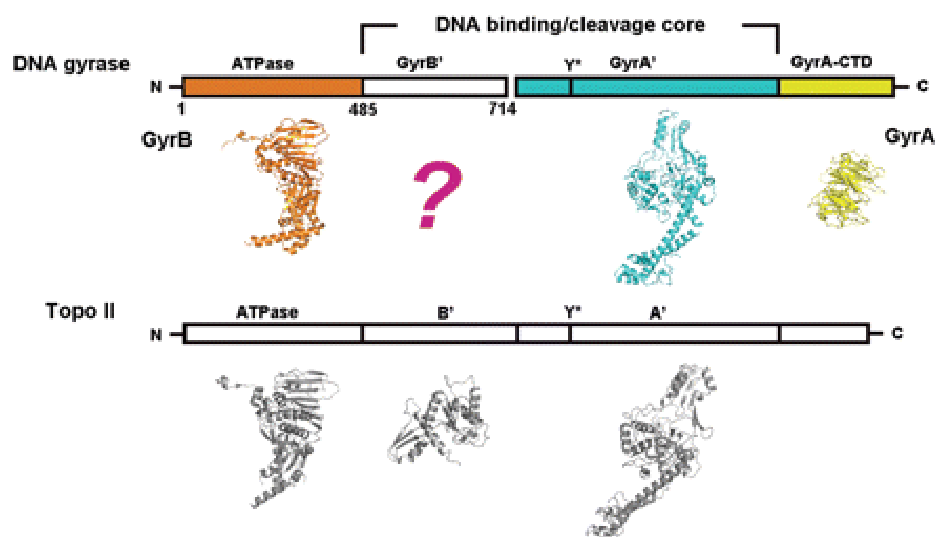
The second line antibiotic dru- Fluoroquilones that interfers with DNA supercoiling in *M. tuberculosis* and inhibits DNA gyrase and therefore induces negative supercoils in circular DNA

2.5.1B Genes encoding for Fluoroquinolones drug resistance (*gyrA* and *gyrB* gene):

The main target of Fuoroquinolones in *M. tuberculosis* is the DNA gyrase, encoded by *gyrA* gene and *gyrB* gene [Ginsburg et al., 2003]. Mutations in two short regions known as "Quinolone resistance-determining regions (QRDR)" have been associated with FQs resistance in *M. tuberculosis* [Ginsburg et al., 2003]. DNA gyrase contains a drug-binding pocket called the quinolone-binding pocket (QBP), which consists of both amino acid residues and DNA nucleotides. Mutations in the two genes change the structure of the QBP and may lead to broad cross-resistance to all FQs.

The genes that encode the DNA gyrase proteins (*GyrA and GyrB*) from *M. tuberculosis* have been cloned and their sequences have been determined [Chang et al., 2010]. The *gyrA* gene is located 36 base pairs downstream of *gyrB* gene. The *M. tuberculosis* GyrB and GyrA proteins share 63% and 69% similarity with the E. coli enzymes, respectively. The *M. tuberculosis* gyrase shows considerable homology with topoisomerase IV from E. coli with 62% similarity between ParC and GyrB, and 59% similarity between ParE and GyrA [Chang et al., 2010].

Figure 2.15: *gyrA* and *gyrB* gene structure



Source: **(http://tuberculist.epfl.ch/quicksearch.php?gene+name=Rv0005)**

The most frequent mutations are found at codons 90 (A90V), 91 (S91P) and 94 (D94G, D94A, D94N and D94Y) of *gyrA* gene [Shi et al., 2006; Wang et al., 2007; Feuerriegel et al., 2009]. Mutation at Codon 95 (Ser95Thr) contains a naturally occurring polymorphism that is not related to FQs resistance, as it occurs in both FQs-susceptible and FQs-resistant strains. Mutations of codons 500, 538, 539 and 540 in *gyrB* gene are also related to resistance to FQs. At present, Arg485 (His, Cys), Ser486Phe, Asp495Asn, Asn533Thr, Asn538 (Thr, Asp), Thr539Pro, Asp500 (Ala, His, Asn), Gly509Ala, Glu540 (Val, Asp) nucleotide substitutions have been described in the QRDR region of the *gyrB* gene [Pitaksajjakul et al., 2005; Feuerriegel et al., 2009]. The regions of *gyrB* gene that encode common mutations that lead to resistance to FQs are also highly conserved between *M. tuberculosis* and other bacteria.

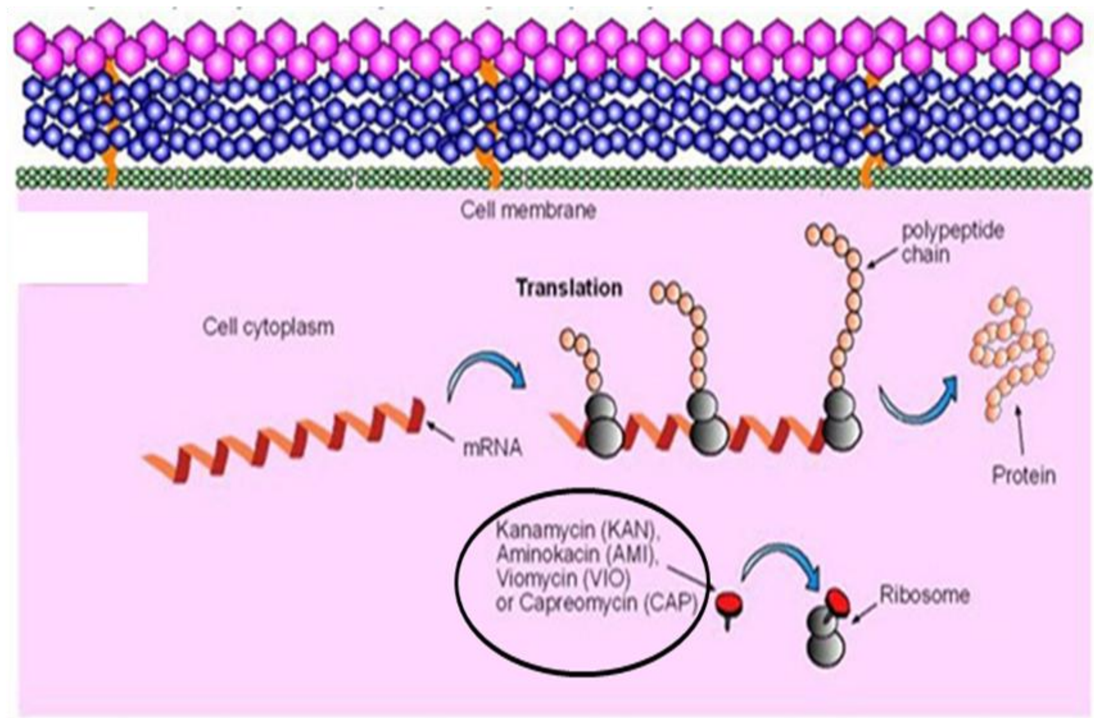
2.5.2 Aminoglycosides:

The Aminoglycosides consist of two or more amino sugars connected to an aminocyclitol nucleus. Aminoglycosides can be differentiated based on their amino sugars. Aminoglycosides that are derived from bacteria of the *Streptomyces* genus are named with the suffix *-mycin*, whereas those that are derived from *Micromonospora* are named with the suffix –micin. The most important aminoglycosides used for anti-tuberculous treatment includes Amikacin, Kanamycin and Capreomycin.

2.5.2A Mode of action of Aminoglycosides (Figure 2.16):

The mode of action of all Aminoglycosides remains the same. Aminoglycosides disrupts the Mycobacterial protein synthesis through binding to ribosomes. Passage of these highly polar molecules across the outer membrane of bacteria is a self promoted uptake process involving the drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules. Penetration through porin channels is unlikely because of the large size of the drug (approximately 1.8 by 1.0 by 1.0 nm). Subsequent transport of aminoglycosides across the inner membrane is dependent upon electron transport and is termed energy-dependent phase I (EDP-I). It is rate limiting and is blocked or inhibited by divalent cations, hyperosmolarity, low pH and anaerobiosis [Alangaden et al., 1998]. In the cytosol, aminoglycosides bind to the 30S subunit of ribosomes, again through an energy-dependent process (energy-dependent phase II [EDP-II]). While this binding does not prevent formation of the initiation complex of peptide synthesis, it interferes with the elongation of the nascent polypeptide chain by disrupting the proofreading process (misreading and/or premature termination). These abnormal proteins may be inserted into the cell membrane, leading to altered permeability and further stimulation of aminoglycoside transport. Leakage of intracellular contents precedes cell death. The extensively rapid kill-rate of the aminoglycoside suggests that lethal events occur prior to the disruption of protein synthesis.

Figure 2.16: Mode of Drug Action Of Aminoglycosides



Source: (http://immunopaedia.org.za/index.php?id=261)

2.5.2B Genes encoding for Aminoglycosides drug resistance:

***tlyA* gene:**

tlyA gene codes for 20-O-ribosemethyltransferase that methylates on the ribose at nucleotide C1402 in helix 44 of 16S rRNA and the ribose at nucleotide C2158 in helix 69 of 23S rRNA [Davies et al., 1997]. These methylated riboses are brought into close proximity upon association of the smaller and larger ribosomal subunits and involved in the transcription.

tlyA gene is the first reported case of a bacterial methyltransferase that modifies specific nucleotides within the rRNAs of both ribosomal subunits. Resistance to ribosome-targeting drugs is generally associated with addition of methyl groups to the rRNA rather than their loss. *tlyA* gene belongs to an exclusive group of methyltransferases that confer antibiotic resistance by losing their function.

The *tlyA* gene (assigned Rv1694 in *M. tuberculosis H37*Rv) is 807bp long. This gene spans the *H37*Rv genome from position 1917924 to 1917927 in the positive strand. The conserved domain sequence of *tlyA* is from the position 1917940 to 1918746.

Although the *tlyA* gene has been found to be a non-essential gene by Himar1-based transposon mutagenesis in *H37*Rv strain [Sassetti et al., 2003], mutations in this gene are found to be resistant to CAP. The *tlyA* gene product methylates the RNA bases within helix 44 of 16S rRNA and within helix 69 of 23S rRNA to form the binding site for CAP, consequently resulting in the inhibition of protein synthesis in *M. tuberculosis*.

TABLE 2.4: Possible hotspot mutations occurring in *tlyA gene* from literature

Polymorphism	Nucleotide position	Codon position	Amino acid
CGA/TGA	7	3	Arg/STOP
del C	26	-	Frameshift
CGA/TGA	52	18	Arg/STOP
CAG/TAG	64	22	Gln/STOP
del A	23	-	Frameshift
GCG/GAG	200	67	Ala/Glu
AAA/GAA	205	69	Lys/Glu
GCA/GAA	272	91	Ala/Glu
del G	310	-	Frameshift
CTG/CCG	353	118	Leu/Pro
GTG/GAG	383	128	Val/Glu
ins C	397	-	Frameshift
del A	400	-	Frameshift
del G	477	-	Frameshift
C/T	548	183	Pro/Leu
C/T	550	184	Gln/STOP
T/G	555	185	Phe/Leu
del G	586	-	Frameshift
del T	653	-	Frameshift
del GT	673-674	-	Frameshift
G/A	712	238	Glu/Lys
del C	758	-	Frameshift

These mutations were obtained by DNA sequencing of the entire *tlyA* gene and the Minimum Inhibitory Concentration (MIC) of the drugs were determined by Proportion method [Maus et al., 2005]. Until now, there have been no confirmations of high

confidence drug resistance mutations in *tlyA* gene and the above putative mutations found in drug resistant clinical TB isolates may be causally related to drug resistance or possibly secondary mutations.

***eis* gene:**

Eis protein has the aminoglycoside acetyltransferase activity and belongs to GCN5-related family of N-acety ltransferases (GNAT). The total gene length is 1209nt and the protein consists of 402aa. It was the over expression of chromosomally encoded aminoglycoside acetyltransferase which confers the kanamycin resistance in *Mycobacterium tuberculosis* [Wenjing Chen et al., 2011]. The main activity of *eis* gene is the aminoglycoside N-acetyltransferase activity. The other function of eis protein includes intracellular survival, association with the cell surface and get secreted, Modulation of cytokine secretion by host immune cells and aminoglycoside antibiotic catabolic process [Wenjing Chen et al., 2011]. It further involves in the subsequent production of tumour necrosis factor (TNF-α) and interleukin-4. It also disturbs the cross regulation of T-cells. It also enhances the intercellular survival in macrophages. It was the over expression of chromosomally encoded aminoglycoside acetyltransferase which confers the kanamycin resistance in *Mycobacterium tuberculosis.* The mutation in the -10 and -35 promoter region of eis protein encodes the uncharacterized acetyltransferase, this must lead to 20-180 fold increase in the amount of *eis* mRNA transcript with corresponding increase in protein expression (Esteban A. Roberts et al). It confers resistance only to kanamycin it doesnot have any cross resistant mechanism with any other drugs.

2.5.3 Para Amino Salicylic Acid (PAS):

The discovery of the antitubercular activity of para-aminosalicylic acid (PAS) by Lehmann in 1943 was followed by two successful clinical trials conducted in 1944 and 1949. The initial success was soon threatened by the emergence of PAS and STR resistance. This was overcome by co-administering PAS and STR, resulting in the advent of combination therapy [Rengarajan et al., 2004]. Although including PAS combination therapy proved efficacious, side effects attributed to PAS were documented as early as 1951 [Rengarajan et al., 2004] . PAS therapy was discontinued after the introduction of Rifampicin and Pyrazinamide. PAS was reintroduced in the United States in 1992, following several

outbreaks of multidrug-resistant (MDR) isolates [Leung et al., 2010]. Since then, the need for new antibiotics for the treatment of MDR TB has led to the development of novel formulations of PAS, which have proven to be less toxic.Recently, PAS is used primarily as a second-line drug to treat MDR TB.

2.5.3A Mode of action of Para Amino Salicylic Acid :

PAS has structural similarities with sulphonamides. Sulphonamides are structural analogues of para-aminobenzoic acid (pABA); the substrate of dihydropteroate synthase (folP1/P2) and hence function as competitive inhibitors. FolP1 and its putative homologue FolP2, catalyzes the condensation of pABA and 6-hydroxymethyl-7,8-dihydropterein pyrophosphate to 7,8-dihydropteroate (DHP), which is converted to dihydrofolate (DHF) and reduced to generate the cofactor tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (dfrA).Thus, PAS mainly involve in the inhibition of folate synthesis of *M. tuberculosis*.

2.5.3B **Gene encoding for PAS resistance (*thyA* gene)**:

Thymidylate synthase A, encoded by *thyA* gene catalyzes the reductive methylation of deoxyuridine 5'-monophosphate (dUMP) to yield deoxythymidine 5'-monophosphate (dTMP), required for de novo dTTP (2'-deoxythymidine 5'-triphosphate) synthesis (12). ThyA requires 5,10-methylene tetrahydrofolate (5,10-MTHF) cofactor both as reductant and carbon-donor in the methylation reaction which are mainly involved in the biosynthesis of thymine in *M. tuberculosis* [Leung et al., 2010].

More recently, using transposon mutagenesis in *M. bovis* BCG, an association of p-amino salicylic acid resistance with mutations in the *thyA* gene that encodes thymidylate synthase A. The same study found that p-amino salicylic acid-resistant clinical isolates of *M. tuberculosis* harboured mutations in *thyA* gene resulting in decreased enzyme activity. Other studies assessing the role of enzymes in the folate pathway determined that p-amino salicylic acid was a pro-drug whose activation required a viable ThyA. However, only 37% of the evaluated clinical isolates of *M. tuberculosis* and spontaneous mutants had mutations in *thyA* gene, suggesting the existence of additional mechanisms for p-amino salicylic acid resistance [Rengarajan et al., 2004]. These studies reported Thr202Ala as the most common mutation associated with p-amino salicylic acid resistance, although a few susceptible isolates have been found to contain this same mutation. These findings have been more

recently challenged by a study that found the Thr202Ala mutation to be a marker for the Latin American Mediterranean (LAM) lineage of *M. tuberculosis* rather than for resistance to p-amino salicylic acid, indicating the need for additional studies to further elucidate the mechanism of action and resistance to p-amino salicylic acid.

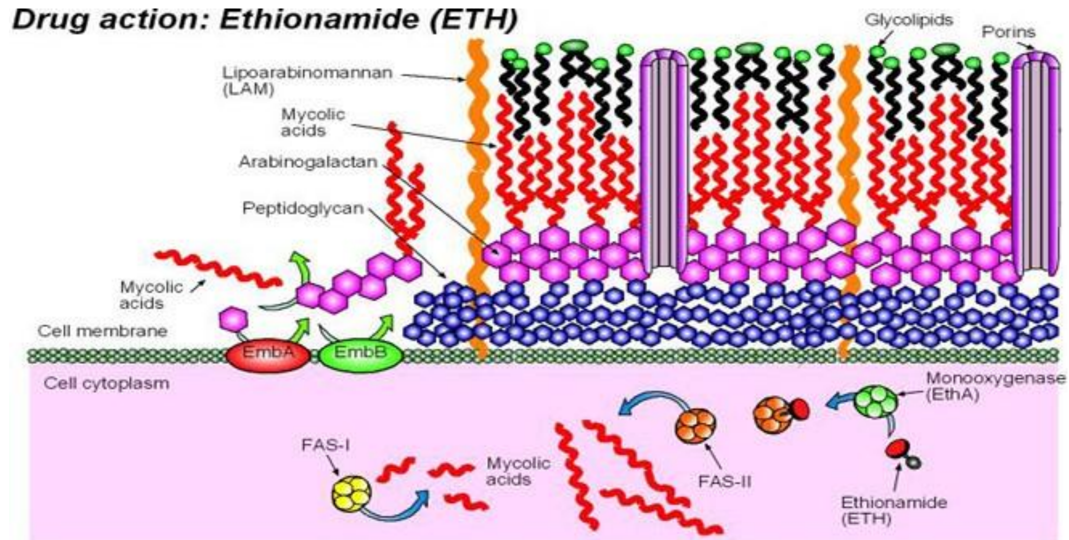
2.5.4 Ethionamide

Ethionamide (ETH), a second-line antituberculous drug, is a structural thioamide analogue of isoniazid (INH) and considered to be the most active antituberculous drug after aminoglycosides and fluoroquinolones and is a component of most of the drug regimens used for treating MDR-TB. Ethionamide (ETH, 2-ethylisonicotinamide) is a derivative of isonicotinic acid and has been used as an antituberculosis agent since 1956.

2.5.4A Mode of action (Figure 2.17):

Ethionamide act as pro-drugs, like isoniazid which is activated by EtaA/EthA (a mono-oxygenase) [Debarber et al., 2000] and inhibits the same target as INH, the InhA of the mycolic acid synthesis pathway [Debarber et al., 2000, Banerjee et al., 1994]. Once delivered into the bacterial cell, ethionamide undergoes several changes. Its sulfo group is oxidized by flavin monooxygenase, and the drug is then converted to 2-ethyl-4-aminopyridine. The intermediate products formed before 2-ethyl-4-aminopyridine seems to be toxic to mycobacteria, but their structures are unknown (may be highly unstable compounds). ETH frequently causes gastrointestinal side effects, such as abdominal pain, nausea, vomiting and anorexia. It can cause hypothyroidism, particularly if it is used with para-aminosalicyclic acid.

Figure 2.17: Mode of Drug Action Of Ethionamide



Source: (http://immunopaedia.org.za/index.php?id=261)

The second line antibiotic drug ETH infers with cell wall biosynthesis in M. tuberculosis. ETH is prodrug converted into monooxygenase and inhibits anoly acly carrier protein reductase and interup the mycolic acid synthesis

2.5.4B Gene encoding for Ethionamide drug resistance (*ethA* and *ethR* genes):

Resistance to ETH has previously been reported to result primarily from (i) mutations altering the activator proteins EthA [Debarber et al., 2000], leading to resistance ETH (ii) mutations in the InhA protein targeted by INH and ETH, which prevents the corresponding activated forms of the drugs from binding the target, leading to cross-resistance to both antibiotics [Debarber et al., 2000]; (iii) mutations in the inhA promoter region that cause overexpression of the target InhA and cross-resistance to the two drugs ; and (iv) mutations in the negative transcriptional regulator EthR, specifically leading to ETH resistance (ETHr).Till now there is no hot spot mutation reported in *ethA* and *ethR* genes.

2.5 GAPS IN EXISTING RESEARCH:

The diagnosis of tuberculosis is difficult and in situations where clinical diagnosis is suggestive but bacteriological proof is lacking. Detection of acid fast bacilli by conventional microscopy is simple and rapid but lacks adequate sensitivity, whereas culture is comparatively more sensitive and specific, but result becomes available after several weeks.

The serological tests available are also marred by a wide range of specificity and sensitivity ratings. Compliance must be maximized to prevent the emergence of drug resistance.

The global threat of drug resistant tuberculosis (MDR-TB and XDR-TB) has great significance for the public health field. Early, accurate diagnosis and immediate, proper curative treatment supported and supervised so that drugs are taken for the appropriate duration, is the key to tuberculosis control. Immediate detection through rapid drug-susceptibility testing is necessary to ensure that patients receive an adequate treatment and that transmission of the disease is thereby interrupted. Hence, there is need for an alternative detection method which is specific, sensitive and rapid, particularly so when bacteriological proof of diagnosis is lacking. Reverse transcriptase PCR (RT-PCR) helps in the detection of actively multiplying *M. tuberculosis* from direct clinical specimens within 24 hours from sample collection. PCR based DNA sequencing method targeting drug resistant genes will help to locate the novel mutational spots occurring in MDR-TB/ XDR-TB isolates which helps in finding out the signature sequence that can be used for developing rapid kit method for the detection of MDR-TB/ XDR-TB strains

Thus, the present study was undertaken mainly to develop rapid, reliable and sensitive molecular techniques for the detection of viable *M. tuberculosis* directly from clinical specimens by nested Reverse Transcriptase PCR (nRT-PCR) and to study the prevalence of MDR-TB and XDR-TB stains in circulating Chennai population for the detection of drug resistant *M. tuberculosis*.

2.6 HYPOTHESIS

Application of Nucleic acid based molecular techniques provide a rapid, sensitive, and reliable tool in the detection of *M. tuberculosis* from clinical specimens and drug resistant tuberculosis isolates. The reverse transcriptase PCR targeting mRNA of some of the specific genes (icl_2, *hspx* and *rRNAP1*) associated with active multiplication of *M. tuberculosis* will result in rapid detection of actively multiplying *M. tuberculosis* directly from clinical specimens.

2.7 OBJECTIVES:

1. Detection of viable *Mycobacterium tuberculosis* directly from clinical specimens using nested Reverse Transcriptase PCR (nRT-PCR) targeting *icl*$_2$*, hspx* and *rRNAP1* genes

   - To standardize nRT-PCRs targeting *icl*$_2$*, hspx* and *rRNAP1* genes for the detection of viable of *M. tuberculosis* directly from clinical specimens.

   - To apply the standardized nRT-PCRs targeting *icl*$_2$*, hspx* and *rRNAP1* genes on direct clinical specimens from clinically suspected tuberculosis patients for the detection of viable of *M. tuberculosis*.

2. Detection of Drug resistance by phenotypic drug susceptibility testing using "micro MGIT BACTEC method" on Multidrug resistant *Mycobacterium tuberculosis* (MDR-TB) [Isoniazid, Rifampicin,] and other first line anti-tuberculosis drugs (Streptomycin, Ethambutol and Pyrazinamide).
3. Application of PCR based DNA sequencing technique for first line anti-tuberculosis drugs for the drug resistant genes targeting
   - *rpoB* gene for detection of mutations, which confer resistance to Rifampicin.
   - *katG, inhA, oxyR* and *ahpc* genes for detection of mutations, which confer resistance to Isoniazid.
   - *embB* gene for detection of mutations, which confer resistance to Ethambutol.
   - *pncA* gene for detection of mutations, which confer resistance to Pyrazinamide.
   - *rpsl*, *rrs* and *gidB* genes for detection of mutations which confer resistance to Streptomycin.
4. Insilico Analysis of Novel Mutation Ala102Pro Targeting *pncA* Gene of *Mycobacterium tuberculosis* using Bioinformatics tools.
5. Detection of Extensively drug resistant tuberculosis (XDR-TB) by phenotypic drug susceptibility testing using "micro MGIT BACTEC method" for second line anti-tuberculosis drugs (Capreomycin, Kanamycin, Moxifloxacin, Ofloxacin, Ethionamide, Para-amino salicylic acid and Amikacin).

6. Standardization and application of PCR based DNA sequencing technique for second line anti-tuberculosis drugs for the drug resistant genes targeting
   - *gyrA* and *gyrB* genes for detection of mutations, which confer resistance to Fluoroquinolones (Ciprofloxacin, Moxifloxacin, Ofloxacin).
   - *tlyA* and *eis* gene for detection of mutations, which confer resistance to aminoglycosides (Kanamycin, Amikacin), Capreomycin,.
   - *ethA and ethR* genes for detection of mutations, which confer resistance to Ethionamide.
   - *thyA* gene for detection of mutations, which confer resistance to Para- amino salicylic acid.

CHAPTER 3

MATERIALS AND METHODS

3.1 Nested Reverse transcriptase PCRs (nRT-PCRs) targeting mRNA of *icl*$_2$, *hspx* and *rRNAP1* genes to detect viable *M. tuberculosis* directly from clinical specimens

3.1.1 Sputum Collection:

A total of 233 Sputum specimens collected from patients attending the out-patient department of Institute of Thoraxic Medicine, Chetpet, Chennai and Stanley hospital, Chennai were included in the study. Among the 233 clinical specimens, 203 specimens were collected from clinically suspected tuberculosis patients and 30 were control specimens (from patients clinically negative for tuberculosis).

Criteria for clinically suspected tuberculosis patients:

- X-ray lesion with abnormal and unstable chest radiograph
- Clinical signs and symptoms with persistant cough lasting for 3 months or more
- bloody sputum, night sweats, weight loss,
- Fever and Past history of the patient and their family

  Source: RNTCP training module for community pharmacist – 2013 report
  http://www.tbcindia.nic.in/pdfs/MODULAR%20TRAINING%20a.pdf

The sputum specimens were collected in a sterile 0.1% Diethyl pyrocarbonate (DEPC) coated containers to prevent RNA degradation. The collected sputum specimens were transported to the laboratory in ice as early as possible.

3.1.2 Processing Of Sputum Specimens:

3.1.2A Ziehl-Neelsen Staining:

A clean glass slide was taken and labeled with the specimen name and reference number on the upper surface. A circle was marked on the lower surface. Smear was made using a loopful of specimen over the circle. It was kept under UV for 15 minutes, dried well and fixed in methanol. Ziehl-Neelsen staining was performed on all Sputum specimens to observe the acid fast bacilli.

3.1.2B Decontamination of Sputum specimens by micro MGIT (Mycobacterium growth indicator tube) BACTEC culture system:

The sputum samples were decontaminated using modified petroff's method (NaLC-NaOH method). Equal volume of clinical specimen and NALC-NaOH was mixed well and incubated at room temperature for 20 minutes. After 20 minutes, sterile PBS was added till the rim of the centrifuge tube and centrifuged at 4,000 rpm for 15 minutes. The supernatant was discarded and again sterile PBS was added till the rim of the centrifuge tube and centrifuged at 4,000 rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile PBS. The pH of the pellet resuspended with PBS was checked to be 7.2. 500µl of pellet was inoculated into 7ml MGIT medium containing 0.8ml of OADC and 0.1ml PANTA. The cultures were incubated at 37ºC for 42 days and readings of the MGIT tubes were taken every day. A smear was done with the pellet and Ziehl-Neelsen staining was performed.

3.1.3 MANUAL EXTRACTION OF RNA BY TRIZOL METHOD:

RNA was extracted from the Sputum specimen by standard TRIzol (Ambion Life technology) method [Desjardin et al., 1996].

- 500µl of TRIZOL reagent was taken in 1.5ml RNase free vial and 200µl of sputum specimen was added and mixed well using cyclomixer.
- 200µl of chloroform was added and mixed well, after completely dissolving the sputum in Trizol reagent and incubated at room temperature for 15 minutes.
- Followed by centrifugation at 12,000 rpm for 15 minutes in cooling centrifuge.
- Transfered the aqueous layer to new sterile 1.5 ml vial and added 500µl of isopropanol. Mixed well with sterile pasteur pipette and centrifuged at 12,000 rpm for 10 minutes in cooling centrifuge.
- Decanted the isopropanol and to the pellet, 1ml of 75% ethanol was added. Mixed well with sterile pasteur pipette and centrifuged at 12,000 rpm for 5 minutes in cooling centrifuge.
- Decanted the 75% ethanol and was air-dried the pellet. After drying, 30µl of sterile DEPC treated water was added and mixed well.

3.1.4 cDNA CONVERSION:

cDNA conversion was done by using Sensiscript Reverse transcription kit (Qiagen, Germany) following the manufacturer's instruction given in table 3.1 below.

Table 3.1 cDNA conversion Protocol

Reagents	Reaction Volume (20 µl)	conditions
dNTP mix (5 mM)	1	37°C for 1 hour
RT buffer (10X)	1	
Oligo dt (10µM)	1	
milliQ	11.5	
RT enzyme	0.5	
RNA	5	

3.1.5 PERFORMANCE OF NESTED REVERSE TRANSCRIPTASE PCR (NRT-PCR) TARGETING mRNA OF *ICL$_2$, HSPX AND RRNAP1* GENES

After cDNA conversion by Sensiscript Reverse transcription kit, the nRT-PCR targeting *icl$_2$, hspx and rRNAP1* genes was performed. **The primer sets (Both inner and outer) used to detect mRNA of *icl$_2$, hspx and rRNAP1* genes were designed indigenously using Primer Blast Software from NCBI.**

3.1.5A Cocktail preparation for nRT-PCR targeting mRNA of *icl$_2$* and *hspx* genes:

A 25 µl PCR reaction includes, 22µl of cocktail consisting of 200 µM of each dNTPs (dATP, dTTP, dGTP, and dCTP), 1 µM of each primer sets, 10X buffers [10 mM Tris - HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$] and 1 unit of *Taq* DNA polymerase was aliquoted into each vial. The negative control was placed inside the PCR machine and 3µl of cDNA was added into respective labeled vials and 2µl of positive control cDNA ($H_{37}Rv$) was pipetted into vial labeled as positive control. For the second round, 2.5µl of the amplified product was transferred to the second round cocktail.

3.1.5B Cocktail preparation for nRT-PCR targeting mRNA of *rRNAP1* gene:

A 25 µl PCR reaction including, 12.5µl of Emerald GT master mix, 10 µl of GT water, 1 µM of each primer sets was prepared. The negative control was placed inside the PCR

machine and 1.5μl of cDNA was added into respective labeled vials and 1μl of positive control cDNA ($H_{37}Rv$) was pipetted into vial labeled as positive control. For the second round, 2 μl of the amplified product was transferred to the second round cocktail. The primer sequence, thermal profile and expected Amplicon size are mentioned below in the table 3.2

Table 3.2: Details of Primer sequence, thermal profile and Amplicon size used to standardize nRT-PCRs.

Primer sequence (5' -3') **(*Indeginously* designed primers)**	**Nucleotide positions of the primers within the genes**	**Thermal profile**	**No. of cycles**	**Amplicon size**
***icl_2* gene (Total length-1287 base pair)**				
First round primer sets ICLP1F:GAAGGCCATCTACCTGTCGG ICLPIR:ACCGGTCTCCATCCAGATCA	255-861	94 °C for 1 min 64 °C for 1 min 72 °C for 1 min	30	607bp
Second round primer sets ICLP5F:GCCTGAAGGCCATCTACCTG ICLP5R:ATCACCACCGTGGGAACATC	251-677	94 °C for 1 min 64 °C for 1 min 72 °C for 1 min	25	427bp
***hspx* gene (Total length-635 base pair)**				
First round primer sets HSPP3F: CGGCTGGAAGACGAGATGAA HSPP3R: CGCCACCGACACAGTAAGAA	145-400	94 °C for 1 min 64 °C for 1 min 72 °C for 1 min	30	261bp
Second round primer sets HSPP1F: TTATGGTCCGCGATGGTCAG HSPP1R: AATGCCCTTGTCGTAGGTGG	440-600	94 °C for 1 min 64 °C for 1 min 72 °C for 1 min	25	161bp
***rRNAP1* gene (Total length-478 base pair)**				
First round primer sets RRNAP1F5: TCACGGAGAACGTGTTCGAG RRNAP1R5: ACAACACGCTTGCTTGTTTCC	53-459	98 °C for 10 sec 60 °C for 30 sec 72 °C for 1 min	35	407bp
Second round primer sets RRNAP1F4: CGTGGAGAACCTGGTGAGTC RRNAP1R4: ACACGCTTGCTTGTTTCCG	291-456	98 °C for 10 sec 62 °C for 30 sec 72 °C for 30 sec	25	166bp

3.1.5C Determination of Analytical Sensitivity of nRT-PCRs/PCRs:

The analytical sensitivity of the PCR was performed by serial ten fold dilutions of cDNA/DNA. Briefly, 10 0.5 ml eppendorf vials were taken and labeled from 10^{-1} to 10^{-10} dilution. 45 µl of sterile millQ water was taken in each vial and 5µl of freshly extracted cDNA/DNA was added to the first vial. It was mixed well and 5 µl was transferred to the next vial. Serial dilutions were performed till the last vial and 5 µl was discarded from the last dilutions followed by performance of nRT-PCR/PCR for determining the sensitivity of the primers.

3.1.5D Determination of Specificity of nRT-PCR/PCRs

The specificity of the primers was tested by amplifying the cDNA/DNA from the following strains of Mycobacterial species: *M. tuberculosis* H37Rv and H37Ra, *M. bovis*, *M. intracellularae* (ATCC 1403), *M. kansasii* (ATCC 1201), *M. xenopi* (ATCC 1432), *M. gordonae*, *M. fortuitum* (ATCC 1529), *M. chelonae* (ATCC 1524), *M. abscessus* (lab isolate), *M. smegmatis* (ATCC 607), *M. phlei*, *M. thermoresistible, M. flavescens* obtained from Tuberculosis Research Centre, ICMR unit, Chennai, and performed nRT-PCR/PCR for determining the specificity of the primers.

3.1.5E Detection of Amplified Products:

After the nRT-PCR, 10µl of the amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5 µg/ml ethidium bromide for visualization by UV trans-illuminator (Vilber Lourmet, France).

3.2 Phenotypic First Line Drug Susceptibility Testing By micro MGIT BACTEC Culture System for First Line Anti-Tuberculous Drugs

3.2.1 Clinical Specimens

A total of 326 *M. tuberculosis* isolates were included in this study. The majority of the *M. tuberculosis* isolates were isolated from Sputum specimens (222 – 68%) followed by pus (29- 8.8%), Fine needle aspirate biopsy (20 – 6.1%), Bronchial wash and Broncho alveolar lavage (15 – 4.6%), Cerebrospinal fluid (7-2.1%), Pleural fluid (4- 1.2%), Gastric juice (3-0.9%), Tissue from abdomen (2- 0.6%), Urine (2-0.6%), Synovial fluid (2-0.6%), cervical

biopsy and Endoscopic biopsy (1- 0.3%). The clinical specimen wise distribution of *M. tuberculosis* isolates is given in Table 3.3. Phenotypic drug susceptibility testing against first line drugs was performed by micro MGIT BACTEC culture system for all 326 *M. tuberculosis* isolates.

TABLE 3.3: Clinical Specimen wise Distribution of *M. tuberculosis* isolates

Clinical specimens	**No. of specimens (n= 326)**
Pulmonary specimens	**(n= 257)**
Sputum	222
Bronchial wash	15
Broncho alveolar lavage	15
Pleural Fluid	4
Pus from chest wall	1
Extra pulmonary specimens	**(n= 69)**
Fine needle aspirate biopsy	20
Gastric juice	3
Cerebrospinal fluid	7
Cervical biopsy	1
Synovial fluid	2
Endoscopic biopsy	1
Pus specimen	28*
Urine	2
Tissue from abdomen	1

*4 from cervical lymphnode, one each from right wrist joint, right axillary node, sinus tract, neck abscess, left neck, necrotic scaline node, pre auricular supparative node, spinal abscess, 16 from unknown source

3.2.2 Reconstitution of First Line Drugs:

Each vial of lyophilized drugs (Streptomycin, Isoniazid, Rifampicin and Ethambutol) were reconstituted with 4 ml of sterile distilled water. Aliquoted in 200µl amounts and stored at –20°C upto 6 months.

3.2.3 Final Concentration of first line drugs

Streptomycin (STR) – 0.8 µg/ml

Isoniazid (INH) – 0.1 µg/ml

Rifampicin (RIF) – 3.5 µg/ml

Ethambutol (EMB) – 100 μg/ml

3.2.4 Preparation of the inoculum:

Day 0 – The day culture turns positive

Day 1 – The next day after culture turns positive

Day 1, 2, 3 cultures can be used as such. If sensitivity cannot be performed before day 3, the isolate was sub-cultured in a fresh MGIT tube. Phenotypic drug susceptibility testing was performed within day 3 after the sub-cultured becomes positive.

3.2.5 Procedure for inoculation:

- The 4ml MGIT tubes were labelled as follows: Growth control, Streptomycin, Isoniazid, Rifampicin and Ethambutol along with the specimen no. and date of inoculation.
- 500 μl of SIRE supplement (OADC) was added to all the tubes.
- 100 μl of respective drugs was added to respective tubes.
- 500 μl of culture was added to all the tubes and incubated at 37°C.
- The inoculated tubes were read in MicroMGIT reader from day 3 till growth control becomes positive (till Day 14).

3.2.6 Interpretation of Results:

After the growth control becomes positive, all the 5 tubes were read for next 48 hours.

- An isolate is considered susceptible if the drug-containing tube fluoresce (indicated between 0-13 in the micro MGIT reader) within 2 days of positivity in the Growth Control tube.
- An isolate is considered resistant if the drug-containing tube fluoresce (indicated between 14-20 in the micro MGIT reader) within 2 days of positivity in the Growth Control tube.

3.2.7 PZA susceptibility testing

Lyophilized powder of Pyrazinamide was reconstituted with 2.5 ml of sterile distilled water. Aliquoted in 200μl amounts and can be stored at –20°C upto 6 months.

Final concentration of PZA = 100 μg/ml

Preparation of inoculums and inoculums procedure was same as mentioned in the sections 3.2.4, 3.2.5 and 3.2.6 above except 800µl of the PZA supplement was added instead of 500µl supplement.

3.3 Phenotypic Second Line Drug Susceptibility Testing By micro MGIT BACTEC Culture System

3.3.1 Clinical isolates:

A total of 29 MDR-TB strains from 402 *M. tuberculosis* isolates were included in this study. Among 29 MDR-TB strains, 26 were from sputum, 2 from bronchial wash and 1 from FNAB given in Table 3.4. Phenotypic drug susceptibility testing against second line drugs was performed by micro MGIT BACTEC culture system for all 29 MDR-TB strains.

Table 3.4: 29 MDR-TB strains included included in this study in this study.

MDR-TB strains n=29	
Sputum	**26**
FNAB	**1**
Bronchial wash	**2**

3.3.2 Phenotypic second line drug susceptibility testing by micro MGIT BACTEC culture system.

Phenotypic drug susceptibility testing for second line drugs by Micro MGIT BACTEC culture system was standardized and applied on 29 MDR-TB strains. The procedure for second line anti-tuberculous drugs was same as that of first line drug susceptibility testing described in sections 3.2.4, 3.3.5,3.3.6 except the OADC supplement was 800µl and the final second line drug concentrations varies (mentioned in the table 3.5 below).

Table 3.5: Second line anti-tuberculous drugs and the final concentration used in this study.

S. no	Drug name (sigma chemicals)	Solvent	Drug concentration
1	Ciprofloxacin (CPX)	Distilled water	1.0 µg/ml
2	Ofloxacin (OFX)	0.1 N NaOH	2.0 µg/ml
3	Levofloxacin (LVX)	0.1 N NaOH	2.0 µg/ml

4	Amikacin (AMK)	Distilled water	1.0 µg/ml
5	Kanamycin (KAN)	Distilled water	1.0 µg/ml
6	Capreomycin (CAP)	Distilled water	2.5 µg/ml
7	Para -amino salicylic ac (PAS)	Distilled water	4.0 µg/ml
8	Ethionamide	ethylene glycol	5.0 µg/ml

3.4 Detection of MDR-TB by Genotypic DST using PCR based DNA sequencing technique targeting drug resistance genes of First line anti-tuberculosis drugs (Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide).

3.4.1 Clinical *M. tuberculosis* isolates:

A total of 242 *M. tuberculosis* isolates from 716 clinical specimens were included in this study. The majority of the *M. tuberculosis* isolates were isolated from Sputum specimens (200 – 82.6%) followed by Fine needle aspirate biopsy (20 – 8.26%), Bronchial wash (12 – 4.9%), pus (6- 2.4%), Pleural fluid (3- 1.2%) and Tissue from abdomen (1- 0.4%). The clinical specimen wise distribution of *M. tuberculosis* isolates were given in Table 4.1. Among 242 *M. tuberculosis* isolates, 151 (61.9%) were sensitive to all the 5 first line anti-tuberculosis drugs, 18 (7.4%) *M. tuberculosis* isolates were MDR-TB, 37(15.2%) were Polyresistant and 43 (17.7%) were Monoresistant strains for the first line anti-tuberculous drugs by micro MGIT BACTEC culture system.

Out of the 242 *M. tuberculosis* isolates, 61 were resistant to Streptomycin (Sputum -52, FNAC -4, Bronchial wash -3 and Pleural fluid- 2), 50 were resistant to Isoniazid (Sputum -41, Bronchial wash -4, FNAC -3, Pleural fluid -1), 20 were resistant to Rifampicin (Sputum -17, Bronchial wash- 2, FNAC -1), 41 were resistant to Ethambutol (Sputum -37, FNAB- 3, Bronchial wash- 1, Pleural fluid-1) and 41 were resistant to Pyrazinamide (Sputum -39, FNAC -2) by phenotypic drug susceptibility testing.

3.4.2 Clinical *M. tuberculosis* isolates used to target *gidB* gene confering resistance to Streptomycin:

A total of 112 *M. tuberculosis* isolates (102-STR resistant and 10-STR sensitive by phenotypic drug susceptibility testing by Micro MGIT BACTEC method) were included in

this study. Among 112 clinical isolates, 102 were sputum, 9 were FNAB and 1 was pus from cold abscess.

TABLE 3.6: Clinical Specimen wise Distribution of *M. tuberculosis* isolates

Clinical specimens	No. of specimens (n= 242)
Pulmonary specimens	**(n= 215)**
Sputum	200
Bronchial wash	12
Pleural Fluid	3
Extra pulmonary specimens	**(n= 27)**
Fine needle aspirate biopsy	20
Pus specimens*	6
Tissue from abdomen	1

*One each from cervical lymphnode, chest wall, right wrist joint, right axillary node, sinus tract, unknown source

3.5.3 DNA EXTRACTION:

3.5.3A Materials required:

Double sterilized MilliQ water, Sterile 1.5 ml vials, Centrifuge, Water bath at 80°C

3.5.3B Procedure for DNA extraction from Micro MGIT BACTEC BACTEC culture system:

- 200 µl of the inoculum from the MGIT medium was taken in a sterile 1.5 ml vial.
- It was heated at 80 ºC for 10 minutes followed by centrifugation at 3000 rpm for 5 minutes.
- The supernatant was used as template DNA for PCR.

3.5.4 PCR PROTOCOL:

3.5.4A Materials required (All the PCR reagents were obtained from Bangalore Genei Pvt. Ltd, India):

- Stock dNTPs dilution: 100 mM concentration of dNTPs- dATP, dCTP, dTTP and dGTP
- Working standard dNTP (200µM): 2 µl of each of the stock dNTP made up to 400 µl using MilliQ water.
- *Taq* DNA polymerase 3 units.

- 0.5M Glycerol (5 µl)
- 2µM $MgCl_2$
- The PCR cocktail contained the following:
- For a 50 µl reaction, the PCR cocktail components are given below in Table 3.7

Table 3.7 PCR cocktail preparation

PCR Reagents	volume
dNTPs	8µl
10x buffer (15mM Mg2+,Tris, Kcl(500 mM)- pH 8.3)	5 µl
Forward primer (1pM)	1 µl
Reverse primer (1pM)	1 µl
Taq polymerase	0.3 µl
MilliQ water	30 µl
DNA	5 µl

3.5.4B PCR protocol for *embB* gene

PCR targeting *embB* gene was standardized with a specialized *Taq* DNA polymerase enzyme called "Z Taq" enzyme (Takara Bio, Ohtsu, Shiga, Japan). The *Z-Taq* polymerase offers unmatched PCR productivity, with a processing speed five times faster than those of other commercially available *Taq* polymerases. The total PCR cycle takes only 29 minutes. All the reagents for PCR (dNTP, 10X, Z Taq) was provided along with the buffer. Each 50 l reaction contained MdNTP, 10X, 1pM of forward and reverse primers and Units of 'Z' Taq enzyme.

3.5.4C Thermal profile:

The concentration of template DNA, primers and thermal profile was calculated by trial and error. The annealing temperature was calculated by using the formula **T_m= [2(A+T) + 4(G+C)]/2.** The combination of all the above stated factors that shows the specific amplification was chosen as the appropriate primer concentration for the PCR. The details of primer targeting second line drug resistance genes, their thermal profile used and the expected amplicon size are given in the Table 3.8

Table 3.8: Primer sets used in the study to sequence the target genes with their thermal profile and expected amplicon sizes:

Target genes / Primer sequence (5'-3' Direction)	Thermal profile	No. of Cycles	Expected Amplicon Size (bp)
Targets genes for Rifampicin resistance			
rpoB gene [Siddiq N et al., 2002] CCACCCAGGACGTGGAGGCGATCACAC AGTGCGACGGGTGCACGTCGCGGACCT	95 ºC-5 min 95ºC-30sec 72ºC-1min 72ºC -5 min	35	286
Targets genes for Isoniazid resistance			
katG1 gene [Siddiq et al., 2002] GCCCGAGCAACACCC ATGTCCCGCGTCAGG	94˚C - 1min 58˚C –1min 72˚C –2 min	35	237
katG2 gene [Siddiq et al., 2002] CGAGGAATTGGCCGACGAGTT CGGCGCCGCGGAGTTGAATGA	94˚C - 1min 55˚C –1min 72˚C –2 min	35	414
katG3 gene [Siddiq et al., 2002] CCGGCACCTACCGCATCCAC GCCCCAATAGACCTCATCGG	95˚C - 1min 60˚C –30sec 72˚C –1 min	30	269
katG4 gene [Siddiq et al., 2002] GAAACAGCGGCGCTGGATCGT GTTGTCCCATTTCGTCGGGG	95˚C - 1min 60˚C –30sec 72˚C –1 min	30	209
inhA gene [Siddiq et al., 2002] CCTCGCTGCCCAGAAAGG A ATCCCCCGGTTTCCTCCGGT	94˚C - 5min 94˚C - 1min 64˚C –1min 72˚C –2 min 72˚C – 4 min	40	248
oxyR-ahpC gene [Siddiq et al., 2002] GCTTGATGTCCGAGAGCAT GGTCGCGTAGGCAGTGCCCC	94˚C - 2min 94˚C - 1min 60˚C –1min 72˚C –2 min 72˚C – 5 min	35	701
Targets genes for Streptomycin resistance			
rpsL gene [Siddiq et al., 2002] GGCCGACAAACAGAACGT GTTCACCAACTGGGTGAC	94˚C - 1min 94˚C - 1min 56˚C –1min 72˚C –1 min 72˚C – 7 min	35	505
rrs gene [Siddiq et al., 2002]	94˚C - 1min		1140

TTGGCCATGCTCTTGATGCCC TGCACACAGGCCACAAGGGA	94˚C - 1min 56˚C –1min 72˚C –1 min 72˚C – 7 min	35	
gidB gene [Laure et al., 2010] CGTAATGTCTCCGATCGAGC CTTTGATGGCGAGCATTCG	94 °C -1 min 58 ºC-1min 72ºC-2min	35	494
Targets genes for Pyrazinamide resistance			
pncA gene [Sekiguchi et al., 2007] GGCGTCATGGACCCTATATC CAACAGTTCATCCCGGTTC	94˚C – 30 sec 60˚C – 30 sec 72˚C – 30 sec 72˚C – 5 min	35	670
Targets genes for Ethambutol resistance			
embB gene [Siddiq et al., 2002] CCGACCACGCTGAAACTGCTGGCGAT GGTGGGCAGGATGAGGTAGT	95˚C – 5 sec 55˚C – 10 sec 72˚C – 10 sec	35	937

The amplified products were detected by electrophoresis, in 2% agarose gel with 0.5µg of ethidium bromide, along with molecular weight marker and documentation was done using a Vilbert Lourmat Gel documentation system (Cedex, France).

3.5.4D PCR interpretation criteria:

The PCR results were only considered valid when the negative control of the reaction was negative without specific amplified product and when the positive control yielded a single specific amplified product. The test strains of *M. tuberculosis* were compared against the positive and negative controls to interpret as positive or negative result for the PCR.

3.5.4E Determination of Specificity of the Primers:

Specificity of the primers was performed as same as above procedure mentioned in the section 3.1.5D

3.5.4F Determination of Analytical Sensitivity of the Primers:

Analytical sensitivity was performed as same as above procedure mentioned in the section 3.1.5C

3.5.5 DNA SEQUENCING OF AMPLIFIED PRODUCTS:

3.5.5.1 Cycle Sequencing Reaction Protocol

3.5.5.1A EXO-SAP Treatment

Reagents (Fermentas Life Sciences):

1. ExoI - Exonuclease I degrades single-stranded DNA in a 3'-5' direction and removes primer from PCR mixtures prior to sequencing.

Source –*Escherichia coli* cells with a cloned *E. coli sbcB* gene.

2) SAP - Shrimp Alkaline Phosphatase catalyzes the release of 5' phosphate groups from DNA and degrades dNTPs in PCR mixtures prior to sequencing.

Source –Arctic shrimp *Pandalus borealis*.

The EXO-SAP reaction mixture contained the following

EXO : 0.5 µl

SAP : 1.0 µl

PCR product : 5 µl

Table 3.9: Thermal Profile for EXO-SAP Treatment

Temperature	Duration
37˚C	15 minutes
85˚C	15 minutes
4˚C	Hold

3.5.5.1B Cycle Sequencing Reaction Protocol:

After PCR was completed, cycle sequencing was performed following the protocol given in Table 5.8. The volume of Ready reaction (RR) mix and buffer was adjusted (together volume should be 4 µl) based on the amplified product size. The primer concentration should be 1 picomole/µl and 1 µl of amplified product was added. Finally, sterile milliQ water was added to make up the volume to 10 µl (table 3.10).

Table 3.10: Cycle sequencing protocol

REAGNETS	VOLUME(µl) <400bp	VOLUME(µl) 400-800bp	VOLUME(µl) 800-1000bp	VOLUME(µl) >1000bp
Ready reaction mix (RR mix)	**1**	**1.5**	**2.0**	**3.0**
5x sequencing buffer	**3**	**2.5**	**2.0**	**1.0**
Forward/Reverse primer (1pmol/µl)	**1**	**1**	**1**	**1**
Milli Q water	**3**	3	3	3
Exo- sap treated Amplified PCR product	**2**	**2**	**2**	**2**
Total reaction volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

3.5.5.1C Reaction conditions for Cycle Sequencing:

Initial Denaturation - 96˚C for 1 minute

Denaturation - 96˚C for 10 seconds

Annealing - 50˚C for 5 seconds } 25 cycles

Extension - 60˚C for 4 minutes

3.5.5.1D Purification step of amplified products for DNA Sequencing:

The extension products were purified to remove the unincorporated dye terminators before the samples were analyzed.

Reagents Required:

125 mM EDTA

3 M sodium acetate
Chilled ethanol
70% ethanol

Procedure:

Sterile 0.5 ml microfuge vials were taken and labelled with the specimen number.

- 10 µl sterile milliQ was added followed by 2 µl of 125 mM EDTA, 2 µl of 3 M Sodium Acetate and 50 µl of chilled Ethanol.
- To this mixture, 10 µl of the cycle sequenced product was added and left at room temperature for 15 minutes.
- Centrifugation was done at 12,000 rpm for 20 minutes.
- Supernatant was discarded and pellet washed thrice with 200 µl of 70% ethanol at 12,000 rpm for 10 minutes.
- Vials were dried at 37˚C before loading into DNA sequencer and 20 µl Formamide was added.
- Denaturation at 95˚C for 3 minutes was done followed by snap cooling and the sample was loaded immediately.

3.5.5.2 Loading of Cycle Sequenced products in the DNA Sequencer:

The sequence of the PCR amplified DNA was deduced with the help of the ABI Prism 3100 AVANT (Applied Biosystems, USA) genetic analyzer that works based on the Sanger di-deoxy sequencing method.

The amplified products with the dye at the terminated 3′ end were subjected to capillary electrophoresis by an automated sample injection. The emitted flurorescence from the dye labels on crossing the laser area were collected at the rate of one per second by a cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing. The sequences were analysed by Bio Edit sequence alignment software.

3.5.5.3 BLAST Analysis:

BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) was done to confirm the sequenced data with the standard strains and to determine the percentage homology.

3.5.5.4 MULTALIN Analysis:

Multiple sequence alignment tool developed based on "Multiple sequence alignment with hierarchical clustering", F.Corpet et al, 1988. Multalin analysis, using genbank (reference

sequence accession number - NC_000962), www.ncbi.nlm.nih.gov/genbank were done to identify the presence of any substitution, addition, deletion or frameshift.

3.6 Detection of XDR-TB by Genotypic DST using PCR based DNA sequencing technique for drug resistance genes coding resistance to Second line anti-tuberculosis drugs (Fluoroquinolones, Aminoglycosides, Para-amino salicylic acid, Ethionamide).

3.6.1 Clinical isolates of *M. tuberculosis*:

A total of 111 *M. tuberculosis* isolates (Poyresistant-82 and MDR strains-29) were included in this study. Majority of the clinical specimens were from Sputum (91), FNAB (11), Bronchial wash (5), Pleural biopsy (1), Pleural fluid (1), pus from cold abscess (1), CSF (1). The clinical specimen wise distribution of *M. tuberculosis* isolates were given in Table 7.2. Among 111 *M. tuberculosis* isolates, 82 were Polyresistant strains [Sputum- 65, FNAB -10, Bronchial wash- 3, Pleural biopsy- 1, Pleural fluid -1, pus from cold abscess- 1, CSF -1], 29 were MDR-TB strains [Sputum- 26, Bronchial wash- 2, FNAB-1].

Out of 82 polyresistant strains, 64 were sensitive and 3 were resistant to all second line anti-tuberculous drug (AMK,KAN,CAP,OFX,CPX,LVX,ETH,PAS), 5 were resistant to CPX, 2 were resistant to PAS, 2 were resistant to and 1 each was resistant to [AMK,KAN,CAP,CPX, ETH,PAS], [AMK,KAN,OFX,CPX,LVX,ETH,PAS], [AMK,KAN,CAPCPX,LVX,ETH,PAS],[CAP, PAS], [CAP,CPX], [CAP, ETH] and [CAP, KAN, ETH, CPX] by phenotypic drug susceptibility testing by micro MGIT BACTEC method.

Among 29 MDR strains, 12 were sensitive and 1 was resistant to all second line anti-tuberculous drug [AMK,KAN,CAP,OFX,CPX,LVX,ETH,PAS], 3 each were resistant to [AMK,KAN,ETH,PAS], [AMK,CAP,OFX,CPX,LVX,ETH,PAS], 1 each was resistant to [CAP,OFX,CPX,ETH,PAS], [AMK,KAN,CPX,, [AMK,CPX], [KAN,PAS], [AMK,CAP,OFX,CPX,LVX,ETH,PAS], [AMK,KAN,CAP,CPX,LVX,ETH,PAS], [ETH,PAS], [AMK,KAN, CPX,ETH,PAS] by phenotypic drug susceptibility testing by micro MGIT BACTEC method.

TABLE 3.11: Clinical specimen wise distribution of *M. tuberculosis* isolates included in this study.

Clinical Specimens	**No. of. clinical specimen n=111**
Polyresistant strains	**n=82**
Sputum	**65**
FNAB	**10**
Bronchial wash	**3**
Pleural fluid	**1**
Peural biopsy	**1**
Pus from cold abscess	**1**
CSF	**1**
MDR-TB strains	**n=29**
Sputum	**26**
FNAB	**1**
Bronchial wash	**2**

DNA extraction and PCR based DNA sequencing protocol was carried out as mentioned in the sections 3.5.4 and 3.5.5 respectively.The primer sequence, thermal profile and expected amplicon size for second line drug resistant genes are mentioned in the table 3.12

TABLE 3.12: Primer sets used in the study to sequence the target genes with their thermal profile and expected amplicon sizes:

Target genes / Primer sequence (5'-3' Direction)	**Thermal profile**	**Expected Amplicon Size (bp)**
gyrA gene **[Siddiqi et al., 2002]** gyrAF: CAGCTACATCGACTATGCGA gyrAR: GGG CTTCGG TGTTACCTCAT	94 °C -1 min 52 ºC-1min 72ºC-2min	320
gyrB gene **[Siddiqi et al., 2002]** gyrBF: CCACCGACATCGGTGGAT T gyrBR: CTGCCACTTGAGTTTGTACA	94 °C -1 min 57 ºC-1min 72ºC-2min	428

	72ºC -10 mins	
***tlyA gene* [Via et al., 2010]** tlyAF: CATCGCACGTCGTCTTTC tlyAR: AATACTTTTTCTACGCGCCG	95˚C - 1min 95˚C - 1min 57˚C –1min 72˚C –2 mins	969
***eis gene* [indigenously designed primers]** eisF: GACTGTGACCCTGTGTAGCC eisR: GACGAAGCAGCTGGGAATCT	94˚C - 5min 94˚C - 1min 62˚C –1min 72˚C –2 mins 72˚C – 10 mins	1153
***rrs* gene [Siddiqi et al., 2002]** rrsF: TTGGCCATGCTCTTGATGCCC rrsR:TGCACACAGGCCACAAGGGA	94˚C - 1min 94˚C - 1min 56˚C –1min 72˚C –1 min 72˚C – 7 min	1140
***inhA* gene [Siddiqi et al., 2002]** inhAF: CCTCGCTGCCCAGAAAGG A inhAR: ATCCCCCGGTTTCCTCCGGT	94˚C - 5min 94˚C - 1min 64˚C –1min 72˚C –2 min 72˚C – 4 min	248
***ethA1 gene* [Morlock et al., 2003]** ETHA1 F: ATCATCGTCGTCTGACTATGG ETHA5 R: ACTACAACCCCTGGGACC3’	95˚C - 15 mins 95˚C - 30 sec 65˚C –1.25 mins 72˚C –5 min 72˚C – 5 min	667
***ethA2 gene* [Morlock et al., 2003]** ETHA4 F:CCTCGACCTTCCCGTGA ETHA9 R:CCTCGAGTACGTCAAGAGCAC	95˚C - 15 mins 95˚C - 30 sec 65˚C –1.25 mins 72˚C –5 min 72˚C – 5 min	692
***ethA3 gene* [Morlock et al., 2003]** ETHA9 F:CCTCGAGTACGTCAAGAGCAC ETHA10 R:CGTTGACGGCCTCGACATTAC	95˚C - 15 mins 95˚C - 30 sec 68˚C –1.25 mins 72˚C – 5 min	342
***ethR gene* [Morlock et al., 2003]** ETHR1: CGCTGACACCGGAGATTCC ETHR4: CGCTCCTATATGACCGCACG	95˚C – 5mins 95˚C – 1min 59˚C – 1 min 72˚C – 1 min 72˚C – 7 min	915
***thyA gene* [Siddiqi et al., 2002]** thyAF: ATCGTGTGCCCCATGGTGATCT thyAR: AATACTTTTTCTACGCGCCG	95˚C – 5mins 94˚C – 30 sec 67˚C – 30 sec 72˚C – 30 sec 72˚C – 2 mins	1122

3.7 Insilico Analysis of Novel Mutation Ala102Pro Targeting *pncA* Gene of *Mycobacterium tuberculosis* using Bioinformatics tools

3.7.1 Phenotypic Drug Susceptibility Testing:

Phenotypic PZA (100µg/ml) susceptibility testing was performed by BACTEC MicroMGIT culture system following manufacturer's instruction (Becton Dickinson, USA) [Salman et al., 2000].

3.7.2 PCR BASED DNA SEQUENCING TARGETING *PNCA* GENE

PCR based DNA sequencing was carried out as mentioned in the section 3.5.4 and 3.5.5 respectively

3.7.3 BIOINFORMATICS ANALYSIS:

3.7.3.1 Data sets

The protein sequence for Pyrazinamidase (Pzase) *pncA* (UniProtKB **id: Q50575**) was obtained from UniProtKb to perform *in silico* sequence analysis. The 3D atomic coordinates (PDB ID:3PL1) was obtained from Protein Data Bank (http://www.pdb.org/pdb/home/home.do) for structure analysis, Mutation modeling (Ala102Pro), Molecular docking and Molecular Dynamics Simulation Studies.

3.7.3.2 Predicting stability change on single amino acid polymorphism based on support vector machine (I-Mutant 2.0)

Protein structural stability of the mutants were assessed using I-Mutant 2.0 server which is a support vector machine (SVM) – based tool for automatic prediction of protein stability changes upon single-point mutations; I-Mutant 2.0 predictions are performed for both the sequence and structure of proteins. The output of this program displays the predicted free energy change value (ΔΔG) which is calculated from the unfolding Gibbs free energy value of the native type (kcal/mol). Positive ΔΔG values infers that the mutated protein possesses high stability and vice versa._(http://gpcr.biocomp.unibo.it/cgi/predictors/ I-Mutant 2.0/ I-Mutant 2.0.cgi)

3.7.3.3 Analysis of secondary structure using Protein Structure Prediction Server (PSIPRED v3.0)

The PSIPRED is a reliable sequence based Protein Secondary Structure Prediction Server and was used in this study to predict the secondary structure of the mutant sequence so as to assess the impact of the novel mutation at the secondary structure elements. The output gives the details of presence of helix (H), sheets (E) and coils(C) in the protein sequence with Graphical representation (http://bioinf.cs.ucl.ac.uk/psipred/).

3.7.3.4 Detection of conserved amino acids in the Human Pathogenic organisms containing Pyrazinamidase/nicotinamidase

Consurf analysis can be utilized to reveal the conservation to residues among the group of organisms which common metabolic functions. Here, in this study, the bacteria which exhibit PZase activity were grouped together in terms of sequence identity and were further analyzed for conserved residues through consurf analysis.

3.7.3.5 Homology modeling of mutant Pzase

Homology modeling helps in predicting the 3D structure of a protein with unknown structure by comparing it with a known structure sharing high sequence similarity. In this study, MODELLER9v7 [Eswar N et al., 2007] was used for modeling the novel mutant protein. Since the crystal structure of the pyrazinamidase *M. tuberculosis* (PDB id: 3PL1) was available, the same was used as template to predict the mutant form. Finally, the stereochemcial property of WT and MT of the protein structure was predicted using SAVS server (http://nihserver.mbi.ucla.edu/SAVES/).

3.7.3.6 Structure Optimization and Validation.

The WT and the modeled MT PZAse structures were subjected to energy minimization by steepest descent using GROMOS96 force field [Hess et al., 2008]. The quality of the protein structure modeled was again checked using Q-Mean server [Hess et al., 2008] and ProQ (http://www.sbc.su.se/~bjornw/ProQ/ProQ.cgi)

3.7.3.7 Active site analysis of Pzase enzyme

The active site of Pzase from *M. tuberculosis* was analyzed using Q-SiteFinder, CASTp (http://sts-fw.bioengr.uic.edu/castp/calculation.php) and Pocket Finder server

(http://www.modelling.leeds.ac.uk/pocketfinder/help.html) to identify the binding site for docking studies

3.7.3.8 Ligand optimization

The initial structure of PZA was obtained from PubChem in 3D SDF format. It was converted to PDB format using Open Babel (http://www.molecularnetworks.com/online_demos/corina_demo.html). Hydrogen bonds were added to the structure and was geometry optimized (Broyden- Fletcher-Golfarb-Shanno line search method set to 1000 steps) using ArgusLab [Stoermer et al., 2006], wherein, the energy minimization was carried out using Universal Force Field (UFF) [Da Silva et al., 2006].

3.7.3.9 Docking of PZA-Pzase

PZA was docked into the structures of WT and MT using AutoDock 4.0 [Huey et al., 2007]. In this docking simulation, semiflexible docking protocols [Morris et al., 1998] were used, in which the protein structures were kept rigid and the PZA being docked was kept flexible. Blind docking was performed using grid point value (X, Y and Z) of 100Å and spacing between the gird point was 0.375 Å. Lamarckian genetic algorithm was selected for ligand conformational searching and default docking parameters were used. The best docked complexes based on the lowest binding energy were further analyzed for hydrogen bonding interactions and the binding energy of WT and MT was compared. Finally, the Protein- ligand complexes were analyzed using Discovery Studio Visualizer [Accelrys Software Inc., 2011] and PYMOL visualization tool (http://www.pymol.org/).

3.7.3.10 Docking Complex Simulation:

To study the stability of PZA ligand with wild and mutant forms, molecular dynamics simulation was carried out for the docked Protein-ligand complexes. All simulations were performed using GROMACS [Du et al., 2001], with GROMOS96 43al force field. The topology file for the ligand was generated using Dundee PRODRG Server [http://davapc1.bioch.dundee.ac.uk/prodrg/]. The system was placed in the centred to a cubic periodic box with a area of 37 x 37 x 37Å and was solvated with a simple point charge (SPC213) water model. The distance between solute and edge of the box was 0.9 Å for the system. It includes water molecules for all the complexes. To neutralize the systems charge to zero, adequate number of Na+ or Cl- ions were added. Each protein-ligand

complex was subjected 2000 steps of energy minimization using steepest descent algorithm. Equilibration of the ensembles was conducted in position restraint for 100ps.A constant temperature of 300K and 1 atm pressure was maintained in the system. For long range electrostatics calculation, Partial Mesh Ewald coulomb type was used with a cut off of 1.0 nm. All bonds including H-bonds were constrained using LINCS algorithm. The MD simulation was carried out for 1ns for each complex. After simulation, the trajectories were analyzed by ngmx software package. All the molecular modeling and simulation studies were carried on OPEN Discovery 2 Linux Platform [Vetrivel et al., 2008].

.

CHAPTER 4

Detection of viable *Mycobacterium tuberculosis* directly from clinical specimens using Nested Reverse transcriptase PCRs (nRT-PCRs) targeting mRNA of *icl_2*, *hspx* and *rRNAP1* genes.

4.1 INTRODUCTION:

The laboratory diagnosis of tuberculosis is difficult and in situations where clinical diagnosis is suggestive but bacteriological proof is lacking. Detection of acid fast bacilli by conventional microscopy is simple and rapid but lacks adequate sensitivity [Alberts et al, 1989]. Culture that is known to be "gold standard" in laboratory diagnosis of TB usually takes 3 to 8 weeks. The identification of the isolates on the culture media and susceptibility testing to anti TB drugs add another 2 to 3 weeks time it takes to make a definitive laboratory diagnosis of TB [Alberts et al., 1989]. In recent years, molecular techniques like Polymerase chain reaction (PCR) methods overcome delays caused by the need to culture sufficient biomass and are amenable to high-throughput analysis, thus improving detection rate. DNA-PCR has been shown to play important role as an alternative diagnostic tool in detection of *M. tuberculosis*.

But DNA-PCR is unable to differentiate between live and dead tubercle bacilli. This can be overcome by use of Reverse transncriptase PCR (RT-PCR) which detects mRNA with a mean half-life of 3–5 min [Alberts et al., 1989] is more prone to destruction than genomic DNA, hence a positive mRNA signal would indicate the presence of viable *M. tuberculosis* directly from clinical specimens. Thus, nRT-PCRs targeting mRNA of *icl_2, hspx and rRNAP1* genes will be a rapid, reliable and diagnostic tool for the detection of actively multiplying *M. tuberculosis* directly from the clinical specimens.

4.2 MATERIALS AND METHODS:

Number of Clinical specimens, RNA extraction, cDNA conversion and standardization of nRT-PCRs targeting *icl_2, hspx* and *rRNAP1* genes are already mentioned in section 3.1

4.3 RESULTS:

4.3.1 Direct Smear Microscopy:

Out of the 203 sputum specimens, 24 (11.8%) showed the presence of acid-fast bacilli by Ziehl-Neelsen stain and 179 were negative.

4.3.2 Mycobacterial Culture by micro MGIT BACTEC method:

Out of the 203 sputum samples collected from clinically suspected tuberculosis patients, 48 (23.6%) sputum samples were culture positive and 155 (76.3%) were culture negative. All *M. tuberculosis* isolates (48) were confirmed by PCR targeting *MPB64*gene [Therese et al., 2013], *IS6110* [Therese et al., 2013] region and TBc ID kit (BD diagnostics). All the 30 control clinical specimens were negative by micro MGIT BACTEC method.

4.3.3 Results of nRT-PCRs targeting mRNA of *icl_2*, *hspx* and *rRNAP1* genes:

4.3.3.1 Optimization of nRT-PCRs targeting mRNA of *icl_2* gene using "indegenously designed" primers:

4.3.3.1.1 Determination of Analytical sensitivity of the primers targeting *icl_2* gene

The initial concentration of *M. tuberculosis H37RV* DNA used for all three nRT-PCRs were 624ng/1.5µl of cDNA.

The analytical sensitivity of nRT-PCR targeting mRNA of *icl_2 gen*e, *hspx* and *rRNAP1* genes of *M. tuberculosis* were 64.2pg, 0.642pg and 0.642pg respectivley to detect viable *M. tuberculosis* H37Rv cDNA (Figure 4.1, 4.2, 4.3).

Figure 4.1: Agarose gel electrophoretogram showing the analytical sensitivity of indigenously designed primers targeting *icl₂* gene

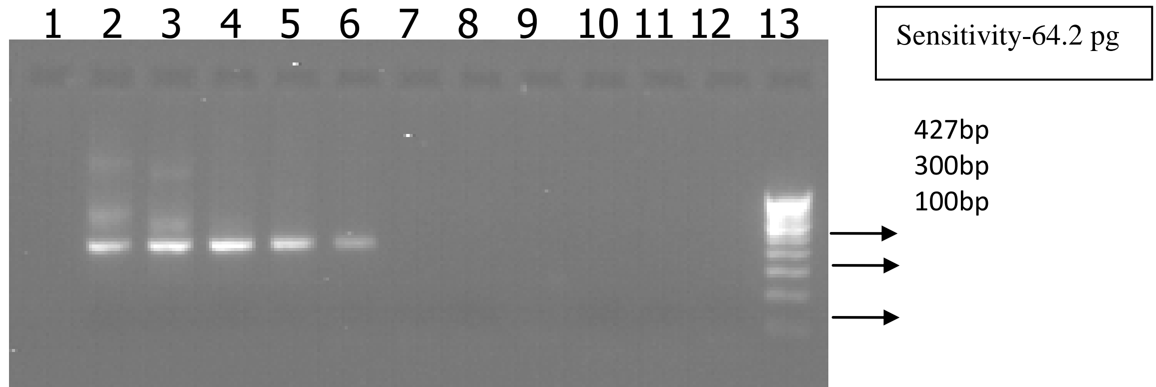


Figure 4.2: Agarose gel electrophoretogram showing the analytical sensitivity of indigenously designed primers targeting *hspx* gene

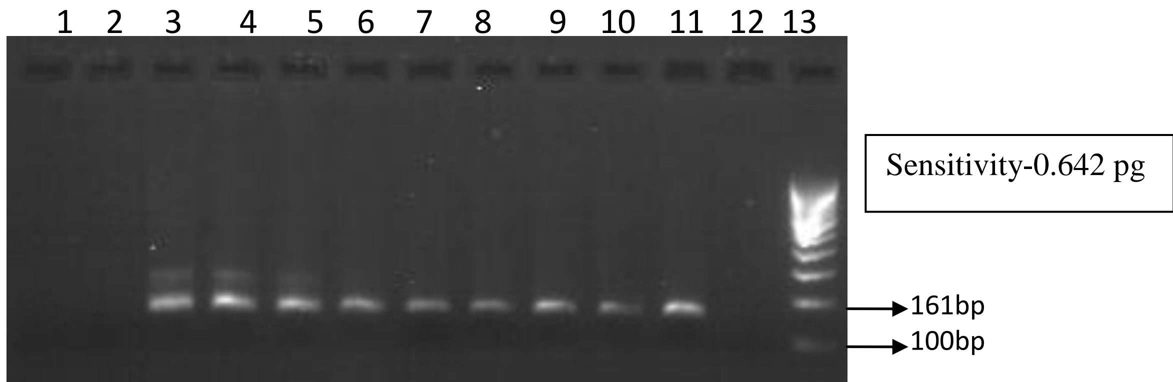


Figure 4.3: Agarose gel electrophoretogram showing the analytical sensitivity of indigenously designed primers targeting *rRNAP1* gene

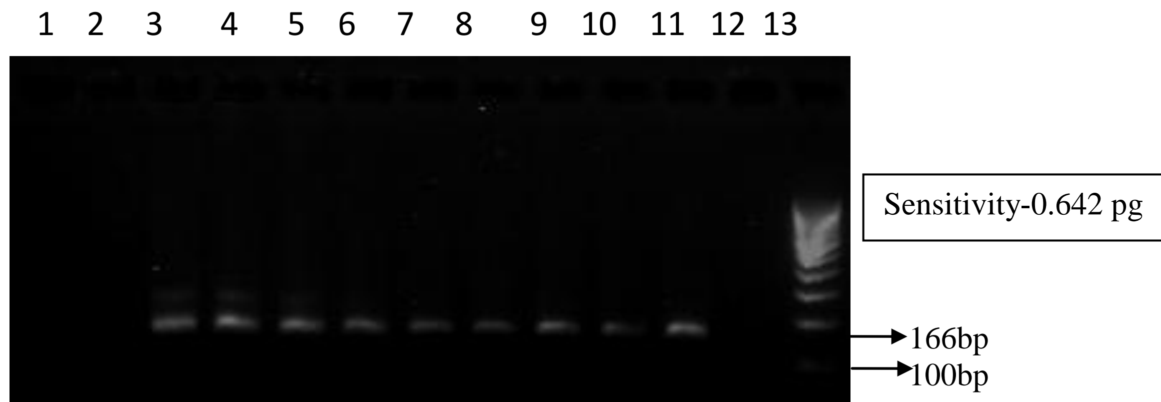


Figure 4.1, 4.2, 4.3 Lane1& 2: Negative controls, Lane 3: positive control -*M .tuberculosis H37Rv ATCC* (positive with specific base pair), Lanes 4-12: 10 fold serial dilutions of *M .tuberculosis H37Rv ATCC*, Lane 13: 100 bp molecular weight marker

4.6.3.1.2 Determination of Specificity of the primers targeting *icl_2* gene

The primers targeting *icl_2, hspx and rRNAP1* gene was specific to detect only *M. tuberculosis* H37Rv (Figure 4.4, 4.5, 4.6).

Figure 4.4: Agarose gel electrophoretogram showing the specificity of indigenously designed primers targeting *icl_2* gene

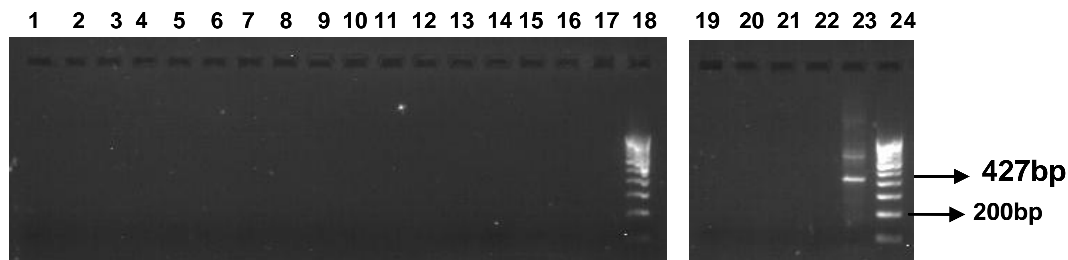


Figure 4.5: Agarose gel electrophoretogram showing the specificity of indegenously designed primers targeting *hspx* gene

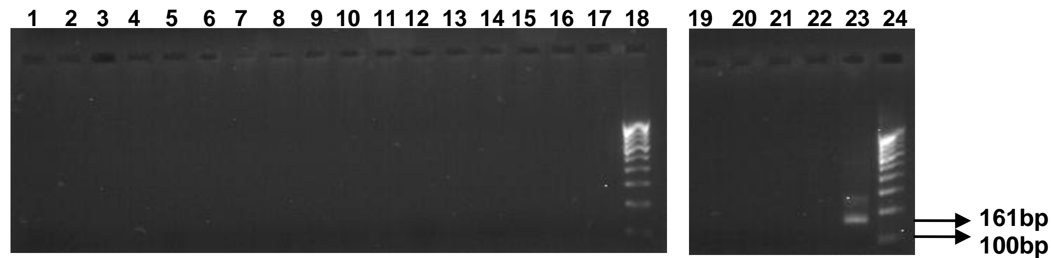
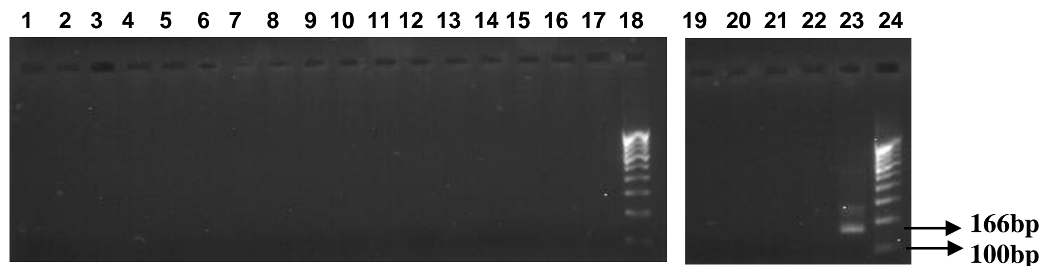


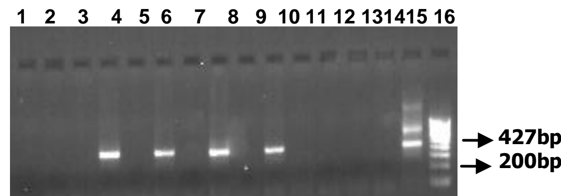
Figure 4.6: Agarose gel electrophoretogram showing the specificity of in- house designed primers targeting *rRNAP1* gene



Lane 1: Negative control (negative), Lane 2:*M. smegmatis* (negative), Lane 3: *M. duvalii* (negative), Lane 4: *M. thermoresistible* (negative), Lane 5: *M. fortuitum* (negative), Lane 6: *M. abscessus* (negative), Lane 7: *M. chelonae* (negative), Lane 8: *M. flavescens* (negative), Lane 9: *M. intracellulara* (negative), Lane 10:*M. phlei* (negative), Lane 11: *M. simiae* (negative), Lane12: *M. kansasii*(negative) , Lane 13: *M. gordonae* (negative), Lane 14: *M. xenopi* (negative), Lane 15: *Nocardia asteroids* (negative), Lane 16: *Actinomyces viscosus* (negative), Lane 17: *Streptomyces spp* (negative), Lane 18: MW DNA Ladder (100bp), Lane 19: Human DNA (negative), Lane 20: *M. Monacense* (negative), Lane 21: *M. bovis* (negative), Lane 22: *M. tuberculosis H37*Ra (negative), Lane 23: *M. tuberculosis H37*Rv (positive with specific base pair product), Lane24: MW DNA Ladder (100bp)

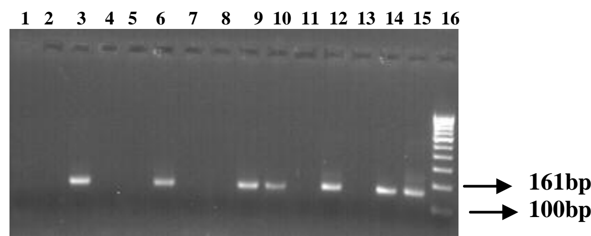
4.6.3.1.3 Results of Amplification of *M. tuberculosis* isolates targeting *icl*$_2$*, hspx and rRNAP1* gene

Figure 4.7: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *icl*$_2$ gene



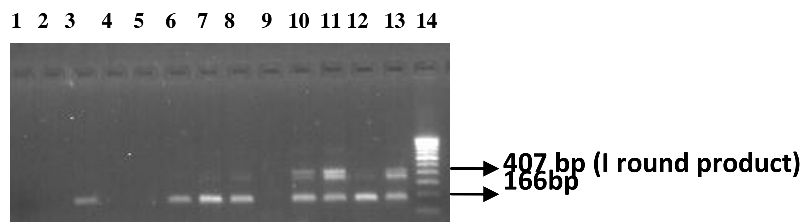
Lane 1: Negative Control1 (Negative), Lane 2:Negative control2 (negative) Lane: 3-14: Clinical specimens, Lane 2: LTITIM 1029 (negative), Lane 3: LTITM 1041 (negative), Lane 4: LTITIM 1060 (positive), Lane 5: LTITIM 1076 (negative), lane 6: LTITM 1077 (positive), Lane 7: LTITM 1064 (negative), Lane 8: LTITM 1078 (positive), Lane 9: LTITM 1079 (negative), Lane 10: LTITM 1080 (positive), Lane 11: LTITM 1081 (negative), Lane 12: LTITM 1083 (negative), Lane13: LTITM 1084 (negative), Lane 14: LTITM 1085 (negative) Lane 15 : PC *M. tuberculosis* H37Rv (Positive with specific 427 bp product), Lane 16 : MW marker 100bp ladder

Figure 4.8: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *hspx* gene



Lane 1: Negative Control1 (Negative), Lane 2:Negative control2 (negative) Lane: 3-14: Clinical specimens, Lane 3: LTITM 1050 (positive), Lane 4: LTITIM 1051 (negative), Lane 5: LTITIM 1052 (negative), lane 6: LTITM 1053 (negative), Lane 7: LTITM 1054 (negative), Lane 8: LTITM 1055 (negative), Lane 9: LTITM 1056 (positive), Lane 10: LTITM 1058 (positive), Lane 11: LTITM 1059 (negative), Lane 12: LTITM 1060 (positive), Lane13: LTITM 1061 (negative), Lane 14: LTITM 1062 (positive) Lane 15 : PC *M. tuberculosis* H37Rv (Positive with specific 161 bp product), Lane 16 : MW marker 100bp ladder

Figure 4.9: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rRNAP1* gene



Lane 1: Negative Control1 (Negative), Lane 2:Negative control2 (negative) Lane: 3-12: Clinical specimens, Lane 3: LTITM 1050 (positive), Lane 4: LTITIM 1051 (negative), Lane 5: LTITIM 1052 (negative), lane 6: LTITM 1053 (positive), Lane 7: LTITM 1054 (positive), Lane 8: LTITM 1055 (positive), Lane 9: LTITM 1056 (positive), Lane 10: LTITM 1058 (positive), Lane 11: LTITM 1059 (positive), Lane 12: LTITM 1060 (positive), Lane 13 : PC *M. tuberculosis* H37Rv (Positive with specific 166 bp product), Lane 14 : MW marker 100bp ladder

4.3.3.1.4 Results of nRT-PCRs targeting mRNA of gene on sputum samples*:*

Out of the 203 Sputum specimens from clinically suspected tuberculosis patients, 111 (54.6%) were positive for nRT-PCR targeting *icl*$_2$ gene. Among these, 24 (21.6%) were AFB smear positive, 42 (36%) were culture positive and 71 (63.9%) were culture negative by micro MGIT BACTEC method, but 6 culture positive sputum specimens were negative for *icl*$_2$ gene. All the 30 controls were negative for for nRT-PCR targeting *icl*$_2$ gene.

4.3.3.1.5 Results of nRT-PCRs targeting mRNA of *hspx* gene on sputum specimens*:*

Out of the 203 Sputum specimens, 107 (52.1%) were positive for nRT-PCR targeting *hspx* gene. Among 107 *hspx* gene positive clinical specimens, 24 (22.4%) were AFB smear positive, 38 (35.5%) were culture positive, 72 (67.2%) were culture negative by micro MGIT BACTEC method, but 10 culture positive sputum specimens were negative for nRT-PCR targeting *hspx* gene. All the 30 controls were negative for nRT-PCR targeting *hspx* gene.

4.3.3.3.3 Results of nRT-PCRs targeting mRNA of *rRNAP1* gene on sputum specimens*:*

Out of the 203 Sputum specimens, 80 (39.1%) were positive for nRT-PCR targeting *rRNAP1* gene. Among these, 24 (30%) were AFB smear positive, 35 (43.75 %) were culture positive, 47 (58.75%) were culture negative, but 13 culture positive sputum specimens were negative for nRT-PCR targeting *icl*$_2$ gene. All the 30 controls were negative for for nRT-PCR targeting *rRNAP1* gene.

The results of nRT-PCRs applied on 203 clinical specimens and the evaluation of nRT-PCRs against culture by micro MGIT BACTEC system is given in table 4.1 and 4.2 respectively.

Table 4.1: Results of nRT-PCRs targeting *icl₂, hspx and rRNAP1* genes on 203 sputum samples

Target genes	Total number of sputum specimens =203		nRT-PCR positive & Culture positive	nRT-PCR negative and culture positiv	AFB Smear positive
	Positive (%)	Negative (%)			
icl_2	111(54.1%)	92 (45%)	42 (20.6%)	6 (2.9%)	24(63.9%)
hspX	107 (52.1%)	96 (48.7%)	38 (18.7%)	10 (4.9%)	24(67.2%)
rRNAP	80 (39.1%)	123(59.6%)	35 (17.2%)	13 (6.4%)	24(58.75%

All the 30 controls were negative for both Mycobacterial culture by BACTEC method and nRT-PCRs targeting *icl_2, hspx and rRNAP1* genes

Table 4.2: Evaluation of nRT-PCRs targeting *icl_2, hspx and rRNAP1* genes results against culture positive *M. tuberculosis* isolates by BACTEC method in 203 Sputum specimens.

Categories of positivity	nRT-PCRs positive results							nRT-PCR*s* targeting *icl_2, hspx and rRNAP1* genes negative
	icl_2 gene alone	*hspx* gene alone	*rRNAP1* gene alone	*icl_2* and *hspx* genes	*icl_2* and *rRNAP1* genes	*hspx* and *rRNAP1* genes	*icl_2, hspx and rRNAP1* genes	
No. of specimens clinically suspected to be tuberculosis (203)	20 (9.8%)	11 (5.4%)	6 (2.9%)	26 (12.8%	4 (1.9%)	9 (4.4%)	65 (32%)	62 (31%)
M.tb culture positive by BACTEC system (48)	6 (2.9%)	2 (0.9%)	1 (0.4%)	2 (1.9%)	1 (0.4%)	nil	33 (16.2%)	3 (1.4%)

All the 30 controls were negative for both Mycobacterial culture by BACTEC method and nRT-PCRs targeting *icl_2, hspx and rRNAP1* genes

4.3.3.3.4 Comparitive results of Culture positive against nRT-PCRs negative clinical specimens:

Among 48 culture positive clinical specimens, 3 were *icl*$_2$ gene negative, 7 were *hspx* gene negative and 10 were *rRNAP1* gene negative. Out of 3 *icl*$_2$ negative clinical specimens, 2 were *hspx* gene positive and 1 was *rRNAP1* gene positive. Among 7 *hspx* gene negative clinical specimens, 5 were *icl*$_2$ gene positive and 2 were *rRNAP1* gene positive. Of 10 rRNAP1 gene negative clinical specimens, 6 were *icl*$_2$ gene positive, 2 were *hspx* gene positive and 2 were *hspx* and *icl*$_2$ genes positive (table 4.3).

Table 4.3: Comparative results of 48 culture positive *M. tuberculosis* isolates against nRT-PCRs (*icl*$_2$*, hspx and rRNAP1* genes) negative clinical specimens:

culture positive clinical specimens	nRT-PCR targeting *icl*$_2$ gene negative clinical specimens		nRT-PCR targeting *hspx* gene negative clinical specimens		nRT-PCR targeting *rRNAP1* gene negative clinical specimens			All nRT-PCRs negative clinical specimens
	hspx gene positive	*rRNAP1* gene positive	*icl*$_2$ gene positive	*rRNAP1* gene positive	*icl*$_2$ gene positive	*hspx* gene positive	*icl*$_2$ and *hspx* gene positive	3
48	2	1	5	2	6	2	2	
Total	3		7		10			3

4.4 DISCUSSION:

Tuberculosis has been reported to be the major cause of death among infectious diseases. Molecular markers of *M. tuberculosis* viability are attractive since results are rapid and there is potential for great analytical sensitivity [Li et al., 2010]. Molecular diagnostic methods like Reverse transcriptase PCR which detects actively multiplying *M. tuberculosis* directly

from the sputum specimens helps the clinicians to initiate proper anti-tuberculous treatment as early as possible, so that the rate of spreading of tuberculosis can be controlled.

The main objective of this study is to standardize nRT-PCRs targeting mRNA of icl_2 *and hspx and rRNAP1* genes to efficiently detect actively multiplying *M. tuberculosis* directly from clinical specimen which pave the way for the early initiation of appropriate anti-tuberculosis drugs.

Previous studies by Dietze *et al* [Dietze et al., 2001] who have demonstrated that DNA assays are not useful in monitoring response to therapy since *M. tuberculosis* DNA persists well beyond the time points that cultures are positive. In another study by Li *et al* [Li et al., 2010] who have compared four different *M. tuberculosis* mRNA targets (*85B, hspX,* icl_2 and *rRNAP1* genes) in the context of an early bactericidal activity study by comparing Isoniazid (INH) with three newer fluoroquinolones . They found that icl_2 mRNA was determined to be the best marker based on high levels of expression in sputum and strong correlation with Colony forming units (CFU) counts, both at baseline and during the 7 days of INH monotherapy. Munos-Elias *et al* [Munos-Elias et al., 2005] demonstrated that the icl_2 gene coding for Isocitrate lyase is essential for *M. tuberculosis* growth and persistence in macrophages. In a study by Haile et al [Haile et al., 2004], assessed the transcription of *hspx* gene isolated from an actively replicating culture of *M. tuberculosis* and found that the expression of *hspX* is enhanced in the actively growing culture at 7 and 10 days of culture of *M. tuberculosis* .

In the present study, nRT-PCRs targeting mRNA of icl_2, *hspx* and *rRNAP1* genes were optimized using **'indeginously designed'** primers using "**primer blast software**" from NCBI and applied on 203 sputum specimens and 30 control specimens. nRT-PCRs targeting icl_2 and *hspx* genes have almost equal positivity with 54.1% and 52.1% respectively to detect from clinically suspected tuberculosis patients than *rRNAP1* gene with 39.1% positivity. In co-relation of nRT-PCRs results with that of the BACTEC culture, icl_2 , *hspx* and *rRNAP1* genes were able to pick up 63.9%, 67.2%, 58.75% more culture negative sputum specimens respectively. None of the controls (30) were positive for both Mycobacterial culture by BACTEC method and nRT-PCRs targeting icl_2, *hspx and rRNAP1* genes. Eventhough in some cases, nRT-PCR was negative for any one of the optimized gene, the remaining three genes were able to pick up the viable *M. tuberculosis* from the clinical specimens. The

statistical analysis using SPSS software was done for the 3 nRT-PCRs and the sensitivity was found to be 36.52% (CI- 27.74%-46.01%), 33.93% (CI- 25.25%-43.48%), and 41.67% (CI- 31%-52.94%) for *icl_2, hspx* and *rRNAP1* genes respectively. Whereas, when all three nRT-PCRs were analysed, the sensitivity was increased to 94% with confidence interval of 82.78%- 98.62%. Therefore, targeting single gene will not be sufficient to detect viable *M. tuberculosis* from clinical specimens. So, all the four genes (*icl_2, hspx*, *rRNAP1* genes) must be targeted simultaneously to improve the sensitivity to 100% to detect viable *M. tuberculosis* from the clinical specimens. However, the nRT-PCR negativity in culture positive clinical specimens in this study was 1.4%. This is one of the significant findings from our study. The 1.4% false negativity in nRT-PCR resulted in our study may be due to the technical error encountered during collection, transportation, and performance of the experiment. From the results obtained in this study, we can also conclude that if the standardized nRT-PCRs were positive for two of the three genes (*icl_2 and hspx and rRNAP1* genes), it will provide 100% sensitivity in the detection of actively multiplying *M. tuberculosis* directly from sputum specimens.

4.5 SUMMARY

Nested RT-PCRs targeting mRNA of *icl_2, hspx and rRNAP1* genes were optimized using Indigenously designed primers (primer blast software) for the rapid detection of actively multiplying *Mycobacterium tuberculosis* and applied on 203 sputum specimens from clinically suspected tuberculosis patients and 30 control specimens. Of 203 sputum specimens, 111 (54.6%), 107 (52.7%) and 80 (39.4%) were positive for *icl_2*, *hspx* and *rRNAP1* genes respectively. In co-relation of nRT-PCRs results with that of the BACTEC culture, *icl_2, hspx* and *rRNAP1* genes were able to pick up 63.9%, 67.2%, 58.75% more culture negative sputum specimens respectively. However, all the three nRT-PCRs were negative in 3 culture positive *M. tuberculosis* isolates. But the false negativity was reduced from 13.8% (unpublished report in our earlier study) to1.4%. The sensitivty for all three nRT-PCRs were analysed to be 94% with confidence interval of 82.78%- 98.62%. Thus, nRT-PCRs standardized in this study when applied together on the suspected clinical specimens will be a rapid, reliable test and potentially serve as a diagnostic marker to detect the actively multiplying *M. tuberculosis* directly from sputum specimens.

4.6 CONCLUSION

In conclusion, nRT-PCRs targeting all the three gene targets optimized in this study needs to be applied simultaneously to increase sensitivity to 100% to detect viable *M. tuberculosis* directly from clinical specimens which helps in initiation of appropriate treatment to the patient. The combinations of indigenously standardized nRT-PCRs are definitely a useful contribution for a development of a rapid molecular diagnostic test for specific and rapid detection of actively multiplying *M. tuberculosis* genome directly from clinical specimens.

4.7 FUTURE ASPECTS

The nRT-PCRs standardized in this study should be applied on more number of clinical specimens to study the significance of the genes (icl_2, *hspX and rRNAP1*) which aid in the early detection of actively multiplying *M. tuberculosis* directly from the clinical specimens. A new set of primers for each gene also can be designed to increase the sensitivity of the nRT-PCR to be applied on clinical specimens for direct detection of actively multiplying *M. tuberculosis.*

CHAPTER 5

Detection of Multidrug resistant tuberculosis (MDR-TB) by phenotypic drug susceptibility testing using "micro MGIT BACTEC method" for first line anti-tuberculosis drugs (Streptomycin, Isoniazid, Rifampicin, Ethambutol and Pyrazinamide).

5.1 INTRODUCTION:

Multidrug resistant tuberculosis is defined as tuberculosis caused by a strain of *M. tuberculosis* that is resistant to Isoniazid and Rifampicin, two of the most important first line drugs used in the treatment of the disease. Drug resistant tuberculosis has been reported since the early days of introduction of anti-tubercular chemotherapy, but recently multi-drug resistant tuberculosis (MDR-TB), has been an area of growing concern, and is posing threat to global efforts of tuberculosis control. Three most important risk factors, identified in the causation of drug resistant tuberculosis are inappropriate previous treatment with anti-tubercular drugs, high prevalence of drug resistant tuberculosis in the community and contact with patients known to have drug resistant tuberculosis [WHO/HTM/TB/2012.402]. However standardized short course chemotherapy carries a little risk of emergence of MDR-TB. Other factors that may be responsible for increased risk of resistant tuberculosis are: Co-infection with HIV, socio economically deprived groups in slums, prisons, correctional facilities, day care centres, intravenous drug abusers and other immuno-compromised states as in transplant recipients, anti-cancer chemotherapy recipients and patients with diabetes mellitus.

Prevalence of MDR-TB, in a community mirrors the functional state and efficacy of tuberculosis control programme and realistic attitude of the community towards implementation of such programmes [WHO/HTM/TB/2012.402]. Management of MDR-TB is difficult, much expensive, challenging and quite often leads to treatment failure. Diagnosis is confirmed by drug susceptibility testing from reliable and reputed laboratories under constant quality control.The present study is undertaken to detect the percentage of MDR-TB circulating in Chennai population by Micro MGIT BACTEC mycobacterial culture system.

5.2 MATERIALS AND METHODS

The clinical isolates and the procedure for phenotypic drug susceptibility testing for first line drugs by micro MGIT BACTEC culture system was detailing explained in section 3.2.

5.3 RESULTS

Among 326 *M. tuberculosis* isolates, 218 (68.4%) were sensitive to all the 5 first line anti-tuberculosis drugs, **18 (5.5%) *M. tuberculosis* isolates were MDR-TB, 37(11.3%) were Polyresistant and 53 (16.2%) were Monoresistant strains for the first line anti-tuberculous drugs.**

Of 18 MDRTB strains, 10 were resistant to SHREZ, 4 were resistant to SHRE, 3 were resistant to HR and 1 was resistant to SHRZ.

Among 37 polyresistant strains, 9 were resistant to SHEZ, 6 were resistant to SZ, 5 were resistant to SHE, 4 were resistant to EZ, 3 each were resistant to SH, SHZ, 2 each were resistant to SE, SR and 1 was resistant to SEZ.

Out of 53 monoresistant strains, 23 were resistant to Streptomycin, 12 were resistant to Isoniazid, 13 were resistant to Pyrazinamide and 5 were resistant to Ethambutol. The results of first line phenotypic drug susceptibility testing for 326 *M. tuberculosis* isolates were shown in the table 5.1.

Table 5.1: First line phenotypic drug susceptibility testing results of 326 *M. tuberculosis* by Micro MGIT BACTEC method

Total no. of isolates tested	Susceptible to all the first line drugs	Monoresistant Strains N=53 (16.2%)	Polyresistant Strains N= 37 (11.3%)	MDR-TB Strains N=15 (5.5%)
326	218 (68.4%)	S- 23 H- 12 Z- 13 E- 5	SHEZ- 9 SZ -6 SHE -5 EZ -4 SH -3 SHZ -3 SR -2 SE -2 SEZ -1	SHREZ- 10 SHRE -4 HR -3 SHRZ -1

S- Streptomycin, H- Isoniazid, R- Rifampicin, E- Ethambutol, Z- Pyrazinamide

5.3.1 MDR-TB Strains:

Among the 326 *M. tuberculosis* isolates, 18 (5.5%) were MDR-TB isolates by Micro MGIT BACTEC method. In this study, majority of MDR-TB strains detected from sputum specimens (16- 88.8%) followed by bronchial wash (2- 11.1%). Of 18 MDRTB strains, 10 were resistant to SHREZ, 4 were resistant to SHRE, 3 were resistant to HR and 1 was resistant to SHRZ. The clinical specimen wise distribution of MDR-TB was shown in the table below (table 5.2 and 5.3)

TABLE 5.2: clinical specimenwise distribution of 18 MDR isolates

CLINICAL SPECIMENS	SHREZ	SHRE	HR	SHRZ
Sputum (16)	10	3	2	1
Bronchial wash (2)	-	1	1	-
TOTAL (18)	**10**	**4**	**3**	**1**

S- Streptomycin, H- Isoniazid, R- Rifampicin, E- Ethambutol, Z- Pyrazinamide

Figure 5.1: Pie Chart showing the distribution of resistance pattern in 18 (5.5%) MDR-TB strains to first line anti-tuberculous drugs

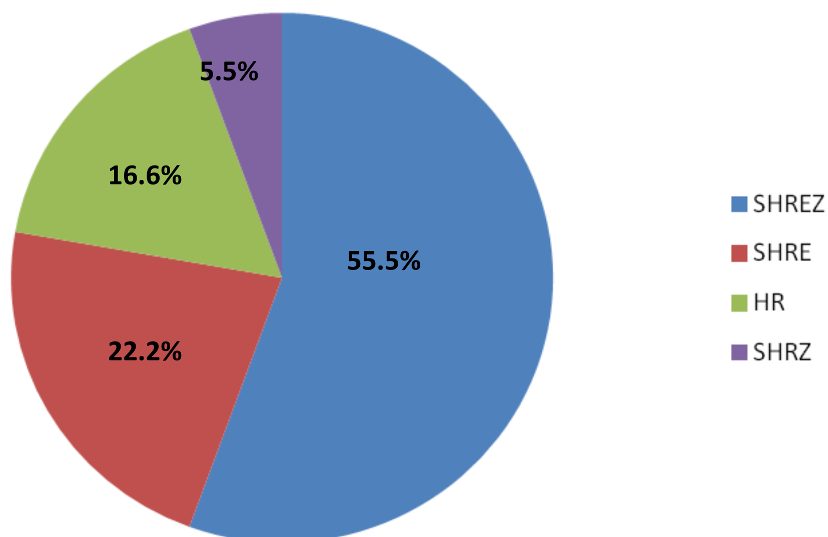


TABLE 5.3: Direct smear, age, sex and first line phenotypic DST results of 18 MDR-TB

S. No	Reference no.	Age/ sex	Clinical specimen	Direct smear	First line phenotypic DST
1	LTITM 480	42/F	Bronchial wash	Negative	HR- Resistant, SEZ- Sensitive
2	LTITM 543	26/F	Sputum	Negative	SHREZ - Resistant
3	LTITM 670	45/M	Sputum	2+	SHREZ - Resistant
4	LTITM 680	49/M	Sputum	3+	SHRZ- Resistant, E-Senstive
5	LTITM 728	30/F	Sputum	3+	SHRE – Resistant Z-Sensitive
6	LTITM 849	24/F	Bronchial wash	Negative	SHREZ- Resistant
7	LTITM 962	23/M	Sputum	Negative	HR- Resistant, SEZ- Sensitive
8	LTITM 1029	64/M	Sputum	1+	SHREZ- Resistant
9	LTITM 1041	30/F	Sputum	Negative	SHREZ- Resistant
10	LTITM 1045	14/F	Sputum	Few	SHRE – Resistant Z-Sensitive
11	LTITM 1052	48/F	Sputum	Negative	SHRE – Resistant Z-Sensitive
12	LTITM 1057	56/M	Sputum	Occ AFB	SHRE – Resistant Z-Sensitive
13	LTITM 1060	46/M	Sputum	3+	SHREZ- Resistant
14	LTITM 1076	19/F	Sputum	3+	SHREZ- Resistant
15	LTITM 1077	33/M	Sputum	2+	SHREZ- Resistant
16	LTITM 1133	40/F	Sputum	Negative	SHREZ- Resistant
17	LTITM 1134	67/M	Sputum	Negative	HR- Resistant, SEZ- Sensitive
18	LTITM 1141	14/F	Sputum	Negative	SHREZ- Resistant

Table (5.3.1) showing grading Chart for ZN Microscopy (1000X) by RNTCP guidelines

ZN staining grading (RNTCP)	Reporting /Grading
>10 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	Positive, 2+
10-99 AFB/100 field	Positive, 1+
1-9 AFB/100 field	Positive, Scanty
No AFB per 100 fields	Negative

Source: RNTCP training module for community pharmacist – 2013 report
http://www.tbcindia.nic.in/pdfs/MODULAR%20TRAINING%20a.pdf

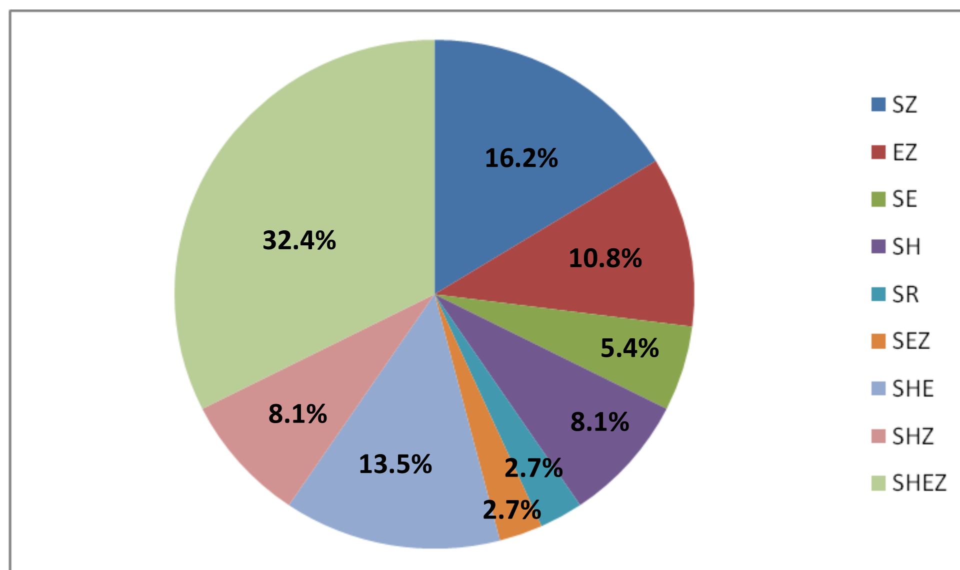
5.3.2 Polyresistant strains

Out of the 326 *M. tuberculosis* isolates, 37 were polyresistant strains. Of 37 Polyresistant isolates, 31(83.7%) were from sputum specimen, 4 (10.8%) were from FNAB and 1 (2.7%) each from bronchial wash and pleural fluid (Table 5.4, Figure 5.2). Among the polyresistant strains, majority were resistant to STR (89.1%), followed by PZA (70.2%), INH (20%), EMB (19%) and RIF (2.7%).

Table 5.4: Clinical specimenwise Distribution of 37 Polyresistant *M. tuberculosis* isolates

CLINICAL SPECIMENS	POLYRESISTANCE TO								
	SZ	**EZ**	**SE**	**SH**	**SR**	**SEZ**	**SHE**	**SHZ**	**SHEZ**
Sputum	6	4	2	3	3	1	2	3	7
Bronchial wash	-	-	-	-	-	-	1		-
Pleural fluid	-	-	-	-	-	-	1		-
FNAB		-					1		1
TOTAL (37)	**6**	**4**	**2**	**3**	**1**	**1**	**5**	**3**	**12**
S- Streptomycin, H- Isoniazid, R- Rifampicin, E- Ethambutol, Z- Pyrazinamide									

Figure 5.2: Pie chart showing the distribution of 37 (11.3%) polyresistant strains of *M. tuberculosis* to first line anti-tuberculosis drugs by micro MGIT BACTEC method included in the study.



5.3.3 Monoresistant strains:

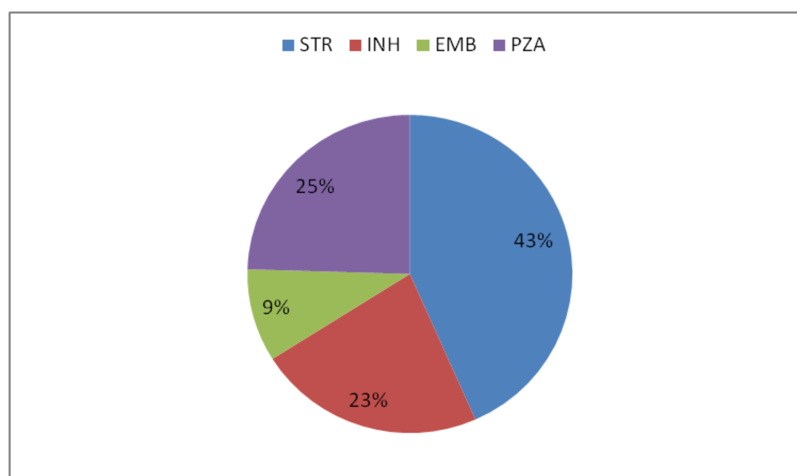
Out of the 326 *M. tuberculosis* isolates, 53 (16.2%) were monoresistant strains (table 5.5). Of 53 monoresistant strains, 40 (80%) were from Sputum, 3(6%) from Broncho alveolar lavage, 2 (4.6%) were from bronchial wash and 1(2.3%) each from Pleural fluid, FNAB and pus (source unknown), Pleural fluid, Gastric juice, Endoscopic biopsy, pus from left neck. Among 53 monoresistant strains, 23 were resistant to Streptomycin [Sputum (17), Bronchial wash (1), Broncho alveolar lavage (3), Pleural fluid (1), FNAB (1)], 12 were resistant to Isoniazid [Sputum (10), Bronchial wash (2), Broncho alveolar lavage (1)], 13 were resistant to Pyrazinamide [Sputum (9), Gastric juice (1), Pus from left neck (1), Endoscopic biopsy (1), Pus (1)] and 5 were resistant to Ethambutol [Sputum (4), Pus (1)]. None of the isolates showed monoresistant to RIF in this study.

Table 5.5: Clinical Specimenwise Distribution of 53 monoresistant *M. tuberculosis* isolates

CLINICAL SPECIMENS (N=53)	MONO RESISTANCE TO				
	STR	INH	EMB	PZA	RIF
Sputum (40)	17	11	4	9	-
Bronchial wash (2)	1	1	-	-	-
Broncho alveolar lavage (3)	2	1	-	-	-
Pleural fluid (2)	2	-	-	-	-
Gastric juice (1)	-	-	-	1	-
FNAB (1)	1	-	-	-	-
Pus (unknown source) (2)	-	-	1	1	-
Pus from neck abscess (1)	-	-	-	1	-
Endoscopic biopsy (1)	-	-	-	1	-
TOTAL (53)	**23**	**12**	**5**	**13**	**-**

S- Streptomycin, H- Isoniazid, R- Rifampicin, E- Ethambutol, Z- Pyrazinamide

Figure 5.3: Pie chart showing the distribution in of 53 (16.2%) Monoresistant strains of *M. tuberculosis* to first line drugs by micro MGIT BACTEC method in this study



5.4 DISCUSSION

Drug resistant tuberculosis still persists as a global health problem and poses challenges for the prevention and control of this deadly disease. In the most recent worldwide survey, the World Health Organization (WHO) documented the highest rates of MDR-TB ever reported. Effective management of drug resistant tuberculosis relies on multiple component including detection, treatment, prevention, surveillance, and continuous program evaluation [Horne et

al, 2013]. Expanding the capacity to diagnose cases of drug-resistant TB is a priority for global TB control, requiring clear policies on the use of diagnostic tests and strengthened laboratories in which testing can be safely and effectively carried out [Champbell et al., 2012].

Phenotypic drug susceptibility testing by BACTEC using proportion method was well studied for first line anti-tuberculosis drugs for treating drug resistant tuberculosis patients [Kim et al., 2005].

Bwaga et al studied the literature review and meta analysis from various reports. The Meta-Disc software was used to analyse the reports and tests for sensitivity, specificity, and area under the summary receiver operating characteristic (sROC) curves. Heterogeneity in accuracy estimates was tested with the Spearman correlation coefficient and Chi-square. They reported 18 direct DST reports were analysed using NRA (4), MODS (6), Genotype MTBDR (3) and Genotype MTBDRplus (5). The pooled sensitivity and specificity for detection of resistance to RIF were 99% and 100% with NRA, 96% and 96% with MODS, 99% and 98% with Genotype MTBDR, and 99% and 99% with the new Genotype MTBDRplus, respectively. For Isoniazid it was 94% and 100% for NRA, 92% and 96% for MODS, 71% and 100% for Genotype MTBDR, and 96% and 100% with the Genotype MTBDRplus, respectively [Bwanga et al., 2009]. Beauty et al performed MODS assay for detection of resistance to INH, RIF and MDR-TB strains. Among directly inoculated samples, re- sistance to INH was detected in 18/27 (67%), to RIF in 18/27 (67%), and to both INH and RIF (i.e., MDR-TB) in 17/27 (63%) by the reference standard. In their study, the MODS assay successfully detected INH and RIF resistance among all six previously cultured (i.e., indirectly inoculated) specimens. Overall sensitivity of the MODS assay for detection of resistance to INH and RIF and MDR-TB was 88% (95% CI, 68–97%), 96% (95% CI, 79–100%), and 91% (95% CI, 72–99%), respectively; specificity was 89% (95% CI, 52–100%), 89% (95 CI, 52–100%), and 90% (95% CI, 56–100%), respectively [Beauty et al., 2013]. Agarwal et al performed the phenotypic DST method using TB 460 reader and MGIT 960 system. Of the 397 study isolates, 114 (28.7%) were resistant to at least one of the four first-line anti-TB drugs tested and 18 (4.5%) were MDR, accounting for 15.8% of the total number of the drug-resistant isolates. The proportion of isolates resistant to each of the four tested anti-TB drugs varied. Resistances to INH (15.5%, 62 of 397) and SM (13.4%, 53 of

397) were almost twice as high as resistances to RMP (6.3%, 25 of 397) and EMB (6.0%, 24 of 397), respectively [Agarwal et al., 2010].

There are only few reports on detection of drug resistance by Micro MGIT BACTEC method from Chennai. In the present study, 326 *M. tuberculosis* isolates were subjected to First line phenotypic drug susceptibility testing by micro MGIT BACTEC method. Among 326 *M. tuberculosis* isolates, 5.5% were MDR-TB strains, 11.3% were polyresistant strains, 16.2% were monoresistant strains to first line anti-tuberculosis drugs. Regarding 18 MDR-TB strains, the majority were from sputum specimens (88.8%) followed by bronchial wash (11.1%).The age group of patients with MDR-TB were between 14-63 and female ratio is higher than the male ration (10:8). Among 242 *M. tuberculosis* isolates, 37 were polyresistant and 53 were monoresistant with maximum of 23 were resistant to STR, followed by PZA (13), INH (12), and EMB (5).

Conventional culture and phenotypic drug susceptibility testing methods require prolonged periods to confirm Mycobacterial growth and detect drug resistance, during which time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Rapid diagnosis of TB and drug resistance will therefore have obvious patient- as well as public health benefits, including better prognosis, increased survival, prevention of acquisition of further drug resistance, and reduced spread of drug resistant strains to vulnerable populations. Early detection of drug resistance in tuberculosis (TB) allows the use of appropriate treatment regimens for patients, which has an important impact for improved TB control.

The development of rapid methods for drug susceptibility testing (DST) is crucial due to increasing rates of multidrug-resistant tuberculosis (MDR-TB) worldwide and the emergence of extensively drugresistant tuberculosis (XDR-TB), with very high reported HIV-associated mortality. Spread of drug resistant strains of *M. tuberculosis* and the management of patients diagnosed with drug resistant disease is one of the most formidable obstacles faced by national tuberculosis control programmes, compounded by a critical lack of appropriate diagnostic tools and vastly inadequate laboratory capacity.

5.7 SUMMARY AND CONCLUSIONS:

Phenotypic drug susceptibility testing by Micro MGIT BACTEC culture system was applied on 326 *M. tuberculosis* isolates. Among 326 *M. tuberculosis* isolates, 5.5% were MDR-TB, 11.3% were Polyresistant and 16.2% were Monoresistant strains.

5.8 FUTURE ASPECTS:

The phenotypic drug susceptibility testing results by micro MGIT BACTEC method should be further validated by performing genotypic method by PCR based DNA sequencing. The genotypic results will further explore the significance of mutations in the drug resistant genes coding for first line drug resistance in *M. tuberculosis* isolates.

CHAPTER 6

Detection of Multi drug resistant tuberculosis (MDR-TB) by Genotypic drug susceptibility testing using PCR based DNA sequencing technique targeting drug resistance genes of First line anti-tuberculosis drugs (Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide).

6.1 INTRODUCTION:

MDR -TB is defined as resistance to the two most effective first line anti-tuberculous drugs, Isoniazid and Rifampicin. Resistance to anti-tuberculous drugs recognized in the very early days of the chemotherapeutic era, has also emerged as a serious problem. Resistance to single anti-tuberculous drug is the most common type; resistance to anti-tuberculous drug is less frequent but of greater concern. Management of MDR-TB relies on strong laboratory support and qualified, dedicated personnel for treatment oversight and supervision. Treatment should be individualized for each patient on the basis of in vitro susceptibility pattern to anti-tuberculous drugs.

Drug resistance in *M. tuberculosis* is caused by mutations in relatively restricted regions of the genome [Musser et al., 1995; Zhang et al., 2000]. Mutations associated with drug resistance occur in *rpo B* gene for RIF, *katG* gene and the promoter region of the *mabA (fabG*1)-*inhA* operon for INH, *embB* gene for EMB, *pncA* gene for pyrazinamide PZA, *rpsl, rrs* and *gidB* genes for STR. PCR based DNA sequencing method targeting first drug resistant genes will help to locate the novel mutational spots occurring in MDR-TB isolates which helps in finding out the signature sequence that can be used for developing rapid kit method for the detection of MDR-TB strains.

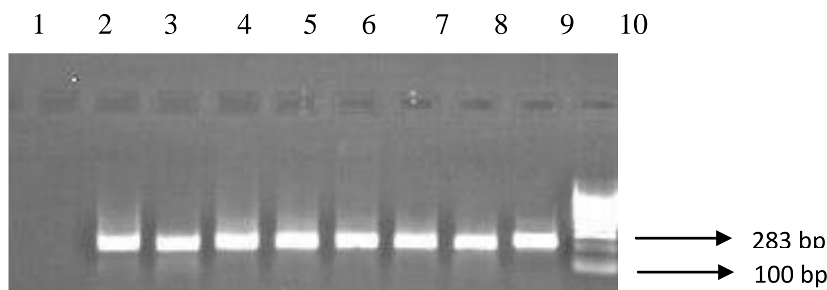
6.2 MATERIALS AND METHODS:

DNA extraction and PCR based DNA sequencing targeting the second line drug resistance was carried out as per mentioned in section 3.3

6.3 RESULTS:

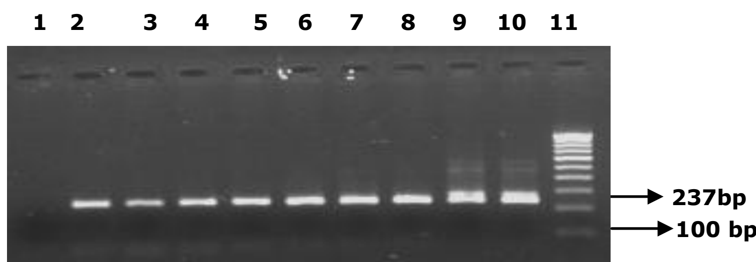
The optimized PCR targeting *rpoB*, *katG, inhA*, *oxyR-ahpC*, *rrs, rpsl, embB* and *pncA* genes for the first line anti-tuberculous drug resistance was applied on 242 in *M. tuberculosis* isolates.

Figure 6.1: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rpo B* gene



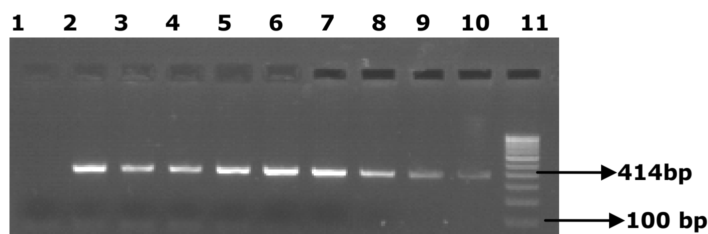
Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positiveecific 283 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: PC- *M. tuberculosis* H37Rv (Positive with specific 283 bp product), Lane 10 : MW marker 100bp ladder

Figure 6.2: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *kaG I* gene



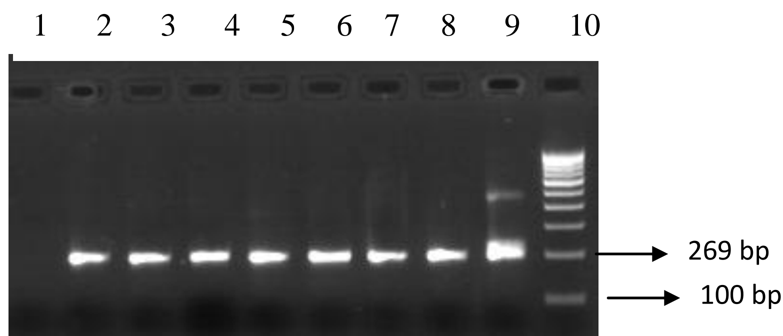
Lane 1: Negative Control (Negative), Lane: 2-9: *M. tuberculosis* isolates (Positive with specific 283 bp product), Lane 2: LTITM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: LTITM 620 (sputum) Lane 10: PC- *M. tuberculosis* H37Rv (Positive with specific 237 bp product), Lane 11 : MW marker 100bp ladder

Figure 6.3: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *kaG II* gene



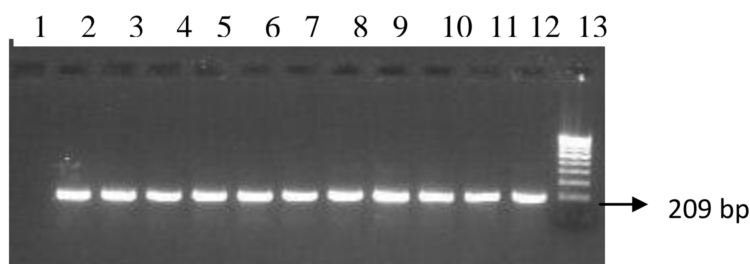
Lane 1: Negative Control (Negative), Lane: 2-9: *M. tuberculosis* isolates (Positive with specific 414 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: LTITM 620 (sputum) Lane 10: PC- *M. tuberculosis* H37Rv (Positive with specific 414 bp product), Lane 11 : MW marker 100bp ladder

Figure 6.4: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *kaG III* gene



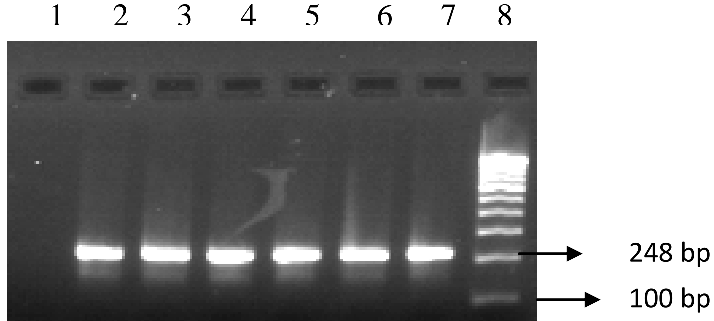
Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positive with specific 269 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: PC- *M. tuberculosis* H37Rv (Positive with specific 269 bp product), Lane 10 : MW marker 100bp ladder

Figure 6.5: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *kaG IV* gene



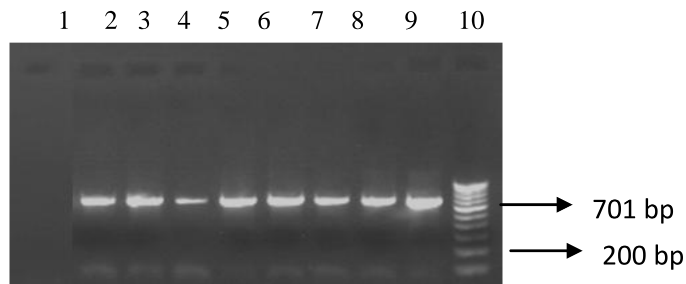
Lane 1: Negative Control (Negative), Lane: 2-11: *M. tuberculosis* isolates (Positive with specific 209 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: LTITM 620 (sputum), Lane 10: LTITM 722 (sputum), Lane 11: LTITM 1134 (sputum) Lane 12: PC- *M. tuberculosis* H37Rv (Positive with specific 209 bp product), Lane 13 : MW marker 100bp ladder

Figure 6.6: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *inhA* gene



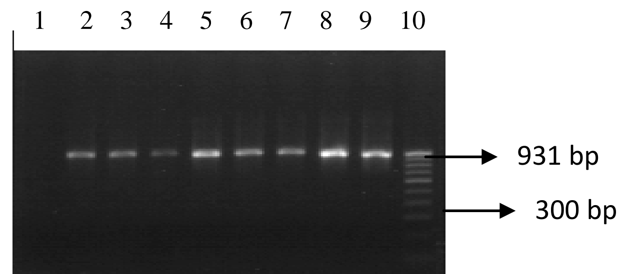
Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 320 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7 : PC *M. tuberculosis* H37Rv (Positive with specific 248 bp product), Lane 8 : MW marker 100bp ladder

Figure 6.7: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *oxyR-aphC* gene



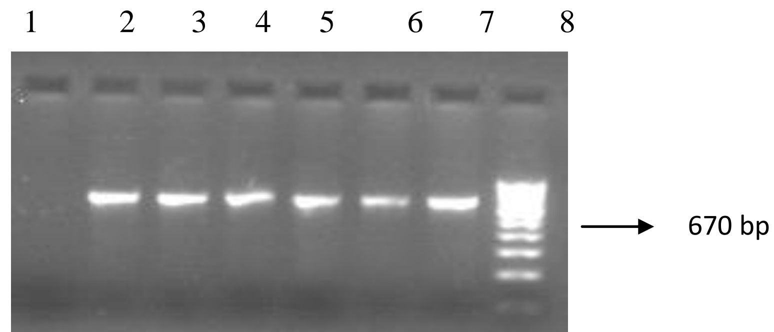
Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positive with specific 283 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: PC- *M. tuberculosis* H37Rv (Positive with specific 237 bp product), Lane 10 : MW marker 100bp ladder

Figure 6.8: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *emb B* gene



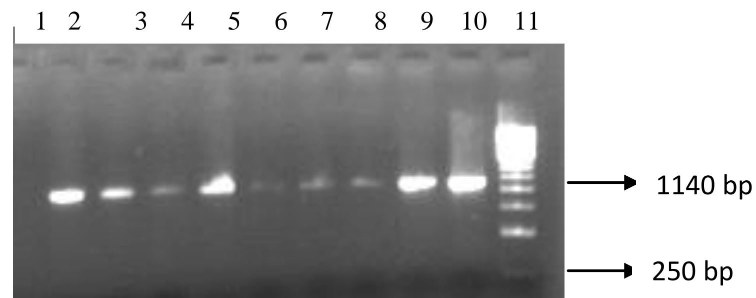
Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positive with specific 931 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: PC- *M. tuberculosis* H37Rv (Positive with specific 931 bp product), Lane 10 : MW marker 100bp ladder

Figure 6.9: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *pnc A* gene



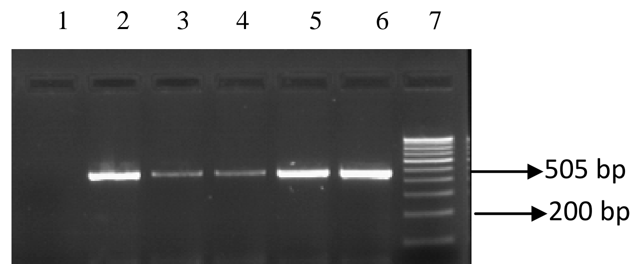
Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 320 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7 : PC *M. tuberculosis* H37Rv (Positive with specific 320 bp product), Lane 8 : MW marker 100bp ladder

Figure 6.10: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rrs* gene for Streptomycin resistance



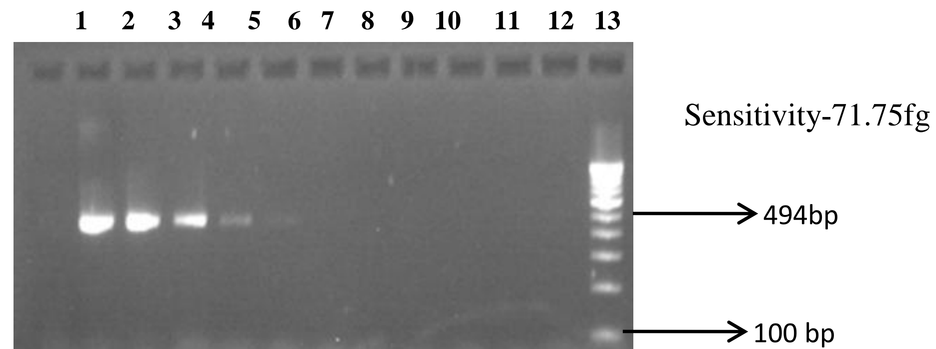
Lane 1: Negative Control (Negative), Lane: 2-9: *M. tuberculosis* isolates (Positive with specific 283 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1141 (sputum), Lane 7 : LTITM 1078 (sputum) Lane 8: LTITM 979 (sputum), Lane 9: LTITM 620 (sputum) Lane 10: PC- *M. tuberculosis* H37Rv (Positive with specific 1140bp product), Lane 11 : MW marker 250bp ladder

Figure 6.11: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *rpsl* gene



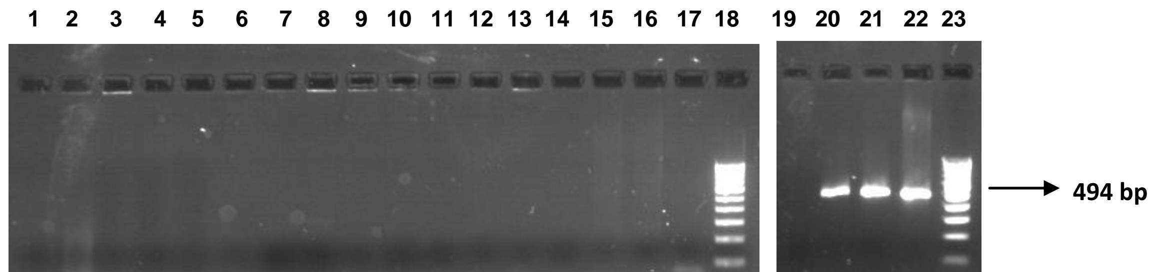
Lane 1: Negative Control (Negative), Lane: 2-9: *M. tuberculosis* isolates (Positive with specific 283 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), Lane 6: PC- *M. tuberculosis* H37Rv (Positive with specific 505 bp product), Lane 7 : MW marker 100bp ladder

Figure 6.12: Agarose gel electrophotogram showing analytical sensitivity of primers targeting *gid B* gene



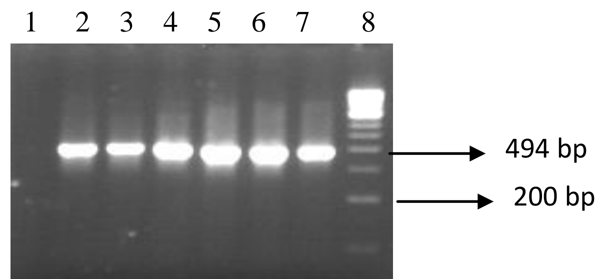
Lane1: Negative control (negative), Lane 2: positive control-*M .tuberculosis H37Rv ATCC* (positive with specific 494 bp product), Lanes 3-12: 10 fold serial dilutions of *M .tuberculosis H37Rv ATCC* 494bp product band was found till 10^{-4} dilution, Lane 13: 100 bp molecular weight marker

Figure 6.13: Agarose gel electrophotogram showing specificity of the primers targeting *gid B* gene



Lane 1: Negative control, Lane 2:*M. smegmatis* (negative), Lane 3: *M. duvalii* (negative), Lane 4: *M. thermoresistible* (negative), Lane 5: *M. fortuitum* (negative), Lane 6: *M. abscessus* (negative), Lane 7: *M. chelonae* (negative), Lane 8: *M. flavescens* (negative), Lane 9: *M. intracellulara* (negative), Lane 10:*M. phlei* (negative), Lane 11: *M. simiae* (negative), Lane12: *M. kansasii*(negative) , Lane 13: *M. gordonae* (negative), Lane 14: *M. xenopi* (negative), Lane 15: *Nocardia asteroids* (negative), Lane 16: *Actinomyces viscosus* (negative), Lane 17: *Streptomyces spp* (negative), Lane 18: MW DNA Ladder (100bp), Lane 19: Human DNA (negative), Lane 20: *M. bovis* (positive with specific 494 bp product), Lane 21: *M. tuberculosis H37*Ra (positive with specific494 bp product), Lane 22: *M. tuberculosis H37*Rv (positive with specific 494bp product), Lane23: MW DNA Ladder (100bp)

Figure 6.14: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *gidB* gene



Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 494 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7 : PC *M. tuberculosis* H37Rv (Positive with specific 494 bp product), Lane 8 : MW marker 100bp ladder

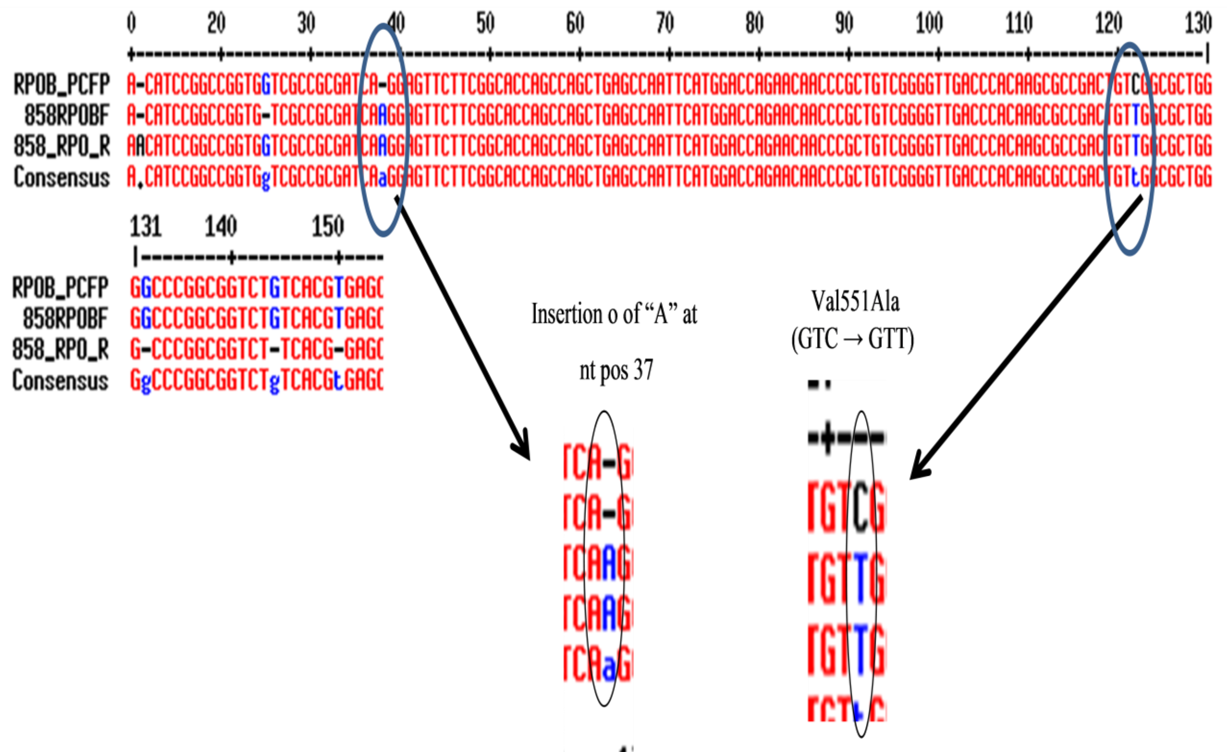
6.3.1 PCR based DNA sequencing results targeting *rpoB* gene encoding for resistance to Rifampicin:

S.no	lab isolate no.	Nucleotide change	mutation type
Rifampicin Sensitive strains			
2	LTITM 842 (Sputum)	**G**TC → **GTT**(Val551Ala)	Substitution
2	LTITM 858 (Bronchial wash)	**G**TC → **GTT(**Val551Al)	Substitution
3	LTITM 1002 (Sputum)	**G**TC → **GTT**(Val551Ala)	Substitution
4	26 strains showed Insertion of "A" at pos 37		Frameshift
Rifampicin resistant strains			
4	6 strains showed Insertion of "A" at pos 37		Frameshift

Among 242 *M. tuberculosis* isolates, 20 were resistant and 222 were sensitive to Rifampicin by phenotypic drug susceptibility testing by Micro MGIT BACTEC method. Novel Insertional mutation of "A" at nucleotide position 37 were observed in 6 (sputum) resistant strains and 26 (Sputum- 15, Pus from chest wall-1, Pleural fluid- 1, FNAC- 4) sensitive strains. Val551Ala Novel substitution mutation was observed in 3 (Sputum-2, Bronchial wash-1) sensitive strains (table 6.1, Figure 6.15).

Table 6.1: Novel mutations in Rifampicin Sensitive and Resistant *M. tuberculosis* isolates in *rpo B* gene

Figure 6.15: Multalin results of PCR based DNA sequencing targeting *rpo B* gene



Row 1: RPOB_PCFP- positive control (*M. tuberculosis H37RV* ATCC DNA) sequence

Row 2: 858RPOF- Forward sequences of *M. tuberculosis* isolates showing novel mutation (Ins of "A" at pos 37)

Row 3: 858_RPO_R- Reverse sequences of *M. tuberculosis* isolates showing novel mutation (Ins of "A" at pos 37)

Row 4: Consensus- The sequence showing the consenses of the isolate with the reference strain

6.3.2 PCR based DNA sequencing results targeting *katG* gene coding for Isoniazid Resistance (Table 6.2):

katG II* region*:

Among 242 isolates, 50 were resistant and 192 were sensitive to Isoniazid by Phenotypic drug susceptibility testing by Micro MGIT BACTEC culture system. Among the 192 sensitive strains, 10 sputum strains showed novel substitution mutation at codon 63

(Gly63Trp). None of the mutations were observed in phenotypically resistant strains. There were no mutation in all the 242 *M. tuberculosis* isolates (50 INH resistant and 192 INH sensitive) targeting katG *I, III* and *IV* genes

Table 6.2: Novel mutations in Isoniazid Sensitive *M. tuberculosis* sputum isolates

S. No	Reference no.	Nucleotide change	Aminoacid change	Mutation type
1	10 strains	**GGG→TGG**	Gly63Trp	Substitution

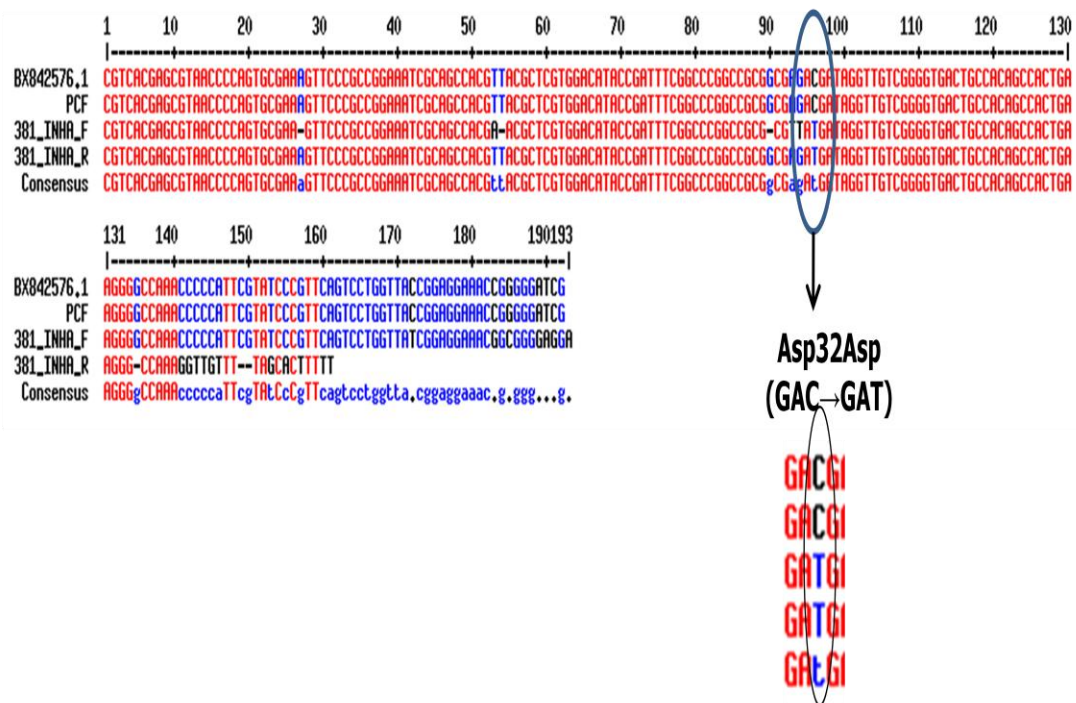
6.3.3 PCR based DNA sequencing results targeting *inhA* gene coding for Isoniazid resistance (Table 6.3, Figure 6.16):

Among 242 *M. tuberculosis* isolates, 50 were resistant and 192 were sensitive to Isoniazid. Asp32Asp novel silent mutation was observed in 2 sputum sensitive strains and 1 sputum resistant strain. In the remaining isolates, there were no mutations observed (**Table 6.3, Figure 6.16**).

Table 6.3: Novel silent mutations in Isoniazid Sensitive and resistant *M. tuberculosis* isolates

S. No	Reference no.	Nucleotide change	Aminoacid Change	Mutation type
Isoniazid resistant strains				
1	2 strains	**GAC→GAT**	Asp32Asp	silent
Isoniazid sensitive strains				
3	1 strain	**GAC→GAT**	Asp32Asp	silent

Figure 6.16: Multalin Results of DNA Sequence Analysis targeting *inhA* gene for isoniazid resistance



Row 1: BX842576.1- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PCF- positive control (*M. tuberculosis H37RV* ATCC DNA) sequence

Row 3: 381_INHA_F- Forward sequence of *M. tuberculosis* isolate showing silent mutation (Asp32Asp)

Row 4: 381_INH_R- Reverse sequence of *M. tuberculosis* isolate showing silent mutation (Asp32Asp)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

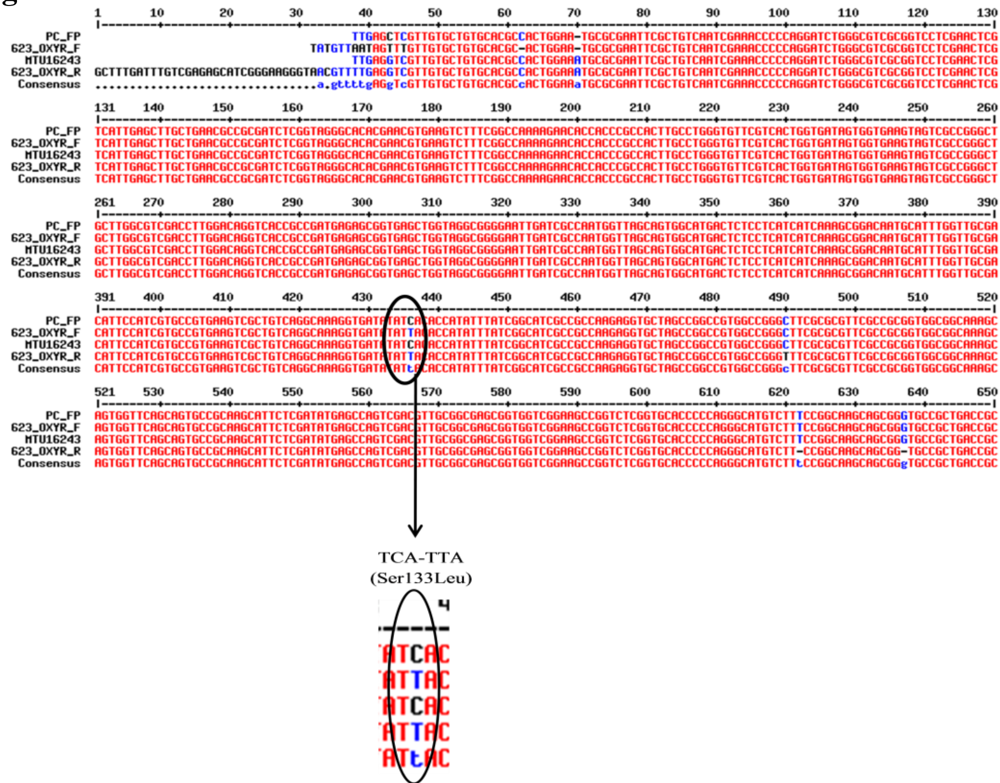
6.3.4 PCR based DNA sequencing results targeting *oxyr-aphc* gene coding for Isoniazid resistance

Among 242 *M. tuberculosis* isolates, 50 were resistant and 192 were sensitive to Isoniazid. Among 192 sensitive strains, 2 (sputum) isolates each showed one novel substitution at codon 133 (Ser133leu) and one novel silent mutation at codon 154 (Gly154Gly).Of 50 phenotypically resistant strains, only one sputum isolate showed novel substitution mutation at codon 149 (Cys149Gly) (Table 6.3, Figure 6.17, 6.18).

Table 6.3: Novel mutations in Isoniazid Sensitive and resistant *M. tuberculosis* sputum isolates

S. No	Reference no.	Nucleotide change	Aminoacid Change	Mutation type
Isoniazid sensitive strains				
1	LTITM 623	**TCA→TTA**	Ser133Leu	Substitution
2	LTITM 646	**CCT→GTT**	Gly154Gly	silent
Isoniazid resistant strains				
3	LTITM 714	**GTG→GGG**	Cys149Gly	Substitution

Figure 6.17: Multalin Results (LTITM 623) of DNA Sequence Analysis targeting *oxyR-aphC* gene



Row 1: PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

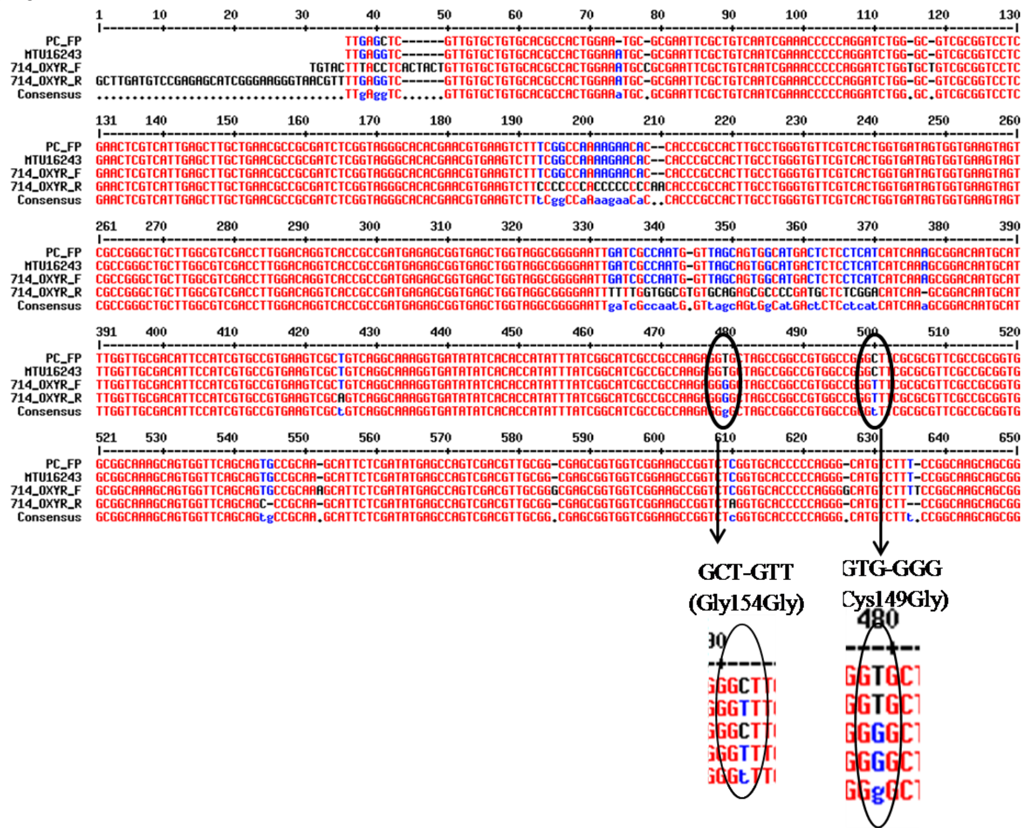
Row 2: 623_OXY_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Ser133Leu)

Row 3: MTU16243- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: 623_OXY_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Ser133Leu)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 6.18: Multalin Results (LTITM 714) of DNA Sequence Analysis targeting *oxyR-aphC* gene



Row 1: PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 2: MTU16243- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 3: PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) sequence

Row 4: 714_OXY_F/- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Cys149Gly)

Row 5: 714_OXY_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation Cys149Gly)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

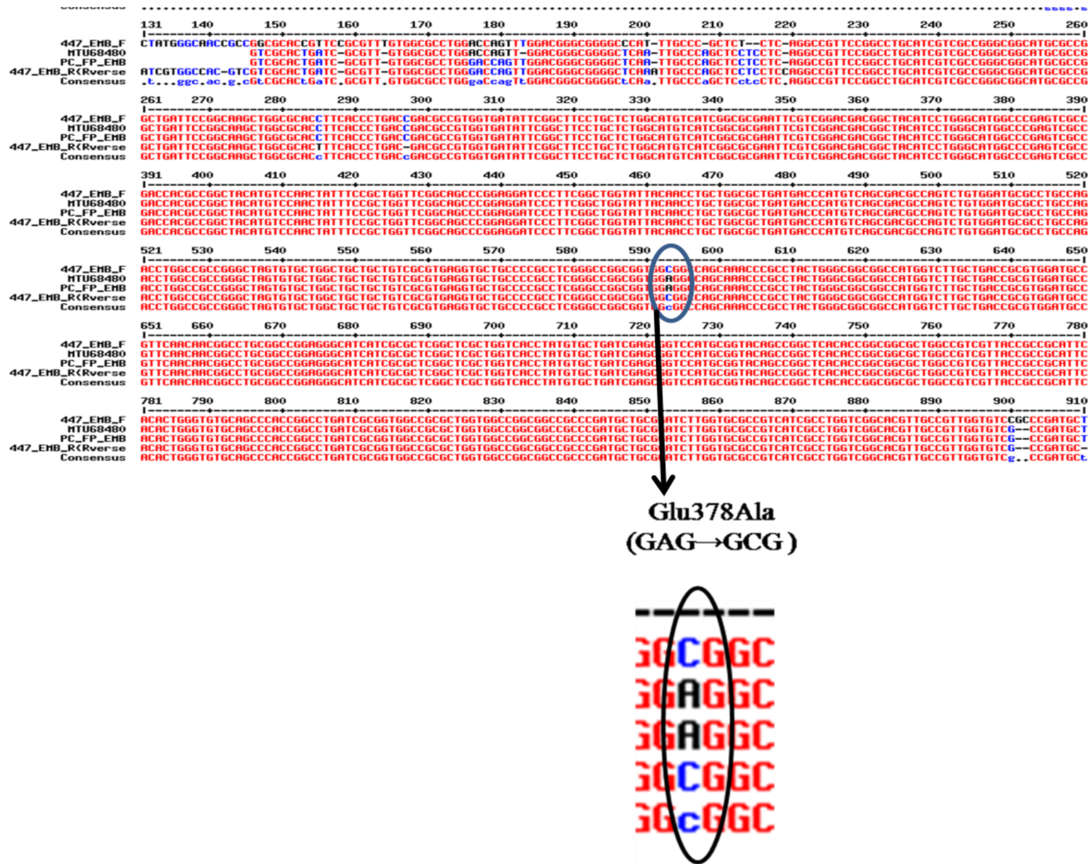
6.3.5 PCR based DNA sequencing results targeting *emb B* gene coding for Ethambutol resistance (Table 6.4, Figure 6.19):

Among 242 *M. tuberculosis* isolates, 201 were sensitive and 41 were resistant to Ethambutol by phenotypic drug susceptibility testing by Micro MGIT BACTEC method. Gly378Ala reported mutation was observed in 2 resistant strains (sputum- FNAB-1) and 7 sensitive strains (sputum-4, FNAB-1, Bronchial wash-1).

Table 6.4: Reported mutations in Ethambutol Sensitive and resistant *M. tuberculosis* isolates

S. No	Reference no.	Nucleotide change	Aminoacid change	Mutation type
Ethambutol resistant strains				
1	2 strains	GAG→GCG	Glu378Ala	Substitution
Ethambutol sensitive strains				
2	8 strains	GAG→GCG	Glu378Ala	Substitution

Figure 6.19: Multalin Results of DNA Sequence Analysis Targeting *emb B* gene for Ethambutol resistance



Row 1: 447_EMB_F/- Forward sequence of *M. tuberculosis* isolate showing reported mutation (Glu378Ala)

Row 2: MTU68480- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 3: PC_FP_EMB- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: 447_EMB_R- Reverse sequence of *M. tuberculosis* isolate showing reported mutation (Glu378Ala)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

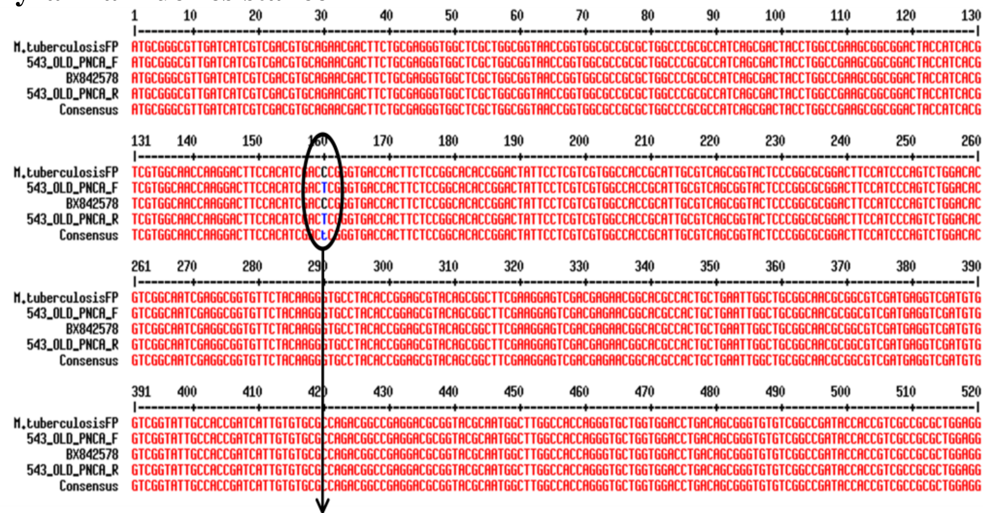
6.3.6 PCR based DNA sequencing results targeting *pncA* gene for Pyrazinamide resistance

Among 242 *M. tuberculosis* isolates, 201 were sensitive and 41 were resistant to pyrazinamide by phenotypic drug susceptibility testing by Micro MGIT BACTEC method. Of 41 resistant strains, 2 sputum isolates showed novel substitution mutation at codon 54 (Pro54Ser) and 1 resistant strain showed novel silent mutation at codon 65 (Ser65Ser). The similar silent mutation (Ser65Ser) was also observed in 2 phenotypically sensitive strains (Table 6.5, Figure 6.20, 6.21).

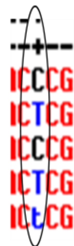
Table 6.5: Novel mutations in Pyrazinamide Sensitive and resistant *M. tuberculosis* isolates

S. No	Reference no.	Nucleotide change	Aminoacid change	Mutation type
Pyrazinamide resistant strains				
1	2 strains	CCG→TCG	Pro54Ser	Substitution
Pyrazinamide sensitive strains				
4	2 strains	TCC→TCT	Ser65Ser	Silent

Figure 6.20: Multalin Results of (LTITM 543) DNA Sequence Analysis Targeting *pncA* gene for Pyrazinamide resistance



Pro54Ser
(CCG→TCG)



Row 1: *M. tuberculosis*_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

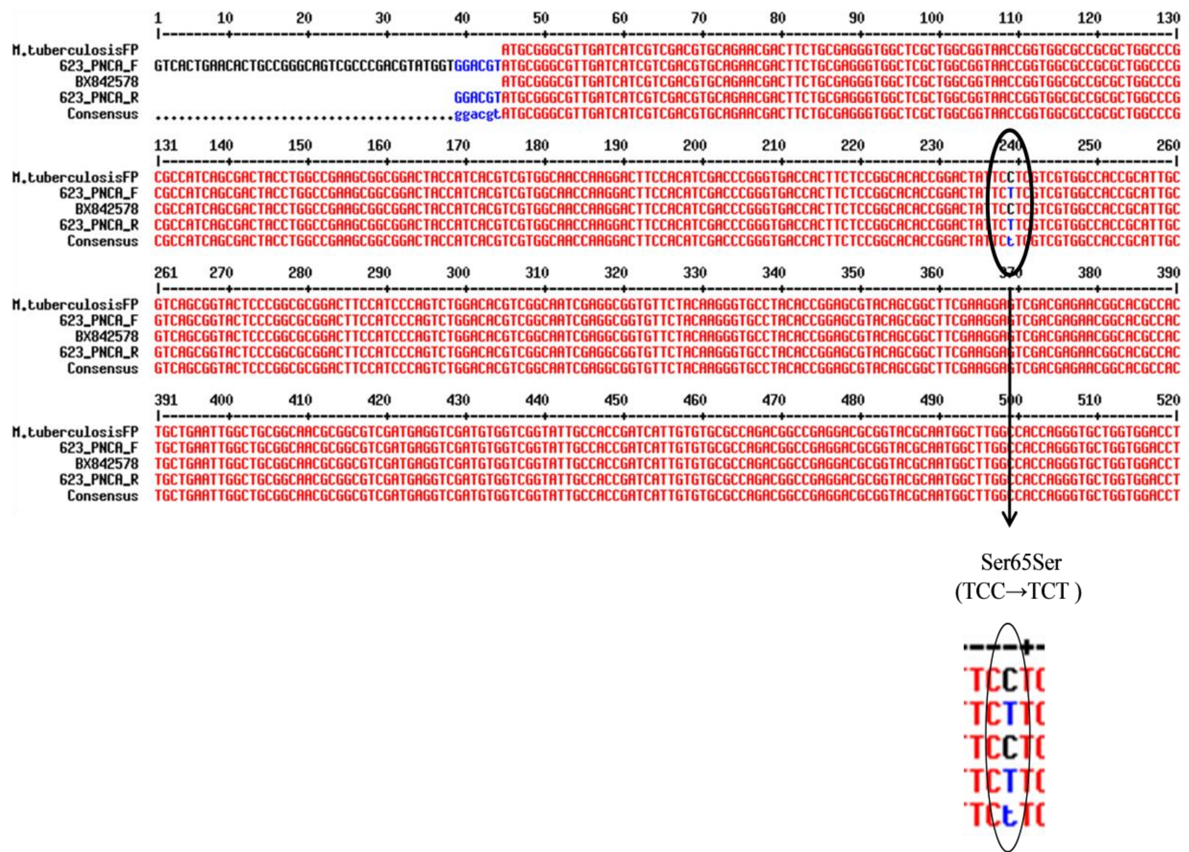
Row 2: 543_PNCA_F/- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Pro54Ser)

Row 3: BX842578- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: 543_PNCA_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Pro54Ser)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 6.21: Multalin Results of (LTITM 623) DNA Sequence Analysis Targeting *pncA* gene for Pyrazinamide resistance



Row 1: *M. tuberculosis*_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 2: 623_PNCA_F- Forward sequence of *M. tuberculosis* isolate showing mutation (Ser65Ser)

Row 3: BX842578- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: 623_PNCA_R- Reverse sequence of *M. tuberculosis* isolate showing silent mutation (Ser65Ser)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

6.3.7 PCR based DNA sequencing results targeting *rrs and rpsl* gene coding for Streptomycin resistance:

Among 242 *M. tuberculosis* isolates, 61 were resistant and 181 were sensitive to Streptomycin by phenotypic drug susceptibility testing by Micro MGIT BACTEC method. None of the 242 isolates showed mutation in *rrs* and *rpsl* genes. The results imply that the phenotypic resistance to STR may be due some other resistant genes which may confer resistance to STR. Thus, in the present study we have newly standardised another gene target "*gid B* gene" from the recent literatures which encode for STR resistance in *M. tuberculosis* isolates (Table 6.6, Figure 6.22-6.26):.

6.3.7 PCR based DNA sequencing results targeting *gid B* gene for Streptomycin resistance :

PCR targeting *gid B* gene encoding resistance to STR was standardised and applied on 102 STR resistant strains and 10 STR sensitive strains. Among 102 resistant strains, 8 sputum isolates each showed the presence of 5 novel substitution mutations (Pro75Leu, Leu59Arg, Trp33Cys Thr93Pro, Ile114Thr), 2 novel deletion mutation of nucleotide" C" at position 105 and 1 novel silent mutation at codon 20 (Arg20Arg). Apart from these mutations, there are 3 reported mutations were observed (1 deletion mutation of nucleotide "G" at position 102 by 2 sputum isolates, 1 substitution mutation at codon 16 [Leu16Arg] by 2 sputum isolates and 2 silent mutaion at codons 110, 141 [Val110Val, Ala141Ala] observed in 24 sputum isolates and 4 FNAB) . None of the STR sensitive strains showed mutations in the *gidB* gene.

TABLE 6.6: Novel and reported mutations in Streptomycin Sensitive and resistant *M. tuberculosis* isolates

S. No	Reference no.	Nucleotide change	Aminoacid Change	Mutation type
Streptomycin resistant strains				
1	LTITM 296 (sputum)	Del of “C” at pos 105	-	Frameshift
	LTITM 553 (sputum)	Del of “C” at pos 105	-	Frameshift
2	LTITM 293 (Sputum)	CCG→CTG	Pro75Leu	Substitution
3	LTITM 447 (Sputum)	CTC→CGC	Leu59Arg	Substitution

4	LTITM 670 (Sputum)	TGG→TGC	Trp33Cys	Substitution
4	LTITM 982 (Sputum)	ACC→CCC	Thr93Pro	Substitution
5	LTITM 979 (Sputum)	ATC→ACG	Ile114Thr	Substitution
6	LTITM 21 (Sputum)	Del of "G" at pos 102	-	Frameshift*
7	LTITM 982 (Sputum)	Del of "G" at pos 102	-	Frameshift*
8	LTITM 28 (Sputum)	CTT→CGT	Leu16Arg	Substitution*
9	LTITM 28 (Sputum)	CTT→CGT	Leu16Arg	Substitution*
10	LTITM 28 (Sputum)	CGG→AGG	Arg20Arg	Silent*
11	25 sputum strains	GTG→GTT	Val110Val	Silent*

*reported mutation

Figure 6.22: Multalin Results (LTITIM 296) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance

Row 1: RV3919C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PC_GIDB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) sequence

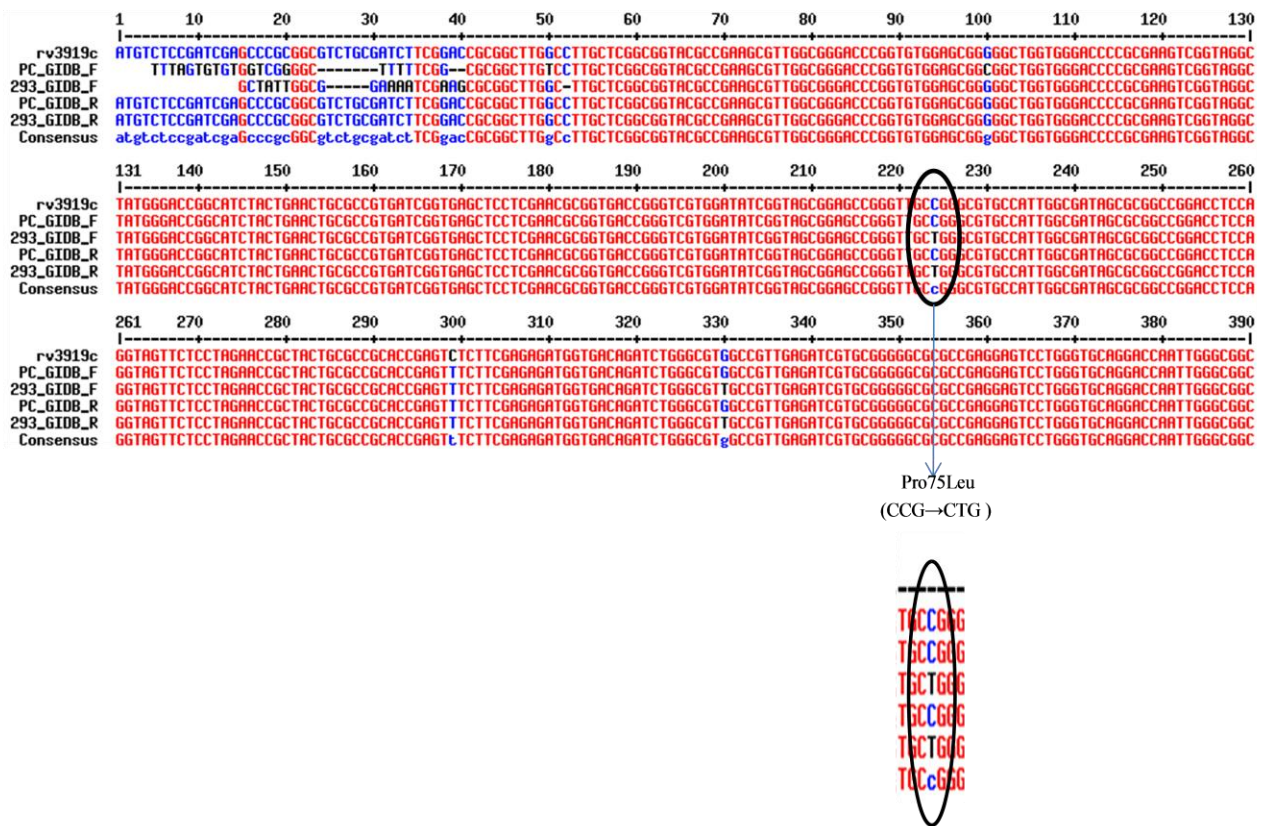
Row 3: 296_GIDB_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Del of “C” at pos 105)

Row 4: PC_GIDB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: 296_GIDB_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Del of “C” at pos 105)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 6.23: Multalin Results (LTITIM 293) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance



Row 1: RV3919C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PC_GIDB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

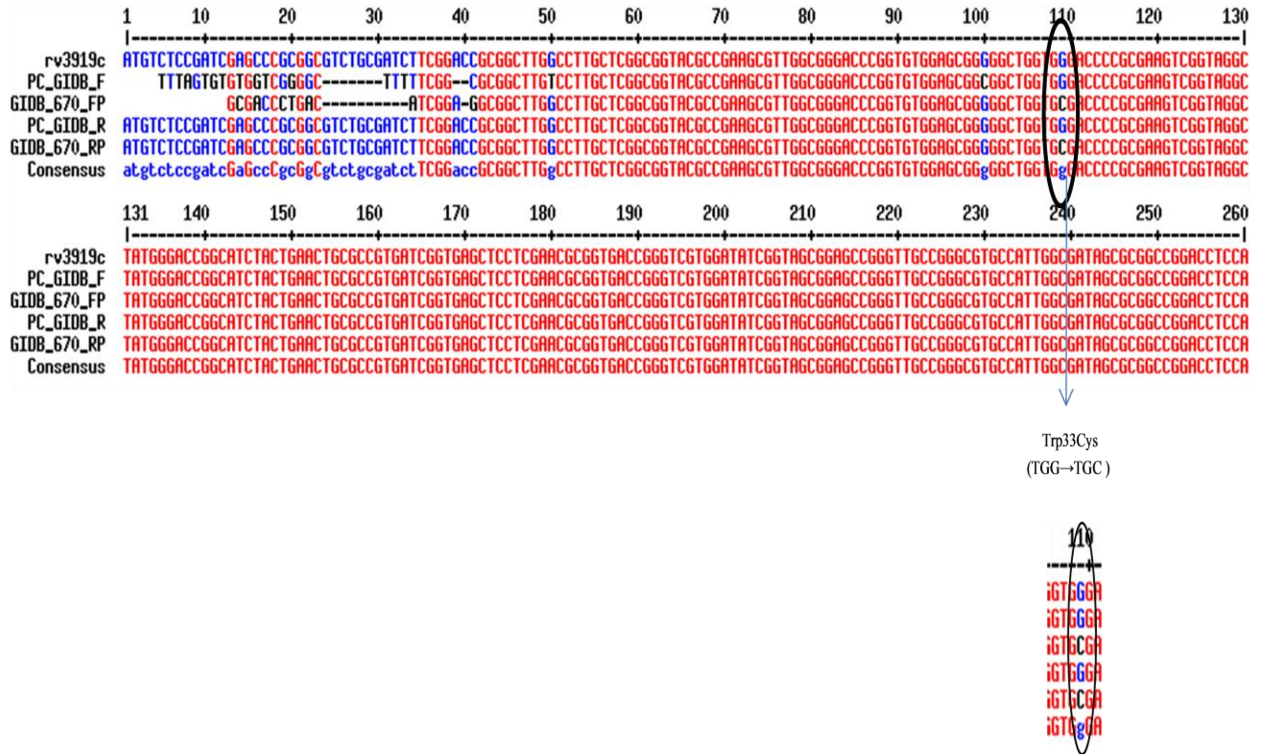
Row 3: 293_GIDB_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Pro75Leu)

Row 4: PC_GIDB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: 293_GIDB_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Pro75Leu)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 6.24: Multalin Results (LTITIM 670) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance



Row 1: RV3919C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PC_GIDB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

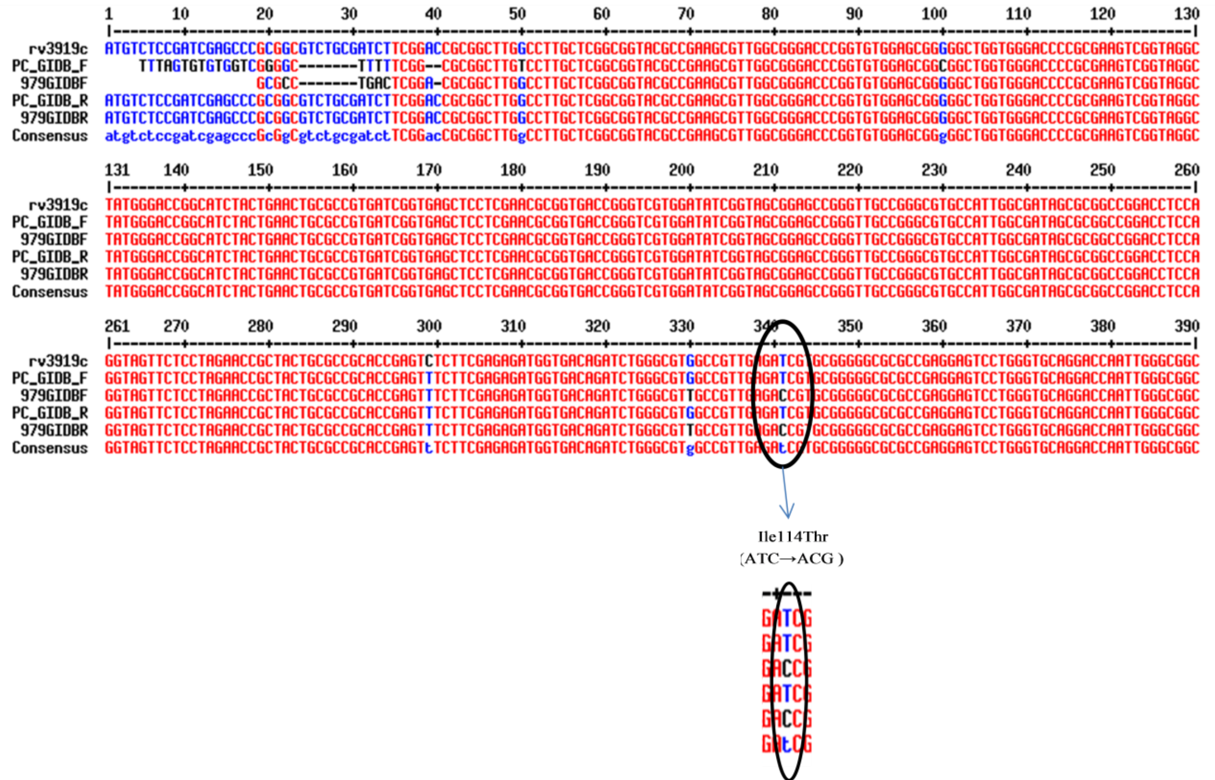
Row 3: GIDB_670_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Trp33Cys)

Row 4: PC_GIDB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: GIDB_670_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Trp33Cys)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 6.25: Multalin Results (LTITIM 979) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance



Row 1: RV3919C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCCDNA) taken from Genbank.

Row 2: PC_GIDB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

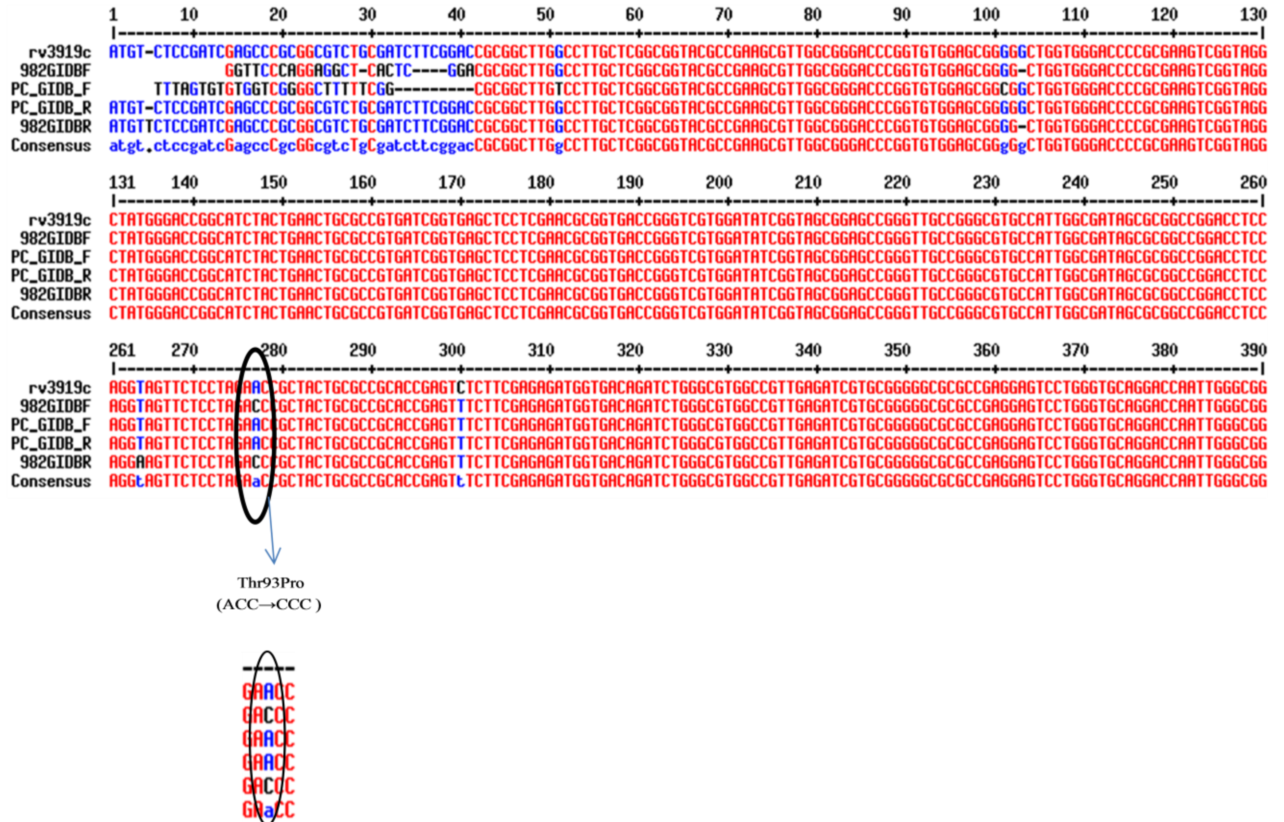
Row 3: 979GIDBF- Forward sequence of *M. tuberculosis* isolate showing mutation (Thr93Pro)

Row 4: PC_GIDB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: 979GIDBR- Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Thr93Pro)

Row 6: Consensus- The sequence showing the consenses of the isolate with the Reference Strain

Figure 6.26: Multalin Results (LTITIM 982) of DNA Sequence Analysis Targeting *gid B* gene for STR resistance



Row 1: RV3919C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: 982GIDBF/- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Ile114Thr)

Row 3: PC_GIDB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: PC_GIDB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: 982GIDBR- Reverse sequence of *M. tuberculosis* isolate showing mutation (Ile114Thr)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

6.4 DISCUSSION:

Diagnostics for drug-resistant tuberculosis (TB) have been identified by the WHO as a key bottle neck in Multidrug-resistant tuberculosis (MDR- TB) control. Important gains have been made in rapid molecular diagnostics for INH and RIF resistance, such as the commercially available INNO-LiPA Rif TB kit, the GenoType MTBDRplus assay, and the Cepheid MTB/RIF assay [Hazbón et al., 2006, Almeida Da Silva et al., 2011]. Clinically, these assays are helpful to rapidly rule in drug susceptibility to the particular drug. However, when MDR-TB is detected, the clinician then immediately wants to know second-line drug susceptibilities as a basis for constructing a therapeutic regimen for the MDR-TB patients [Winder et al., 1982]. However, there are limited diagnostic tools that address this need.

World Health Organization (WHO) guidelines recommend the diagnosis of tuberculosis (TB) by smear microscopy in all new TB cases and smear microscopy, culture and phenotypic drug susceptibility testing (DST) in retreatment cases. If a new case fails to convert after 2 to 3 months of first line therapy, culture and phenotypic DST is requested. Routine phenotypic DST methods are culture based and are initially done to detect isoniazid (INH) and rifampicin (RIF) resistance. If resistance to INH and RIF is found, DST for ethambutol (EMB) is requested. DST usually takes between 3 to 6 weeks, resulting in long diagnostic delays. These delays are further exacerbated in new cases with primary drug resistance, given that DST is only initiated after 2 to 3 months of first line therapy. Such long delays and the administration of inappropriate therapy during the delay period may lead to the further acquisition of drug resistance, as well as the dissemination of drug resistant strains through transmission. Thus, to improve the outcome and prevent transmission of drug resistant TB, robust and effective alternative diagnostic tests are required that will enable the identification of drug resistant TB within a few days after Mycobacterial culture turns positive. In the present study, Genotypic DST by PCR based DNA sequencing was applied on first line drug resistant genes to find out the mutations conferring resistance particularly in Chennai population was carried out. The molecular mechanism of resistance to RIF is the most completely understood of the resistance mechanisms of all the drugs used in the treatment of tuberculosis, and it has been well established that mutations within the RRDR of *rpoB* occur in 95% or more of RIF-resistant isolates. Within the RRDR, three specific mutations predominate (Ser531Leu, His526Tyr, and Asp516Val), presumably because these

mutations minimally impact the fitness of the bacilli [Sherman et al., 1996]. In this study, novel mutations like Val551Ala and insertion mutation of "A" at nucleotide position 37 was observed in 3 and 32 (6- resistant, 26 sensitive) clinical isolates respectively. Since these mutations are far less frequently encountered, their association with RIF resistance has not been well established.

The mode of action of Isoniazid, though extensively investigated, remains incompletely understood. Several different loci are known to be involved in resistance, especially *katG* and *inhA* genes. Mutations in codon 315 of *katG* gene and the *inhA* promoter are proven mechanisms of INH resistance. None of the INH susceptible isolates in this study had a mutation in *katG* codon 315. But novel mutation occured in 10/192 sensitive strain at codon 63 (Gly63Trp). Regarding *inhA* gene, Asp32Asp novel silent mutation was observed in 2 sensitive and 1 resistant strains and in case of *oxyr-aphc* gene, each sputum isolate showed mutation at codons 133, 149, 154.

Until recently, the significance of mutations within the *M. tuberculosis embB* gene, especially those at codon 306, had been controversial because such mutations had been reported in both EMB-sensitive and resistant isolates [Perdigao et al., 2009]. Allelic exchange experiments have now convincingly shown that mutations at *embB* codons 306, 406, and 497 confer EMB resistance [Safi et al., 2010]. The most frequent *embB* substitution mutation observed in this study was Glu378Ala. This was the only *embB* mutation in 9 isolates, of which 7 were EMB sensitive. Therefore, the data obtained from this study indicates that the mutation resulting in the Glu378Ala substitution is a naturally occurring polymorphism and possible lineage marker that does not confer EMB resistance. Functional genetic analysis of this mutation is needed to definitively validate this conclusion.

The presence of a mutation within *pncA* gene has been shown to correlate well with PZA resistance [Scorpio et al., 1996], and a diverse and widely distributed array of *pncA* mutations has been reported [Sreevatsan et al., 1997]. In the present study, there was Pro54Ser novel substitution mutation in 2 resistant strains and Ser65Ser mutation in 2 sensitve and 1 resistant strain.

STR-resistance in *Mycobacterium tuberculosis* is known to be reported in *rpsl* and *rrs* genes, although 50% of STR-resistant *M. tuberculosis* strains show no mutation in either of these

genes. Recently, mutations within the *gidB* gene have been reported in STR resistance in *M. tuberculosis*. In Previous study by Okomoto *et al,* demonstrated that mutations within the gene *gidB* confer low-level streptomycin resistance and an important cause of resistance found in 33% of STR resistant *M. tuberculosis* isolates [Okamoto et al., 2007]. In 2008, Spies *et al* evaluated the possible role of the efflux mechanism and *gid B* gene mutation as a molecular basis of STR resistance in clinical isolates of *M. tuberculosis*, using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and verapamil as efflux pump inhibitors (EPI) and finally concluded that mutation in the *gidB* gene and an EP could act synergistically to confer low STR resistance [Spies et al., 2008]. Recent report by Wong *et al* observed between clinical isolates harboring a *gidB* mutation and a low-level STR-resistant phenotype in. In the present study, *gidB* gene mutations in drug target loci of patients resistant and sensitive to streptomycin was well characterised. PCR based DNA sequencing performed on the 112 *M. tuberculosis* isolates indicated the presence of 5 novel substitution mutations at positions 75,59,33,93,114 and 1 silent mutation at position 20 and 1 deletion of nucleotide C at position 105. There were 3 reported mutations identified (2 silent and 1 substitution mutations).None of the STR sensitive strains showed mutations.

6.5SUMMARY:

In the present study, genotypic drug susceptibility testing by PCR based DNA sequencing was applied on 242 *M. tuberculosis* isolates. Val551Ala novel mutation and insertion mutation of “A” at nucleotide position 37 was observed in 3 and 32 strains respectively. In the *katG II* region, Gly63Trp mutation was observed in 10 INH sensitive strains. Asp32Asp mutation was observed in 2 sensitive and 1 resistant strains in *inhA* gene and in case of *oxyr-aphc* gene, each sputum isolate showed mutation at codons 133, 149, 154. Regarding *emb B* gene, Glu378Ala mutation was observed in 9 strains. Pro54Ser novel substitution mutation in 2 resistant strains and Ser65Ser mutation in 2 sensitve and 1 resistant strain was observed when targeted *pnc A* gene. The STR resistance encoded by *gid B* gene was standardized by PCR based DNA technology and applied on 112 *M. tuberculosis* isolates. Of which 5 novel substitution mutations at positions 75, 59, 33, 93, 114 and 1 silent mutation at position 20 and 1 deletion of nucleotide C at position 105. There were 3 reported mutations identified (2 silent and 1 substitution mutations) were observed in STR resistant strains.

6.6 CONCLUSION:

The present study was aimed to target drug resistant genes encoding resistance to first line anti-tuberculous drugs to detect mutations targeting the reported genes encoding for resistance to first line anti-tuberculous drugs in *M. tuberculosis* isolates from local population by PCR based DNA sequencing technology. The above developed genotypic method will inevitably produce more rapid results for drug-resistant isolates, which will lead to rapid identification of MDR-TB strains, more tailored treatment regimens, and a reduction in the transmission of drug resistant tuberculosis.

6.7 FUTURE ASPECTS

The novel and reported mutations obtained from this study can be used to design diagnostic tests utilizing other mutation detection technologies such as the line probe assay or DNA microarrays will facilitate the optimization of the use of new-generation of anti-tuberculous drug to treat patients with MDR-TB. Further studies like structural and functional analysis are needed to elucidate the actual molecular mechanism in the drug resistant *M. tuberculosis* strains using bioinformatics tool.

CHAPTER 7

Insilico Analysis of Novel Mutation Ala102Pro Targeting *pncA* Gene of *Mycobacterium tuberculosis* using Bioinformatics tools in a phenotypically and genotypically Pyrazinamide resistant strain.

7.1 INTRODUCTION:

Pyrazinamide (PZA) is the first-line drug used in the treatment of tuberculosis along with Isoniazid and Rifampicin and it inhibits semi-dormant Mycobacteria only at low pH *invitro*. The *pncA* gene encodes pyraziniamidase (PZase), and mutations in *pncA* gene are associated with resistance to PZA [Dalmer et al., 1936, Yeager et al., 1952] or loss of PZase activity. PZA acts by targeting the fatty acid synthase/synthetase enzyme, and is responsible for the killing of persistent tubercle bacilli in the initial intensive phase of chemotherapy. It is a prodrug that is converted to its active form namely, pyrazinoic acid (POA) by the catalytic action of PZase enzyme, encoded by the *pncA* gene in *M. tuberculosis.* Interestingly, PZA is active only at low pH since acidic environment favours accumulation of POA in the cytoplasm due to an ineffective efflux pump, thereby leading to improper efflux out of the amidase from the cell to the exterior [Yeager et al.,1952, Morlock et al.,2000]. The present objective was to study the structural and functional basis of pyrazinamide (PZA) resistance conferred by a novel mutation Ala102Pro in *pncA* gene by PCR based DNA sequencing technique from a PZA resistant *M. tuberculosis* strain using bioinformatics tools.

7.2 MATERIALS AND METHODS:

PCR based DNA sequencing and bioinformatic procedures were carried out as per the protocol given in section 3.4

7.3 RESULTS:

7.3.1 Phenotypic drug susceptibility testing:

Phenotypic drug susceptibility testing by Micro MGIT BACTEC method showed the *M. tuberculosis* isolate to be resistant to PZA (100 μg/ml).

7.3.2 PCR targeting the *pncA* gene and DNA sequencing

PCR targeting the *pncA* gene resulted in a 670-bp amplified product. DNA sequencing of the amplified product with forward and reverse primers followed by MultAlin analysis of the *M. tuberculosis* strain revealed the presence of novel substitution mutation Ala102Pro (**G**CG→CCG).

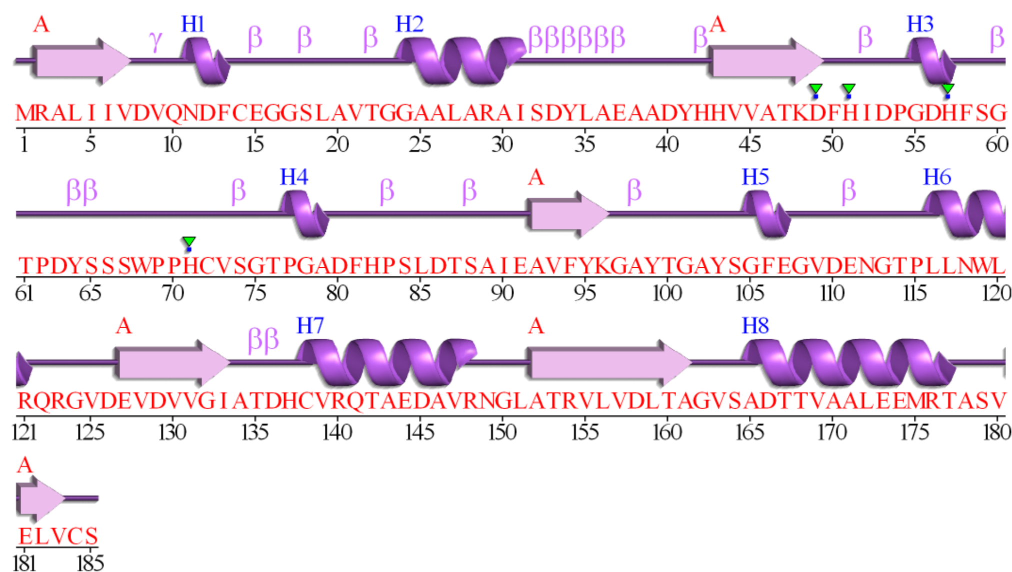
7.3.3 SNP Analysis of I-Mutant Server.

In this study, wild type sequence of *pncA* at 102nd position Ala was replaced by Pro to predict protein stability changes through I-mutant server. The results infer loss of stability by the Mutant protein with negative Gibbs free energy value of -1.10 at pH 5.5 and 37ºC.

7.3.4 Secondary structure analysis for Wild and Mutant Type of PZA (Pdbsum SERVER)

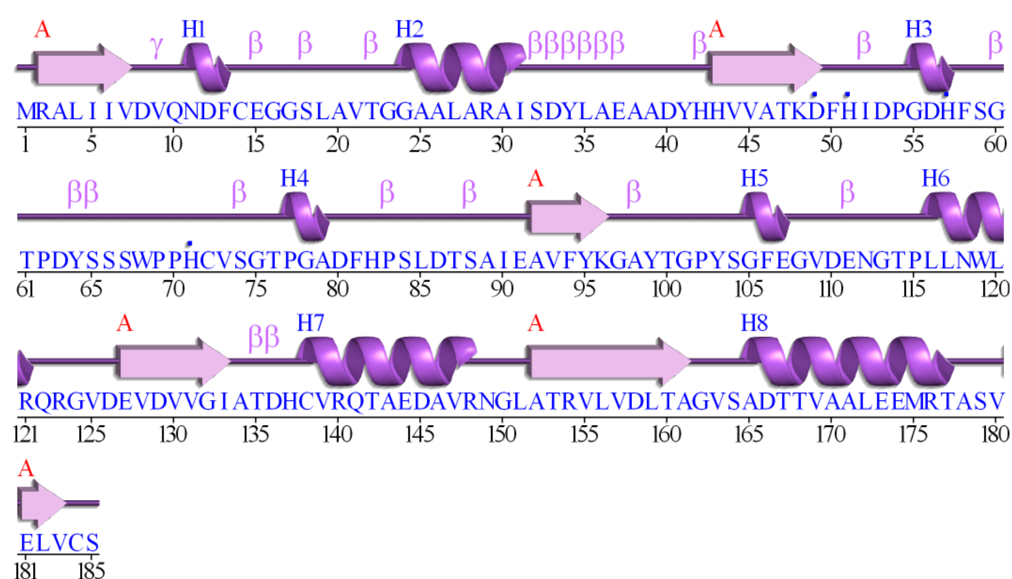
PSIPred results for the MT shows no significant change when compared to WT and MT. (Figure7.1&7.2).

Figure 7.1: secondary structure prediction of Wildtype of PZA using pdbsum sever



This illustrated picture shows secondary structure with 6 strands (A), 8 helices (H), 21 beta turns (β) and 1 gamma turn (γ) of Wildtype of PZA

Figure 7.2: Secondary structure prediction of Mutant type (Ala102 Pro) of PZA using pdbsum sever



This illustrated picture shows secondary structure with 6 strands (A), 8 helices (H), 21 beta turns (β) and 1 gamma turn (γ) of Mutant type of PZA

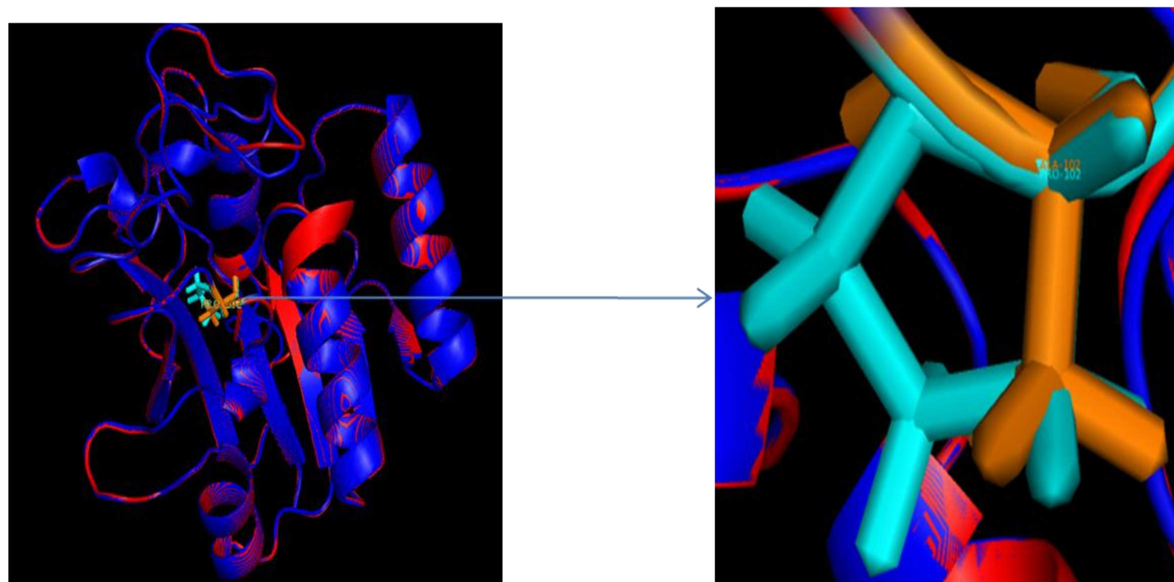
7.3.5 ConSurf analysis:

Consurf analysis revealed the following residues as conserved across all the selected Pzase activity exihibiting pathogenic organisms: Asp8, Phe13, Val44, Asp49, His51, His71, Gly78, Lys96, Tyr103, Thr114, Leu120, Gly132, Ala134, Asp136, Cys138, Val139, Ala146, Val155, Ala171, and Met175 and shall contribute to structural and functional aspects. As the novel mutation Ala102Pro was predicted to occur near the Tyr103 conserved region, it shall also contribute in the modulation of activity of the protein. Moreover, proline insertion shall also confer structural deviation as it forms partial peptide bond.

7.3. 6 Homology modeling and loop refinement of MT Pzase

The structure of MT Pzase (Ala102pro) was modeled using Modeler9v7 with WT structure as template. The modeled structure was found to be highly plausible as it had 99% sequence identity with that of the template. Morever, the ramachandran plot also showed 83.5% of residues in most favored regions with no residues in disallowed region (Figure 7.3)

Figure 7.3: Superimposition of WT and MT PZase 3D structure. WT –blue & mutant-red. Residue at position 102 is shown in stick form.



7.3.7 Structure optimization and Validation:

The structures of WT and MT were energy minimized using GROMOS96 force field by Gromacs software package. The potential energy of WT and MT were found to be -2.6391059e+04 Kcal/mol and MT -2.3403371e+04 Kcal/mol, repectively. The optimized structure was validated using Q-mean and ProQ Server (Table 7.1 and Figure 7.4 and Figure 7.5).

Table 7.1: Energy minimization and Protein structure Quality assessment of WT and MT of Pzase.

SAVS (structure analysis and validation server)	Before energy minimization		After energy minimization	
	Wild	Mutant	Wild	Mutant
most favoured regions	84.3%	90.5%	87.3 %	91.9%
additional allowed regions	12.6%	7.0%	12.7 %	8.1%

generously allowed regions	1.3%	1.9%	0%	0%
disallowed regions	1.9%	0%	0%	0%
ProQ LG score	5.167	5.340	6.064	5.006
Max sub	0.187	0.228	0.594	0.511
Q mean score	-	-	0.82	0.79

Figure 7.4: Q-mean scores of WT of PZase

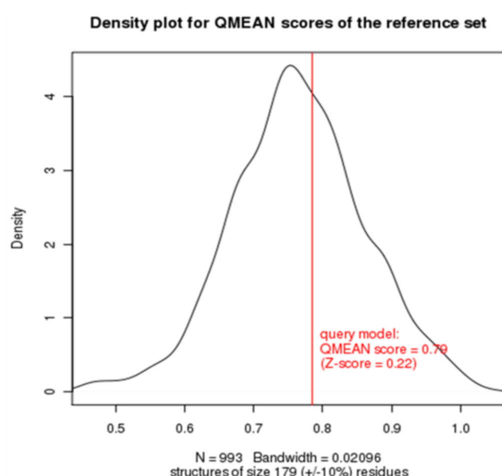
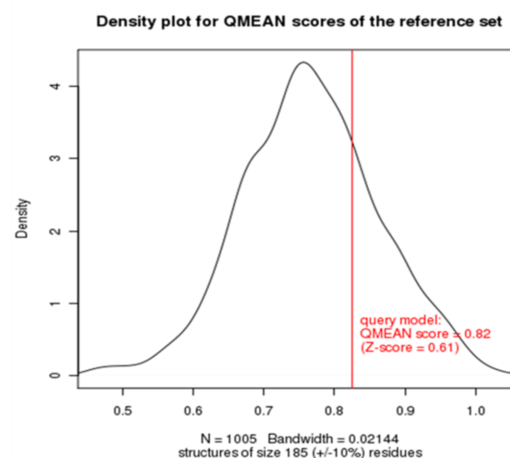


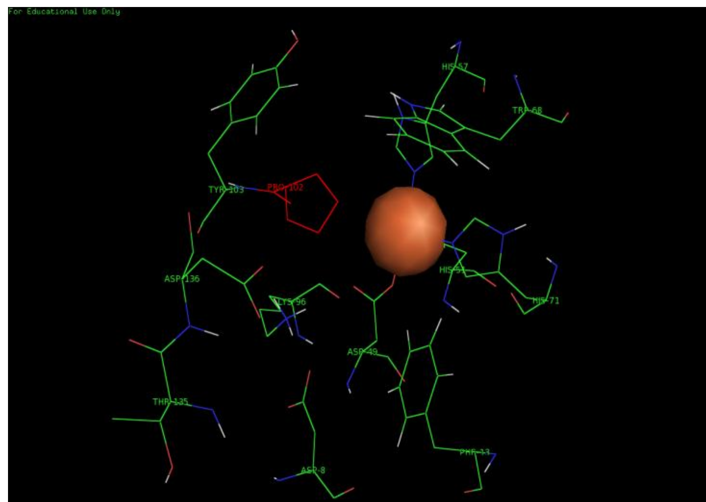
Figure 7.5: Q-mean scores of MT of PZase



7.3.8 Active site analysis of PZA:

Three different methods namely, CASTp, Q-site and pocket finder were implemented to predict the active site residues of PZase. All the predictions were found to be synonymous and representing the same amino acid residues: Asp8, Phe13, Leu19, Asp49, His57, Trp68, His71, Lys96, Ala102, Tyr103, Thr135, Asp136, and Cys138 (Figure 7.6)

Figure 7.6: CASTp Pocket prediction: Heme is indicated as brown sphere shape and Ala 102 Pro mutant position in Red and other pocket residues.



Ala102 was found to structurally orient near the active site along with other heme binding residues: ASP49, HIS51, HIS57, HIS71. Hence, the mutation in Ala102Pro shall affect the PZA activity in terms of substrate binding.

7.3.9 Docking studies of WT &MT type of Pzase with PZA

The docking studies were performed using the Autodock software. The aminoacid residues Asp8, Phe13, Thr61, Pro69, Lys96, Tyr103, and Cys138 were assigned as catalytic region as these residues were proven to be potential active site [Du et al., 2001, Lemaitre et al., 2001].

The WT had binding energy of -4.21Kcal/mol and theoretical inhibitory constant (Ki) of 822.42µM (table 5.2). The residues Asp8, Lys96, and Ala102 interacted through 4 hydrogen bonds with PZA. The interaction was found to span within the chosen active site [Du et al., 2001, Lemaitre et al., 2001].

The MT had binding energy of –4.1 Kcal/mol and theoretical inhibitory (Ki) constant of 990.0µM (Table 5.2). The results of docking study infer that both WT and MT interact with PZA at the same binding pocket region. However, MT showed deviation in terms of interaction with PZA by excluding hydrogen bond formation with Lys96 and Ala l02 and including Ala134 and Ile133. (Figure 7.7, 7.8 and table 7.3).

Figure 7.7: Docking interaction of WT PZAse with PZA.PZA represented in green colour

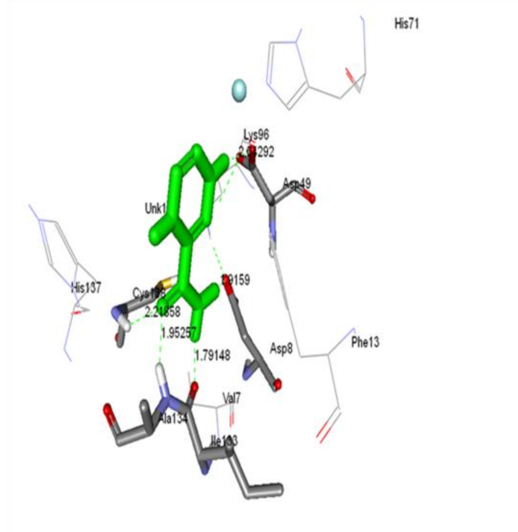
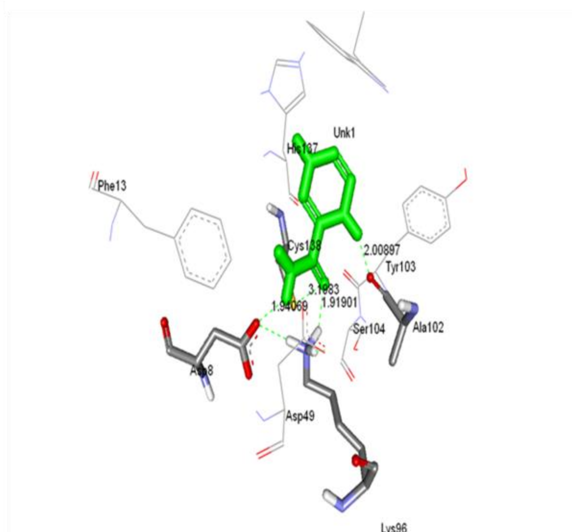


Figure 7.8: Docking interaction of MT PZAse with PZA.PZA represented in green colour



.

Table 7.3: Docking Studies of Pzase with PZA

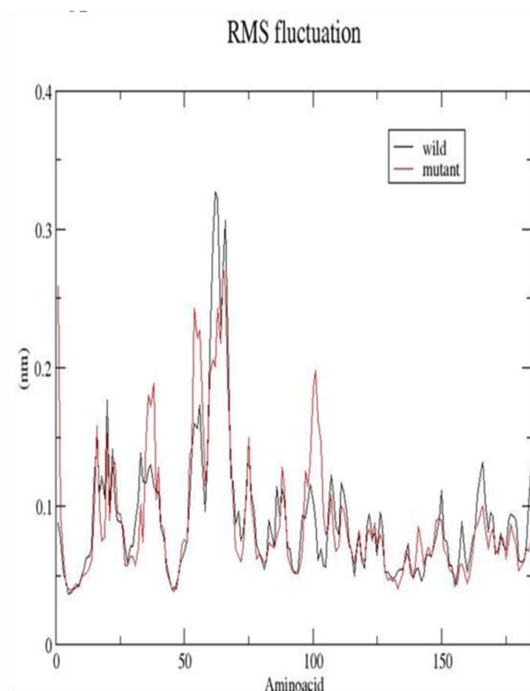
Type	Binding energy	Ki value: μm	Hydrogen bond with PZA
Wild	-4.21	822.42	Ala102, Lys96, Asp8,Cys138
Mutant (Ala102Pro)	-4.1	990.0	Asp8, Asp49, cys138, Ala134, Ile13

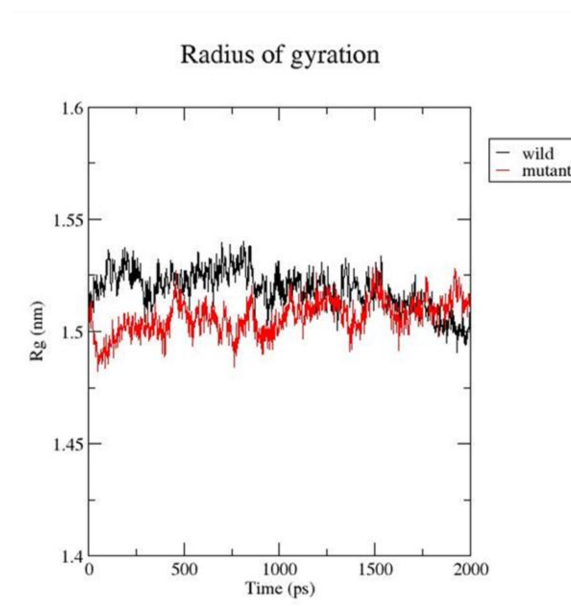
7.3.10 Dynamics Simulation analysis:

To further validate the molecular docking studies, high performance molecular dynamics simulation protein-ligand complex were performed and from the resultant trajectory, root mean square fluctuation (RMSF) and gyration analysis were performed. The RMSF results showed that the MT has high fluctuation when compared to WT due to the acquired mutation. (Figure 5.6.1). Further, the Radius of gyration analysis also showed MT to be slightly compact than WT which shall interfere with the flexibility of the protein (Figure 7.9&7.10)

Figure 7.9: Root mean square fluctuation of residues in WT and MT type of Pzase. Black lines indicate WT and red line indicates MT

Figure 7.10: Radius of gyration in WT and MT type of Pzase. Black lines indicate WT and red line indicates MT





7.4 DISCUSSION:

The anti-bacterial activity of pyrazinamide (PZA), one of the first-choice drugs in the chemotherapy of tuberculosis, requires an enzyme, pyrazinamidase (PZase), which converts PZA into pyrazinoic acid (POA). Recent reports have established clearly that mutations in the *pncA* gene encoding PZase lead to the loss of PncA activity and are the main mechanism of PZA resistance in *M. tuberculosis* [Doustdar et al., 2009].

A large diversity and a wide distribution along the entire length of the *pncA* gene are the two main features of the resistance-associated mutations. However, the recent reports emphasise that a significant proportion of the substitutions are clustered in three regions in the *M. tuberculosis pncA* gene [Doustdar et al., 2009]. At the protein level, these regions were found to be well conserved among the amino acid sequences of PncA proteins from different bacterial species. The overall architecture of the pyrazinamidase of *M. tuberculosis* is similar to that reported for the other pyrazinamidases of *A. baumanii* and *P. horikoshii*. However, several striking structural variations that have been specifically detected in the crystal structure of MtPncA, are of particular note. First, analysis of the metal ion content of MtPncA by ICP-MS analysis indicated that the enzyme preferentially contains iron.

The novel mutation Ala102Pro was found to span in the putative active site (Lys96-Tyr103) region of PZase. The SNP anlaysis by I-mutant server showed that the stability of the protein was affected with negative Gibbs free energy value -1.10 at pH 5.5 and 37ºC. The aminoacids predictions found in this study well correlates with the already documented studies by Lemaitre et al [Lemaitre et al., 2001] where they determined the activity of nine PZA mutant and strongly suggest that the amino acids found at positions 8, 13, 61, 69, 96, 103, 104 and 146 are functionally and}or structurally important in PncA. In addition, the level of activity displayed by the PncA mutants seems to be well correlated with the location of the mutated residues and the structural modifications they can cause in the vicinity of the putative active-site cysteine residue found at position 138. Molecular modeling and docking analysis showed that MT has a higher inhibitory constant than WT in terms of PZA binding, which indicates that the drug affinity is highly affected in the MT due to structural changes. The molecular dynamics simulation and gyration analysis together infer the change in flexibility at the active site cavity of MT. Hence, this study gives insight on the impact of novel mutation on the activity of this protein which can be attributed to the drug resistance observed.

7.5 SUMMARY

The novel mutation Ala102Pro was found to span in the putative active site (Lys96-Tyr103) region of PZase. The SNP anlaysis by I-mutant server showed that the stability of the protein was affected with negative Gibbs free energy value -1.10 at pH 5.5 and 37ºC. In addition, the level of activity displayed by the PncA mutants seems to be well correlated with the location of the mutated residues and the structural modifications they can cause in the vicinity of the putative active-site cysteine residue found at position 138. Molecular modeling and docking analysis showed that MT has a higher inhibitory constant than WT in terms of PZA binding, which indicates that the drug affinity is highly affected in the MT due to structural changes. The molecular dynamics simulation and gyration analysis together infer the change in flexibility at the active site cavity of MT.

7.6 CONCLUSION

In conclusion, the crystal structure of the pyrazinamidase from *M. tuberculosis* described in this study has unveiled important structural features of the enzyme and so, represents a

valuable tool to decipher the structure-function relationships and investigate the molecular mechanisms of resistance to PZA stemming from the point mutations identified in clinical isolates. Finally, it highlights the underestimated importance of protein folding and thermal stability in PZase activity of the PncA mutants produced in clinical isolates, a parameter which has to be taken into account to fully understand resistance to PZA.

7.7 FUTURE ASPECTS:

The present study emphasize on the protein structure and its altered activity of PZase in PZA resistant isolates. Further the *in-silico* analysis on structural and functional activity in more number of PZA isolates should be carried out to explore the exact mechanism exhibited by these isolates in responds to PZA.

CHAPTER 8

Detection of Extensively drug resistant tuberculosis (XDR-TB) by phenotypic drug susceptibility testing using "micro MGIT BACTEC method" for Second line anti-tuberculosis drugs (Fluoroquinolones, Aminoglycosides, Para-amino salicylic acid, Ethionamide).

8.1 INTRODUCTION:

Tuberculosis (TB) is the leading cause of death in the world and humans are the only reservoir of this organism [Collee et al., 2002]. *M. tuberculosis* strain that is resistant to Isoniazid and Rifampicin, two of the most important first line drugs used in the treatment of the disease is called as multi drug resistant tuberculosis (MDR-TB). Drug resistant tuberculosis has been reported since the early days of introduction of anti-tubercular chemotherapy, but recently MDR-TB is posing threat to global efforts of tuberculosis control programmes. Three most important risk factors, identified in the causation of drug resistant tuberculosis are inappropriate previous treatment with anti-tubercular drugs, high prevalence of drug resistant tuberculosis in the community and contact with patients known to have drug resistant tuberculosis [Collee et al., 2002].

MDR-TB cases threaten the effectiveness of chemotherapy for both treatment and control of TB and require the use of second-line drugs (SLDs) that are more expensive, toxic, and less effective than first-line anti-TB drugs [Zumia et al., 2001]. While assisting MDR-TB treatment programs worldwide, and ensuring the proper use of SLDs in resource limited countries the committee encountered reports of multiple cases of TB with resistance to virtually all SLDs. This led to the emergence of new terminology in relation to drug-resistant TB, i.e., extensively drug-resistant TB (XDR-TB). XDR-TB is a form of TB caused by *M. tuberculosis* that are resistant to Isoniazid and Rifampicin (i.e. MDR-TB) as well as any one of the fluoroquinolone and second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin) [Masjedi et al., 2006, Banerjee et al., 2008]. Because XDR-TB is resistant to first line and second line drugs, treatment options are seriously limited.

The emergence of XDR-TB has re-focused attention on TB as a disease of continuing significance in the developed and developing world [Pooja singla et al., 2014]. Approximately 9% of MDR-TB cases detected to have XDR-TB. So, there is an urgent need for the detection of second line drug resistance pattern to treat XDR-TB patients.

8.2 MATERIALS AND METHODS:

Phenotypic drug susceptibility testing using "micro MGIT BACTEC method" for second line anti-tuberculosis drugs was carried out as per the protocol given in the section 3.5

8.3 RESULTS

Among 29 MDR-TB strains, 10 (2.4%) were XDR-TB strains. Regarding the resistance to individual second line drug, 20, 19, 18, 16, 15, 14, 9, 9 were resistant to ETH, PAS, CPX, AMK, CAP, Kan, OFX and LVX respectively (table 6.3).

8.3.1 XDR-TB strains:

Of 10 XDR-TB strains, 2 (sputum) were resistant to all eight second line drugs [AMK,CAP,OFX.CPX,LVX,ETH,PAS], 1 (sputum) each were resistant to [AMK, KAN, PAS, ETH], [CAP, CPX, OFX, PAS, ETH], [AMK, CAP,OFX, CPX, LVX, PAS, ETH] and [AMK, KAN,CAP, CPX, PAS, ETH], [AMK, KAN, OFX, CAP, LVX, PAS, ETH], [CAP, OFX, CPX, ETH, PAS, AMK, KAN], [AMK, KAN, CPX] and [AMK, CPX].

8.3.2 Polyresistant strains:

Among 4 (sputum) polyresistant strains, 1 each were resistant to [PAS, ETH] , [AMK, KAN, ETH], and [KAN, PAS].

Table 8.1: Details of age, sex, direct smear, first line and second line drug DST results for XDR-TB strains from this study

S. No	Reference no.	Age/ sex	Clinical specimen	Direct smear	First line phenotypic DST	Second line phenotypic DST
1	LTITM 3	45/M	Sputum	Occasional AFB	SHR- Resistant, EZ-Sensitive	AMK, KAN, CF, ETH, PAS - Resistant, CPM, OFX, LEV- Sensitive
2	LTITM 136	22/M	Sputum	Negative	SHREZ- Resistant	CAP, OFX, CPX, ETH, PAS - Resistant, AMK, KAN, LVX- Sensitive
3	LTITM 232	45/F	Sputum	1+	SHRE Z– Resistant	AMK, KAN, CPX-resistant ETH, PAS ,CAP, OFX, LVX- Sensitive
4	LTITM 254	25/M	Sputum	3+	SHREZ – Resistant	AMK, CPX - Resistant, KAN, CAP,OFX, LVX, ETH, PAS – Sensitive
5	LTITM 1029	65/M	Sputum	1+	SHREZ – Resistant	AMK,CAP,OFX.CPX,LVX,ETH,PAS-Resistant, KAN-Sensitive
6	LTITM 1041	31/F	Sputum	Negative	SHREZ- Resistant	AMK,KAN,CAP,CPX,LVX,ETH,PAS-resistant,OFX-sensitive
7	LTITM 1076	19/F	Sputum	3+	SHREZ- Resistant	AMK,CAP,OFX.CPX,LVX,ETH,PAS-Resistant, KAN-Sensitive
8	LTITM 1077	33/M	Sputum	2+	SHREZ- Resistant	AMK,CAP,CPX,ETH,PAS-Resistant, KAN, OFX,LVX-Sensitive
9	LTITM 1045	14/F	Sputum	Negative	SHRE-resistant,Z-sensitive	AMK,CAP,KANOFX,LVX,ETH,PAS-Resistant, CPX-Sensitive
10	LTITM 1057	56/M	Sputum	Occasional AFB	SHRE-resistant,Z-sensitive	AMK.KAN,CAP,OFX.CPX,LVX,ETH, PAS-resistant

8.4 DISCUSSION:

The important aspects to control tuberculosis are prompt identification of new cases and rapid implementation of effective treatment regimens to interrupt transmission of the disease. The chance of incidence of XDR-TB is on the rise due to improper use of second line anti-tubercular drugs leading to drug resistance. Delay in the diagnosis of XDR-TB is mainly due to slow growth of the organisms which eventually reflects further identification of XDR-TB strains and therefore delays in performing drug susceptibility testing for the second line drugs. Since conventional drug susceptibility testing using liquid and solid medium takes nearly about 22 and 68 days respectively, there is a need for the rapid diagnostic drug susceptibility testing methods to identify XDR-TB and further prevent the spread and emergence of drug resistant tuberculosis which results in early initiation of the treatment.

The first outbreak of XDR-TB occurred in South Africa in 2006 studied by Gandhi et al [Gandhi et al., 2006], who reported1428 patients with suspicion of tuberculosis. Of 1428 patients analysed, 475 (33%) of 1428 patients were culture-positive for *Mycobacterium tuberculosis*. Among the confirmed culture positive cases, they found that the prevalence of MDR tuberculosis was 39% (185/475) and of XDR tuberculosis was 6% (30/475) by proportional method on Middlebrook 7H10 agar. In 2005 Zarir Udwadia from Hinduja Hospital reported first case of XDRTB in India. Of 3,904 samples included in their study, 409 samples (32.35%) were found to be MDR-TB, out of which 33 (8%) were XDRTB [Deepak et al., 2005].Jain et al [Jain et al., 2009] in 2008 reported on the High Prevalence of XDR TB from a Tertiary Care Hospital in India. Of 3904 samples (sputum, pleural fluid, CSF etc.) included in their study, 1264(32.3%) samples were culture positive for *Mycobacterium tuberculosis*. 409 samples (32.35%) were found to be MDR-TB, out of which 33 samples (8%) were XDR-TB. In 2010, Balaji et al [Balaji et al., 2010] studied the clinical and demographic risk factors associated with the isolation of XDR-TB in a tertiary hospital laboratory in South India by performing drug susceptibility testing. Datta et al [Datta et al., 2010] studied the prevalence of MDR-TB and XDR-TB in Kashmir valley of India, during the period of March 2003 to February 2007.Out of 910 cases of pulmonary tuberculosis they found 52 (5.7%) cases of MDR-TB, among which 8 (15.3%) were diagnosed as XDR-TB by BACTEC MGIT 960 method.

A recent study from Hinduja hospital (2011), Udwadia et al [Udwadia et al., 2011] reported four cases of "total drug resistant tuberculosis" (TDR-TB).According to this report, 12 patients have shown resistance to all the first line TB drugs (isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin) and to seven second line anti-TB drugs (ofloxacin, moxifloxacin, kanamycin, amikacin, capreomycin, para-aminosalcyclic acid and ethionamide). However, within a couple of weeks the Indian health authorities had rejected their claim, saying that all the cases were in fact extensively drug resistant, that is XDR-TB. So far, three of the TDR-TB patients died, one of them after lung surgery. One of the patients passed on the infection to her daughter. The doctors were pessimistic saying that they have little to offer those 12 patients except for drastic surgery and medication for some relief. It was also said that TDR TB had emerged because of the failure of the overall health system as these patients received erratic, unsupervised second line drugs, added individually and often in incorrect doses, by multiple private practioners [Udwadia et al., 2011].

In the present study, the standardized phenotypic drug susceptibility testing for second line drugs by Micro MGIT BACTEC culture system showed 17 /111 (15.3%) *M. tuberculosis* strains (29- MDR strains, 82 – Polyresistant strains to first line drugs) were XDR-TB strains, 6.3% were Polyresistant strains and 8.1% were Monoresistant strains to second line anti-tuberculous drugs. Amomg 111 *M. tuberculosis* isolates, the highest resistance were observed for ETH (18%), followed by PAS (17%), CPX (16%), AMK (14%), CAP (13%), KAN (12%) and lowest resistance (8.1%) were observed for OFX and LVX.

Regarding 17 XDR-TB strains, the majority were from sputum specimens (94.4%) followed by FNAB (0.56%).The age group of patients with XDR-TB were between 14-75 and male ratio is higher than the female ration (10:7). Among 111 *M. tuberculosis* isolates, 7 were polyresistant and 9 were monoresistant with maximum of 4 were resistant to CPX, followed by PAS (3), ETH (2). In case of 29 MDR-TB strains, 10(2.4%) were XDR-TB strains.

8.5 SUMMARY

In the present study, Optimization of second line drugs susceptibility testing by phenotypic method using micro MGIT BACTEC culture system was done and applied on 111 *M. tuberculosis* isolates. Among 111 isolates, 2.4% were XDR-TB strains, 6.3% were

Polyresistant strains and 8.1% were Monoresistant strains to second line anti-tuberculous drugs. Over all the highest drug resistance was observed for Ethionamide (18%).

8.6 CONCLUSION

In conclusion, the phenotypic DST results for second line anti-tuberculous drugs obtained from this study needs to be validated by performing the genotypic method of DST by PCR based DNA sequencing targeting drug resistant genes to find out mutations that confers resistance to second line anti-tuberculous drugs.

8.7 FUTURE ASPECTS

The above standardized method for second line drug susceptibility testing should be applied on more number of MDR-TB isolates to study the significance of resistance pattern and prevalence of XDR-TB cases circulating in Chennai population.

CHAPTER 9

Detection of Extensively drug resistant tuberculosis (XDR-TB) by Genotypic drug susceptibility testing using PCR based DNA sequencing technique for drug resistance genes coding resistance to Second line anti-tuberculosis drugs (Fluoroquinolones, Aminoglycosides, Para-amino salicylic acid, Ethionamide).

9.1 INTRODUCTION:

Resistance to anti-tuberculous drugs has been noted since the drugs were first introduced, and occasionally outbreaks of drug-resistant tuberculosis have been reported worldwide. But recent outbreaks of XDR-TB have differed considerably from the previous outbreaks of drug-resistant tuberculosis and even multi-drug resistant tuberculosis outbreaks. WHO emphasizes that proper tuberculosis control prevents the emergence of drug resistance in the first place and that the proper treatment of multi-drug resistant tuberculosis prevents the emergence of XDR-TB.The rate of XDR-TB drug resistance have been increasing steadily especially in the developing countries. Therefore, there is an urgent need for the detection of mutations in the second line drug resistant gene encoding for drug resistance will pave way for the early detection of XDR-TB and further the treament can be modified to minimize the rate of drug resistance. The principal etiology of drug-resistant TB especially XDR-TB remains inadequate and/or incomplete treatment due to poor medical adherence to the standard treatment regimen. The increasing global prevalence of XDR-TB is primarily important to understand the mode of action of each drug as well as the molecular basis of drug resistant genes encoding for resistance to second line drugs. Genotypic drug susceptibility testing method by PCR based DNA sequencing targeting drug resistance genes of second line drug resistance will be a biomarker to study the prevalence of drug resistance in XDR-TB cases in chennai population

9.2 MATERIALS AND METHODS:

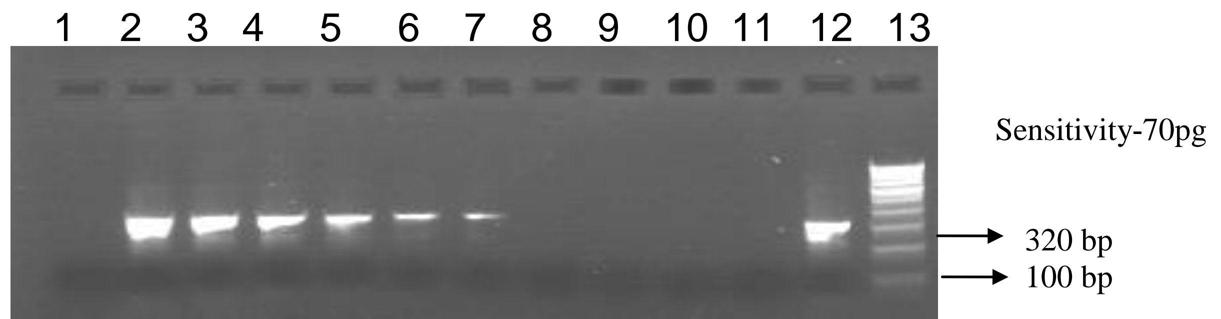
PCR based DNA sequencing targeting second line drug resistance was carried out as per protocol given in 3.6

9.3 RESULTS:

9.3.1 Results of optimized PCRs targeting the second line drug resistance genes in *M. tuberculosis* isolates:

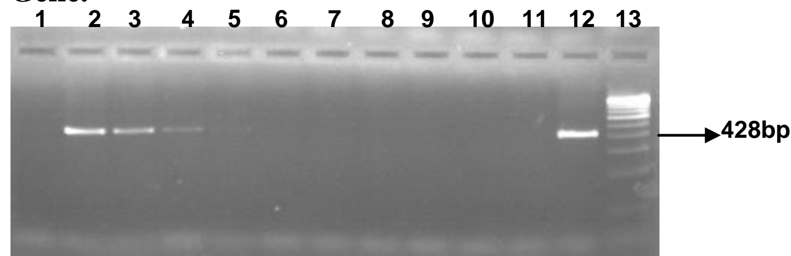
Determination of Analytical sensitivity of the primers targeting the second line drug resistance genes in *M. tuberculosis* isolates:

Figure 9.1: Agarose gel electrophotogram showing analytical sensitivity of the primers targeting *gyr A* gene. .



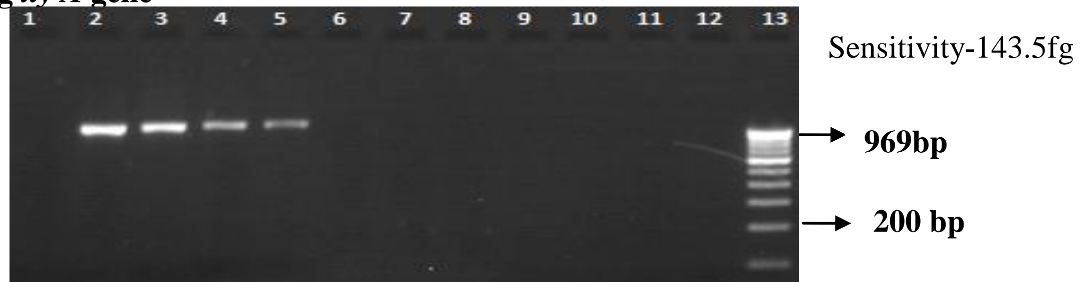
Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 7µg in 5µl of DNA

Figure 9.2: Agarose Gel Electrophotogram Showing Analytical Sensitivity Of Primers Targeting *gyr B* Gene.



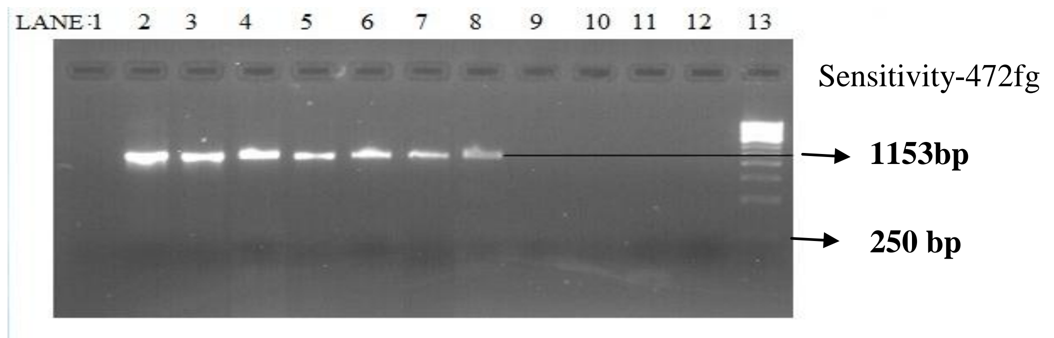
Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 60ng in 5µl of DNA.

Figure 9.3: Agarose gel electrophotogram showing analytical sensitivity of primers targeting *tly A* gene



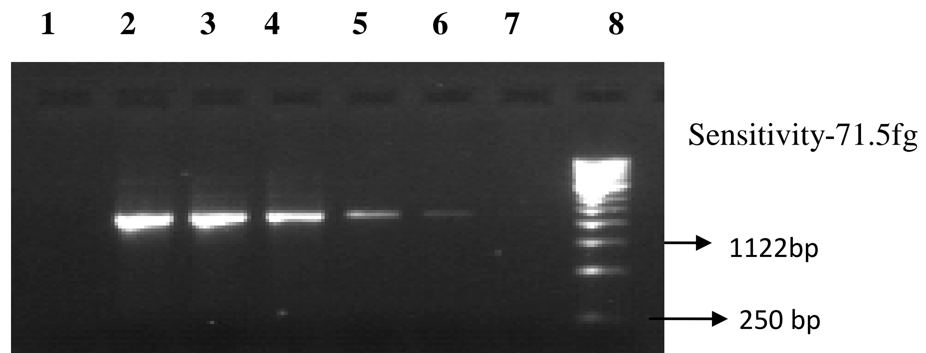
Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 143.5pg in 5µl of DNA

Figure 9.4: Agarose gel electrophotogram showing analytical sensitivity of primers targeting *eis* gene .



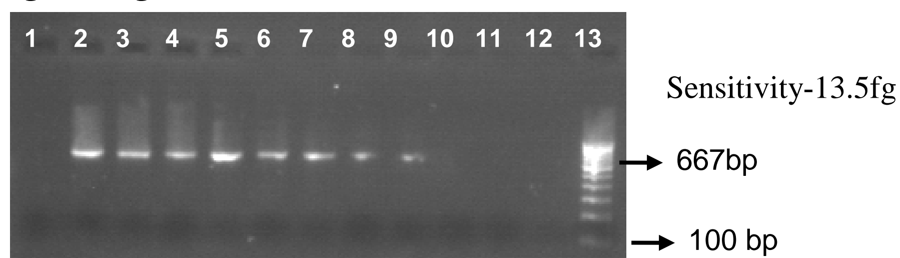
Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 472µg in 5µl of DNA

Figure 9.5 : Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *thyA* gene.



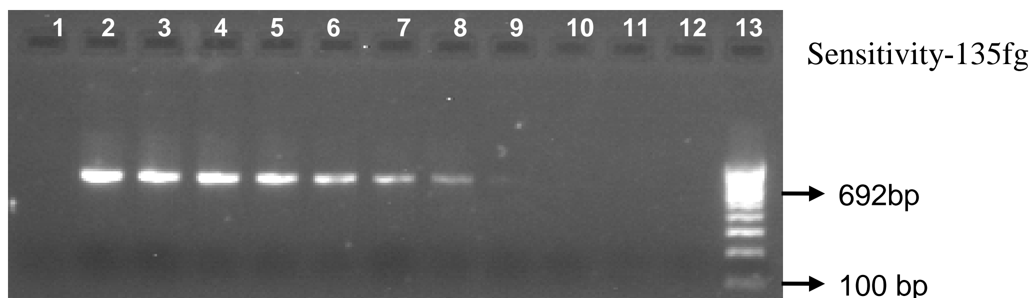
Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 7.15pg in 5µl of DNA

Figure 9.6: Agarose gel electrophoretogram showing the analytical sensitivity of primers targeting *ethA1* gene.



Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 135µg in 5µl of DNA

Figure 9.7: Agarose gel electrophoretogram showing the sensitivity of primers targeting *ethA2* gene.

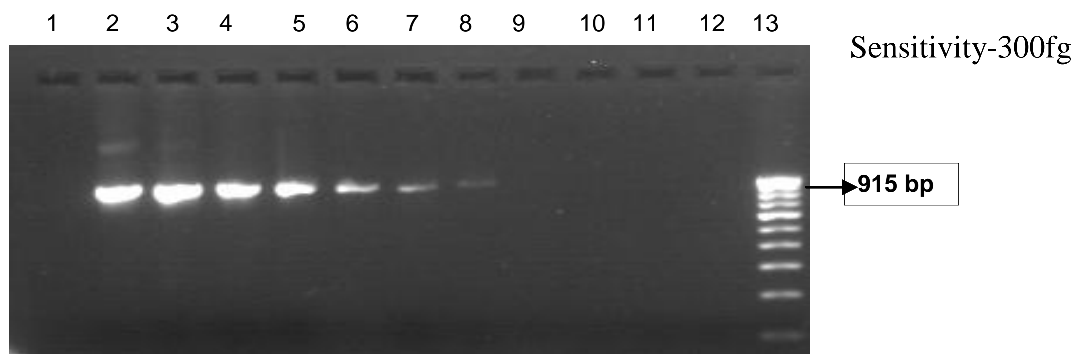


Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 13.5ng in 5µl of DNA

Figure 9.8: Agarose gel electrophoretogram showing the sensitivity of primers targeting *ethA3* gene.

Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 135µg in 5µl of DNA

Figure 9.9: Agarose gel electrophoretogram showing the sensitivity of primers targeting *ethR* gene.



Initial DNA concentration used for the serial dilution (10^{-1} to 10^{-10}) of the positive control (*M. tuberculosis* H37RV) DNA was 300µg in 5µl of DNA

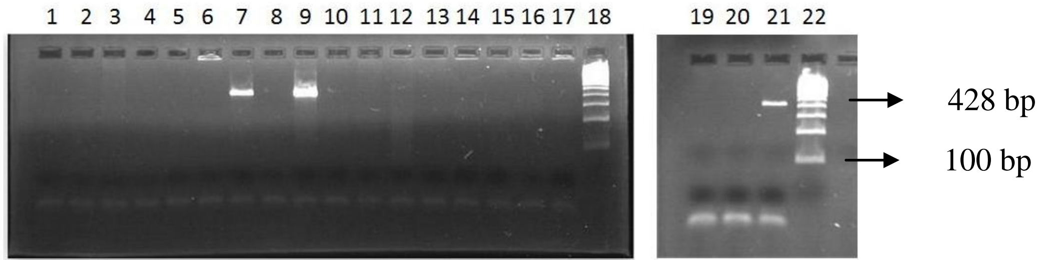
Lane1: Negative control (negative), Lane 2: positive control- *M .tuberculosis H37Rv ATCC* (positive with specific bp product), Lanes 3-12: 10 fold serial dilutions of *M .tuberculosis H37Rv ATCC* , Lane 13: 100 bp molecular weight marker

9.3.2 Determination of Specificity of the primers targeting *gyrA* gene for fluoroquinones resistance:

.**Figure 9.10: Agarose gel electrophotogram showing specificity of the primers targeing *gyr A* gene**

Lane 1: negative control, Lane 2 :*M. phlei* ATCC (negative), Lane 3: *Nocardia asteroides*(lab isolate) (negative), Lane 4: *M. gordonae* ATCC (negative), Lane5: *M. thermoresistible* ATCC (negative), Lane 6:*M. duvalii* (lab isolate) (negative), Lane 7:*M. kansasii* ATCC (negative), Lane 8:*M. xenopi* ATCC (negative), Lane9: *M. intracellularae* ATCC (negative), Lane 10:*M. fortuitum* ATCC (negative), Lane 11: *M. flavescens* ATCC (negative), Lane12:, *M. chelonae* ATCC (negative), Lane 13: ***M.bovis*** ATCC (negative), Lane 14: *M .tuberculosis* H*37*Ra ATCC (negative), Lane15: *M. abscessus* (lab isolate) (negative), Lane:17:*M.simiae* (negative), Lane 19:*M. smegmatis* ATCC (negative), Lane 20: ***M .tuberculosis* H*37*Rv ATCC** (positive with specific 320 bp product),Lane 18&21:100bp molecular weight ladder

Figure 9.11: Agarose gel electrophotogram showing specificity of the primers targeting *gyr B* gene



Lane 1: negative control, Lane 2 :*M. phlei* ATCC (negative), Lane 3: *Nocardia asteroides*(lab isolate) (negative), Lane 4: *M. gordonae* ATCC (negative), Lane5: *M. thermoresistible* ATCC (negative), Lane 6:*M. duvalii* (lab isolate) (negative), Lane 7: ***M. tuberculosis* H37Ra ATCC** (positive with specific 428bp product), Lane 8:*M. xenopi* ATCC (negative), Lane9: ***M.bovis* ATCC** (positive with specific 428bp product), Lane 10:*M. fortuitum* ATCC (negative), Lane 11: *M. flavescens* ATCC (negative), Lane12:, *M. chelonae* ATCC (negative),Lane 13: *M. intracellularae* ATCC (negative), Lane 14: *M. kansasii* ATCC (negative), Lane15: *M. abscessus* (lab isolate) (negative), Lane:17:*M.simiae* (negative), Lane 19:*M. smegmatis* ATCC (negative), Lane 20: ***M .tuberculosis* H37Rv ATCC** (positive with specific 428bp product), Lane 18&21:100bp molecular weight ladder

Figure 9.12: Agarose gel electrophotogram showing Specificity of the primers targeting *tly A* gene

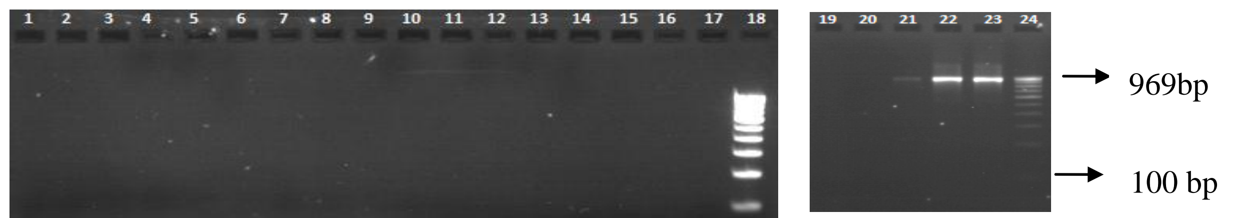


Figure 9.13: Agarose gel electrophoretogram showing the specificity of primers targeting *thyA* gene

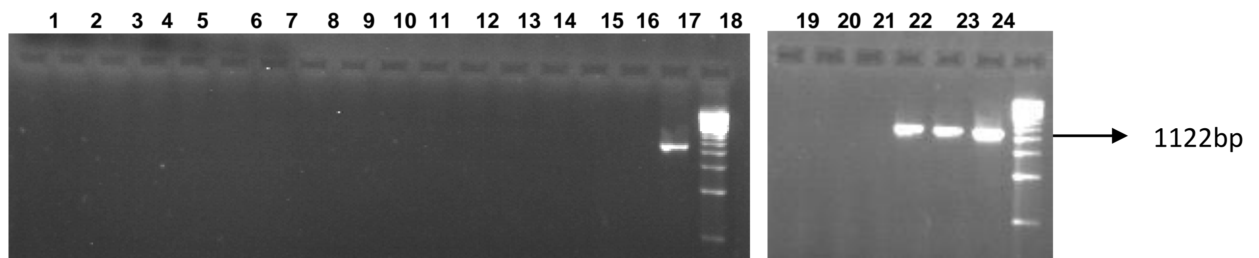


Figure 9.14: Agarose gel electrophoretogram showing the specificity of primers targeting *ethA1* gene

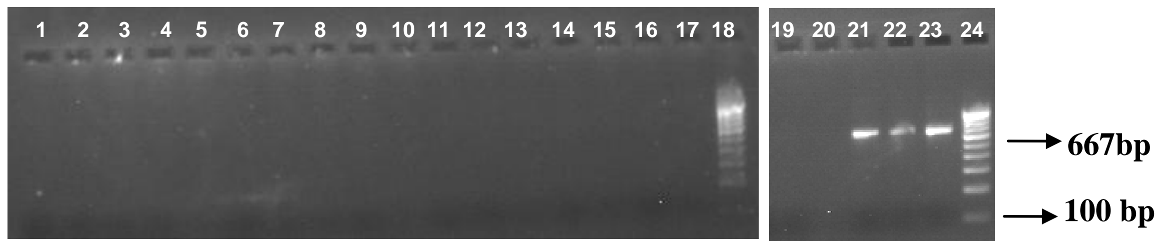


Figure 9.15: Agarose gel electrophoretogram showing the specificity of primers targeting *ethA2* gene

Figure 9.16: Agarose gel electrophoretogram showing the specificity of primers targeting *ethA3* gene

Figure 9.17: Agarose gel electrophoretogram showing the specificity of primers targeting *ethR* gene

Figure 9.12-9.17: Lane 1: Negative control (negative), Lane 2:*M. smegmatis* (negative), Lane 3: *M. duvalii* (negative), Lane 4: *M. thermoresistible* (negative), Lane 5: *M. fortuitum* (negative), Lane 6: *M. abscessus* (negative), Lane 7: *M. chelonae* (negative), Lane 8: *M. flavescens* (negative), Lane 9: *M. intracellulara* (negative), Lane 10:*M. phlei* (negative), Lane 11: *M. simiae* (negative), Lane12: *M. kansasii*(negative) , Lane 13: *M. gordonae* (negative), Lane 14: *M. xenopi* (negative), Lane 15: *Nocardia asteroids* (negative), Lane 16: *Actinomyces viscosus* (negative), Lane 17: *Streptomyces spp* (negative), Lane 18: MW DNA Ladder (100bp), Lane 19: Human DNA (negative), Lane 20: *M. Monacense* (negative), Lane 21: *M. bovis* (), Lane 22: *M. tuberculosis H37*Ra (positive), Lane 23: *M. tuberculosis H37*Rv (positive with specific base pair product), Lane24: MW DNA Ladder (100bp)

Figure 9.18: Agarose gel electrophotogram showing specificity of the primers targeting *eis* gene

Lane 1: Negative control (negative), Lane 2:*M. Smegmatis* (negative), Lane 3: *M. duvalii* (negative), Lane 4: *M. thermoresistible*(negative) , Lane 5: *M. fortuitum* (negative), Lane 6: *M. abscessus* (negative), Lane 7: *M. chelonae* (negative), Lane 8: *M. flavescens* (negative), Lane 9: *M. intracellulara* (negative), Lane 10:*M. phlei* (negative), Lane 11: *M. simiae* (negative), Lane12: *M. kansasii* (negative), Lane 13: *M. gordonae* (negative), Lane 14: *M. xenopi* (negative), Lane15*: Nocardia species* (negative), Lane 16: *M. bovis* (negative), Lane 17: *M. tuberculosis H37*Ra (negative), Lane 18: DNA Ladder (100bp), Lane 19: *M. tuberculosis H37*Rv (positive with specific 1153 bp), Lane20: DNA Ladder (250bp)

Figure 9.19: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *gyr A* gene

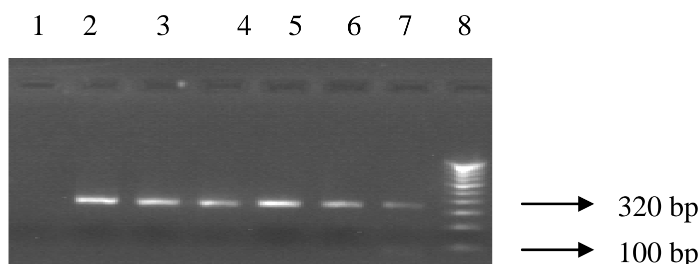
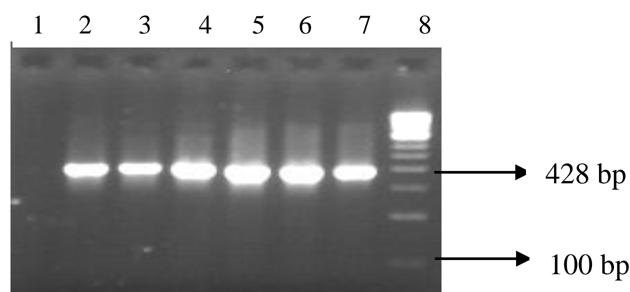
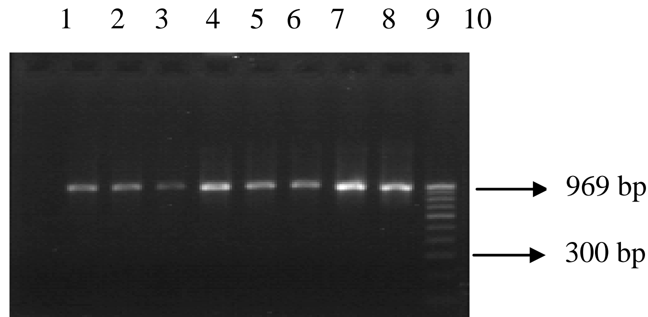


Figure 9.20: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *gyr B* gene



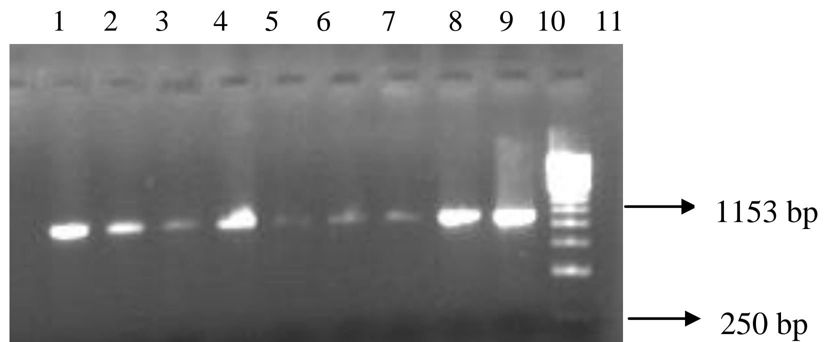
Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 428 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1133 (sputum), Lane 4: LTITIM 1141 (sputum), Lane 5: LTITIM 1131 (sputum), lane 6: LTITM 1076 (sputum), Lane 7 : PC *M. tuberculosis* H37Rv (Positive with specific bp product), Lane 8 : MW marker 100bp

Figure 9.21: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *tly A* gene



Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positive with specific 969 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7: LTITM 1028 (sputum), Lane: 8: LTITM 1062 (sputum), Lane 9 : PC -*M. tuberculosis* H37Rv (Positive with specific 969 bp product), Lane 10 : MW marker 100bp ladder

Figure 9.22: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *eis* gene



Lane 1: Negative Control (Negative), Lane: 2-9: *M. tuberculosis* isolates (Positive with specific 1153 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7: LTITM 1028 (sputum), Lane 8: LTITM 979 (sputum), Lane 9: LTITIM 1141 (sputum) Lane 10 : PC *M. tuberculosis* H37Rv (Positive with specific 1153 bp product), Lane 11 : MW marker 250bp ladder,

Figure 9.23: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethA1* gene

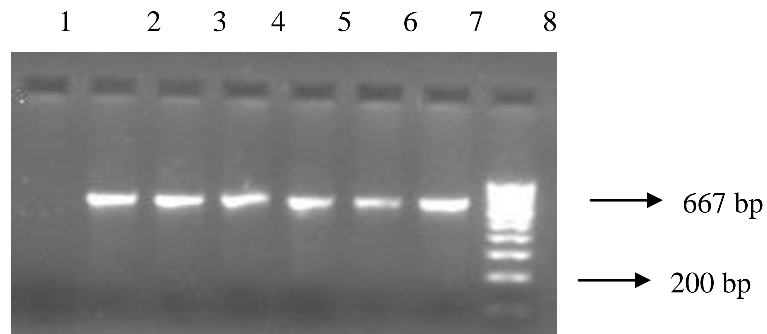


FIGURE 9.24: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethA2* gene

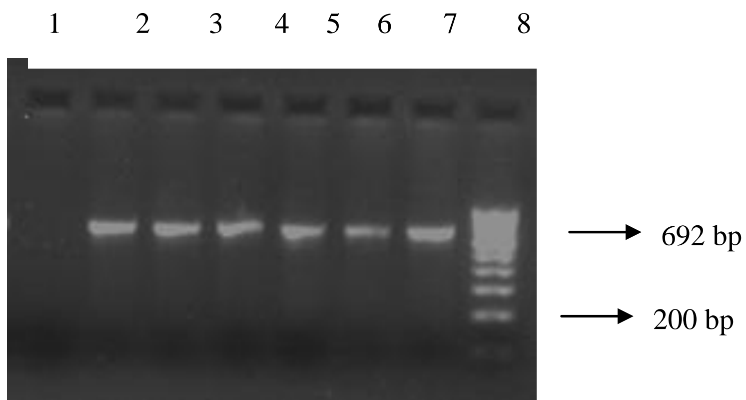
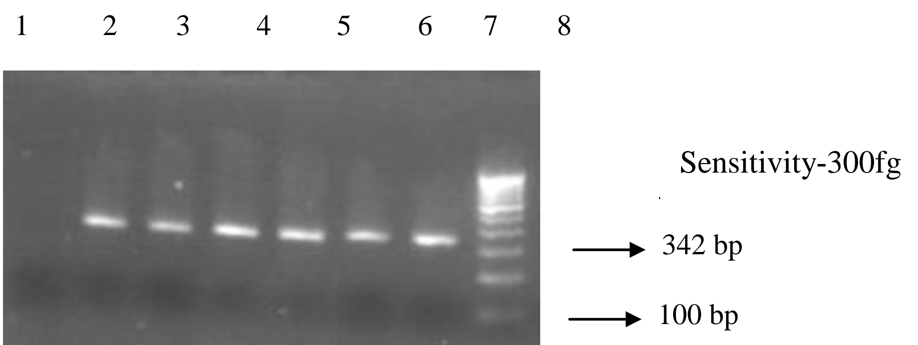
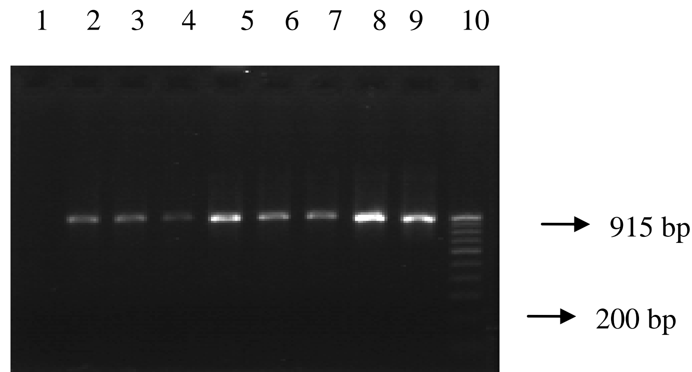


Figure 9.25: Agarose gel electrophoretogram showing the amplification of *M . tuberculosis* isolates targeting *ethA3* gene



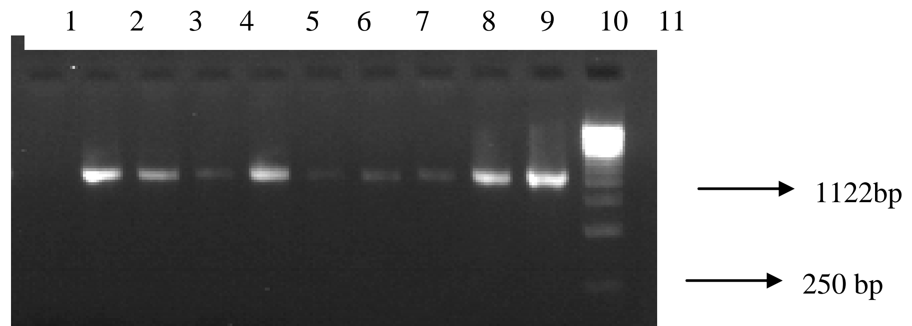
Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 692 bp product), Lane 2: LTITIM 1028 (sputum), Lane 3: LTITM 1062 (sputum), Lane 4: LTITIM 1003 (sputum), Lane 5: LTITIM 989 (sputum), lane 6: LTITM 1078 (sputum), Lane 7: PC- *M. tuberculosis* H37Rv (Positive with specific base pair product), Lane 8: MW marker 100bp ladder

Figure 9.26: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *ethR* gene



Lane 1: Negative Control (Negative), Lane: 2-8: *M. tuberculosis* isolates (Positive with specific 915 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum),Lane 7: LTITM 989 (sputum), Lane 8: LTITM 1003 (sputum), Lane 9 : PC *M. tuberculosis* H37Rv (Positive with specific 915 bp product), Lane 10 : MW marker 100bp ladder,

Figure 9.27: Agarose gel electrophoretogram showing the amplification of *M. tuberculosis* isolates targeting *thyA* gene



Lane 1: Negative Control (Negative), Lane: 2-6: *M. tuberculosis* isolates (Positive with specific 320 bp product), Lane 2: LTITIM 1029 (sputum), Lane 3: LTITM 1041 (sputum), Lane 4: LTITIM 1060 (sputum), Lane 5: LTITIM 1076 (sputum), lane 6: LTITM 1077 (sputum), Lane 7 : PC *M. tuberculosis* H37Rv (Positive with specific 1122 bp product), Lane 8 : MW marker 250bp ladder

9.3.4 PCR based DNA sequencing results targeting *gyrA* gene encoding for Fluoroquinolone resistance (table 9.1, figure 9.28):

Polyresistant strains:

Among 82 Polyresistant strains, 69 were phenotypically sensitive and 13 were phenotypically resistant to Fluoroquinones by Micro MGIT BACTEC method. Out of 13 phenotypically Fluoroquinones resistant strains, only 2 sputum isolates showed reported mutation at 95th codon (Ser95Thr). No mutations were observed in remaining 11 resistant isolates.Of 69 Phenotypically sensitive strains, 8 (7- Sputum, 1- FNAB) isolates showed reported mutation at 95th codon (Ser95Thr) and remaining isolates, there were no mutations observed .

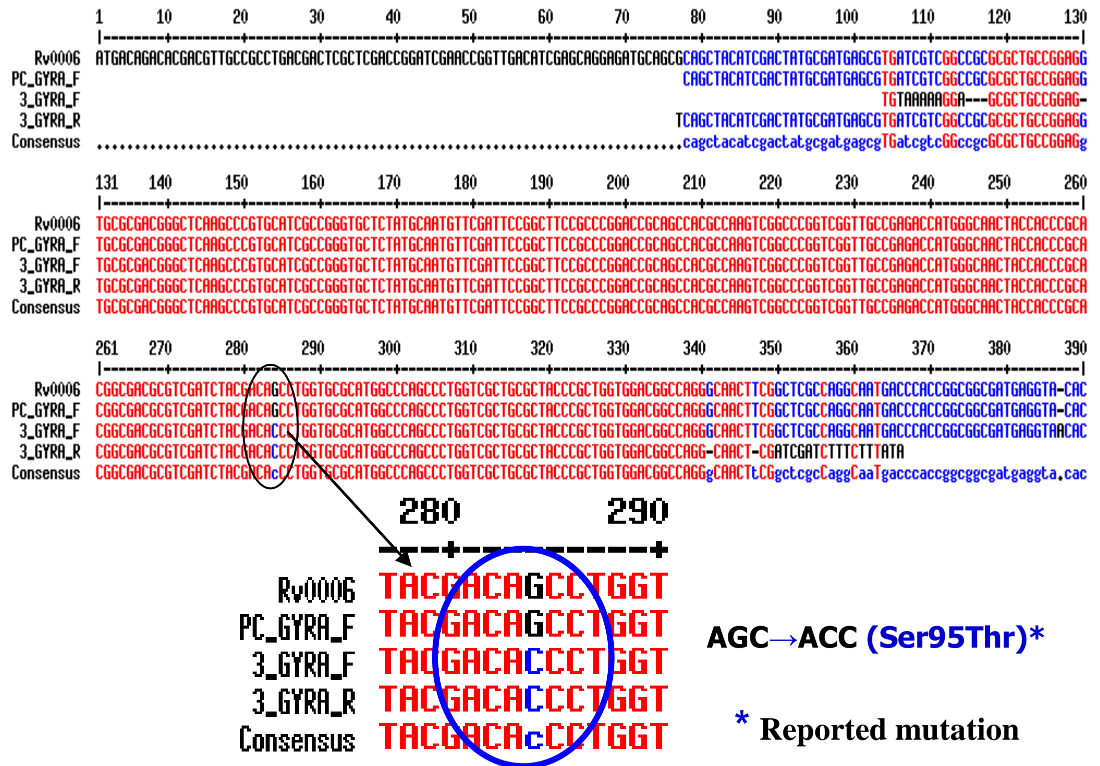
MDR-TB strains:

Among 29 MDR strains, 9 were phenotypically resistant and 20 were phenotypically sensitive to Fluoroquinones by Micro MGIT BACTEC method. Five sputum (2-resistant strain, 3- sensitive strains) isolated showed reported mutation at 95th codon (Ser95Thr).

Table 9.1: Reported mutations in FQs sensitive and resistant *M. tuberculosis* isolates in Polyresistant and MDR-TB strains

S.no	lab isolate no.	Nucleotide change	Aminoacid change	Type of mutation
POLYRESISTANT STRAINS				
Fluoroquinones resistant strains				
1	2 sputum strains	**AGC → ACC**	Ser95Thr	Substitution
Fluoroquinones sensitive strains				
2	8 sputum strains	**AGC → ACC**	Ser95Thr	Substitution
MDR-TB STRAINS				
Fluoroquinones resistant strains				
3	2 sputum strains	**AGC → ACC**	Ser95Thr	Substitution
Fluoroquinones sensitive strains				
4	3 sputum strains	**AGC → ACC**	Ser95Thr	Substitution

Figure 9.28: Multalin Results of DNA Sequence analysis targeting *gyr A* gene



Row 1: RV006- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PC_GYRA_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: 3_GYRA_F- Forward sequence of *M. tuberculosis* isolate showing reported Mutation (Ser95Thr)

Row 4: 3_GYRA_R- Reverse sequence of *M. tuberculosis* isolate showing reported mutation (Ser95Thr)

Row 5: Consensus- The sequence showing the consenses of the isolate with the reference strain

9.3.5 PCR based DNA sequencing results targeting *gyrB* gene encoding for Fluoroquinolones resistance:

Polyresistant strains:

Among 82 Polyresistant strains, 69 were phenotypically sensitive and 13 were phenotypically resistant to Fluoroquinones by Micro MGIT BACTEC method. Of 13 phenotypically Fluoroquinones resistant strains, only 1 sputum isolate showed 7 Novel Substitution mutations (Cys4Gly, Arg5Gln, Ser6Phe, Ser9Ile, Lys20Asp,Val26Gly, Ala133Glu), 2 novel deletion mutations at nucleotide positions 12, 15 and 1 Novel silent mutation (Gly8Gly). No mutations were observed in remaining 12 resistant isolates. None of the mutation was observed in sensitive strains (Table 9.2, Figure 9.29, 9.30, 9.31, and 9.32).

MDR-TB strains:

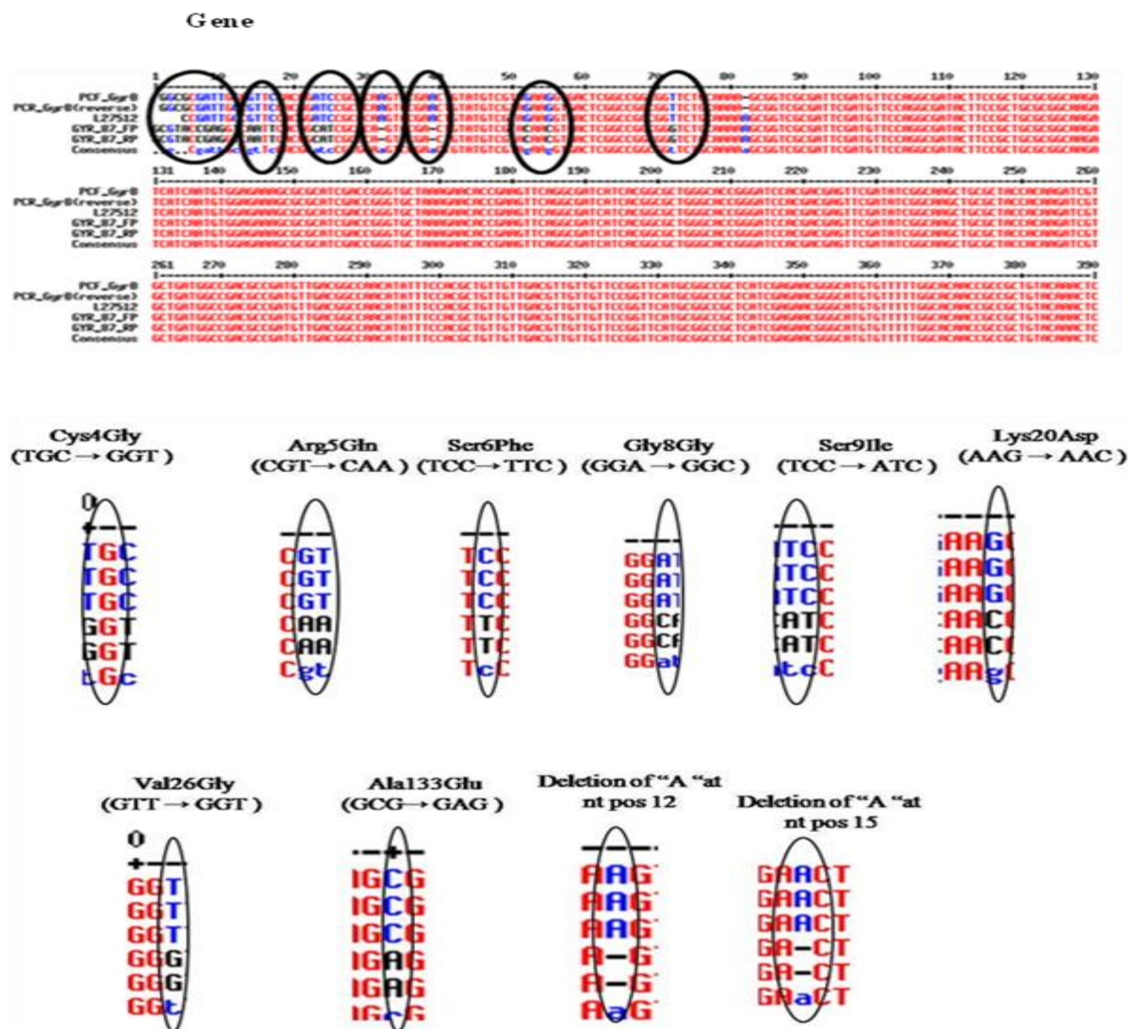
Among 29 MDR strains, 9 were phenotypically resistant and 20 were phenotypically sensitive to Fluoroquinones by Micro MGIT BACTEC method. Of 9 resistant strains, only one Sputum isolate showed 19 Novel substitution mutation (Asp455Gly, Arg457Asp, Thr459Ala, Asp460Val, Pro461Ala, Glu470Gln, Gly471Arg, Lys479Stop, Gly494Val, Lys495Ala, Asn498Ala, Iso504Met, Lys509Gln, Thr522Pro, Asp529His, Lys532Glu, Tyr535Ala, Pro578Arg, Lys583Glu) and 9 Novel silent mutations (Ser458Ser, Gly476Gly, Leu492Leu, Iso497Iso, Arg506Arg, Phe528Phe, Gly531Gly, Arg561Arg, Arg586Arg). Among 20 sensitive strains, only one sputum isolate showed 20 Novel substitution mutations (Lys479Stop, Gly494Val, Val499Glu, Lys495Ala, Iso504Met, Val507Gly, Leu508Pro,Lys509Gln, Gln514Arg, Iso517Phe, Thr518Ala, Asp526Glu, Gly531Val, Lys532Glu,Arg534Leu, Tyr535Ala, Gly548Asp, Leu579Arg, Tyr580Stop) , 5 Novel silent mutation (Gly476Gly, Ala502Ala, Gly521Gly, Ala542Ala, Arg586Arg) and 1 reported mutaion (Glu512Asp).

9.2: Novel and reported mutations targeting *gyr B* region in Polyresistant and MDR-TB strains

S.no	lab isolate no.	Nucleotide change	Aminoacid change	type of mutation
		Polyresistant Strains		
Fluoroquinones resistant strains				
1	LTITM 87 (Sputum)	**TGC → GGT**	Cys4Gly	Substitution
		CGT→ CAA	Arg5Gln	Substitution
		TCC→ **TTC**	Ser6Phe	Substitution
		GGA → GGC	Gly8Gly	Silent
		TCC → ATC	Ser9Ile	Substitution
		AAG → AAC	Lys20Asp	Substitution
		GTT → GGT	Val26Gly	Substitution
		GCG→ GAG	Ala133Glu	Substitution
		Deletion of A pos 12	-	Deletion
		Deletion of A pos 15	-	Deletion
		MDR-TB strains		
Fluoroquinones resistant strains				
2	LTITM 232 (Sputum)	**GAT→GGA**	Asp455Gly	Substitution
		CGT→GAT	Arg457Asp	Substitution
		TCC→TCA	Ser458Ser	Silent
		ACG→GCC	Thr459Ala	Substitution
		GAT→GTT	Asp460Val	Substitution
		CCG→GCG	Pro461Ala	Substitution
		GAA→CAA	Glu470Gln	Substitution
		GGT→CGT	Gly471Arg	Substitution
		AAA→TAA	Lys479Stop	Substitution
		GGT→GGG	Gly476Gly	Silent
		CTG→TTG	Leu492Leu	Silent
		GGC→GTA	Gly494Val	Substitution
		AAG→GCG	Lys495Ala	Substitution
		ATC→ATT	Iso497Iso	Silent
		AAT→GCT	Asn498Ala	Substitution
		ATC→ATG	Iso504Met	Substitution
		CGG→CGC	Arg506Arg	Silent
		AAG→CAG	Lys509Gln	Substitution
		ACC→CCC	Thr522Pro	Substitution
		TTC→TTT	Phe528Phe	Silent
		GAT→CAT	Asp529His	Substitution

		GGC→GTT	Gly531Gly	Silent
		AAG→GAG	Lys532Glu	Substitution
		TAC→GCC	Tyr535Ala	Substitution
		CGG→CGA	Arg561Arg	Silent
		CCG→CGG	Pro578Arg	Substitution
		AAG→CAA	Lys583Glu	Substitution
		CGC→AGG	Arg586Arg	Silent
Fluoroquinones sensitive strains				
3	LTITM 480 (Sputum)	GGT→GGG	Gly476Gly	Silent
		AAA→TGA	Lys479Stop	Substitution
		GGC→GTA	Gly494Val	Substitution
		GTG→GAG	Val499Glu	Substitution
		AAG→GCG	Lys495Ala	Substitution
		GCG→GCC	Ala502Ala	Silent
		ATC→ATG	Iso504Met	Substitution
		GTG→GGG	Val507Gly	Substitution
		CTA→CCC	Leu508Pro	Substitution
		AAG→CAG	Lys509Gln	Substitution
		GAA→GAT	**Glu512Asp**	Substitution*
		CAG→CGG	Gln514Arg	Substitution
		ATC→TTC	Iso517Phe	Substitution
		ACG→GCG	Thr518Ala	Substitution
		GAC→GAG	Asp526Glu	Substitution
		GGC→GTT	Gly531Val	Substitution
		AAG→GAG	Lys532Glu	Substitution
		CGC→CTC	Arg534Leu	Substitution
		TAC→GCC	Tyr535Ala	Substitution
		GCC→GCG	Ala542Ala	Silent
		GGC→GAC	Gly548Asp	Substitution
		CTG→CGG	Leu579Arg	Substitution
		TAC→TAA	Tyr580Stop	Substitution
		CGC→AGG	Arg586Arg	Silent
		GGC→GGG	Gly521Gly	Silent

Figure 9.29: Multalin results of LTITM 87- resistant strain targeting *gyr B* gene



Row 1: PC_GYRB_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 2: PC_GYRB_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

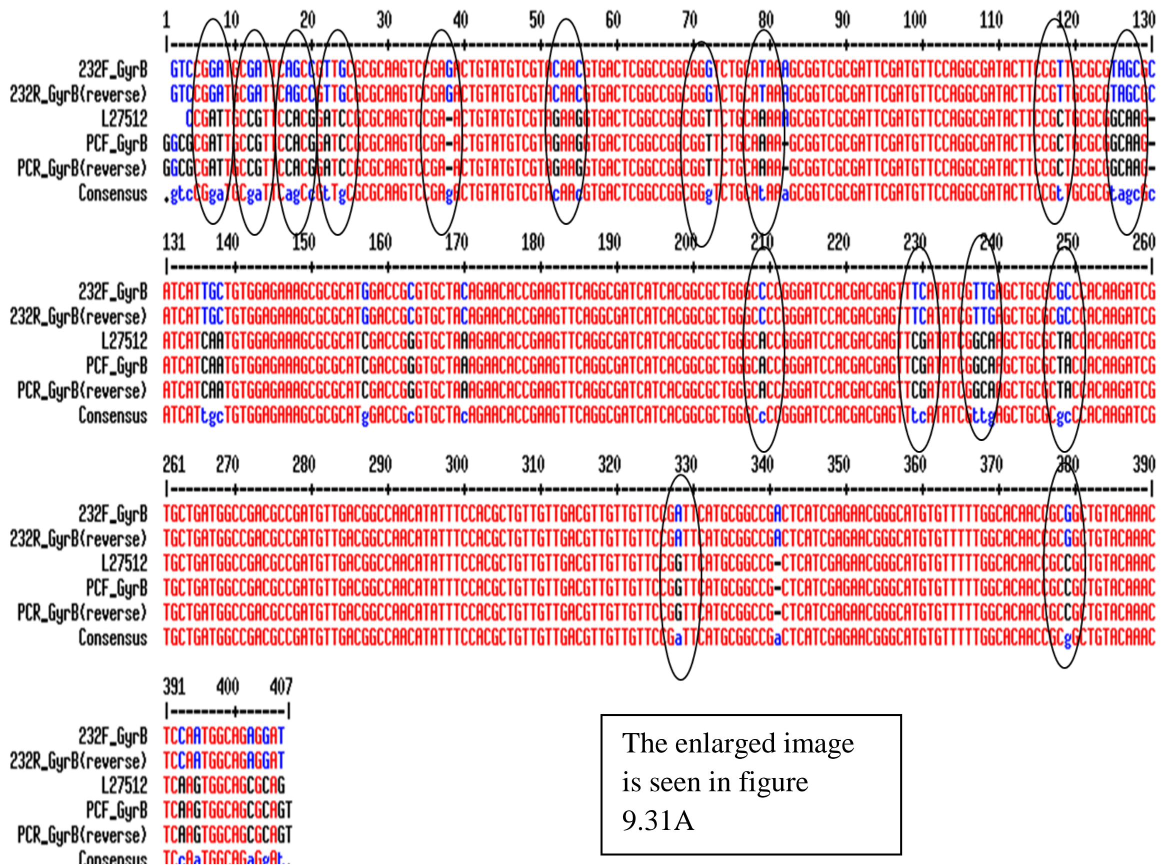
Row 3: L27512- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: 87_GYRB_F- Forward sequence of *M. tuberculosis* isolate showing novel mutations

Row 5: 87_GYRB_R- Reverse sequence of *M. tuberculosis* isolate showing novel mutations

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.30: Multalin results of LTITM 232- resistant strain targeting *gyr B* gene



Row 1: 232F_GYRB - Forward sequence of *M. tuberculosis* isolate showing novel mutations

Row 2: 232_GYRB (REVERSE)- Reverse sequence of *M. tuberculosis* isolate showing novel mutations

Row 3:L27512- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: PC_GYRB- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: PC_GYRB (REVERSE)- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.31A: Multalin results of LTITM 232- resistant strain targeting *gyr B* gene

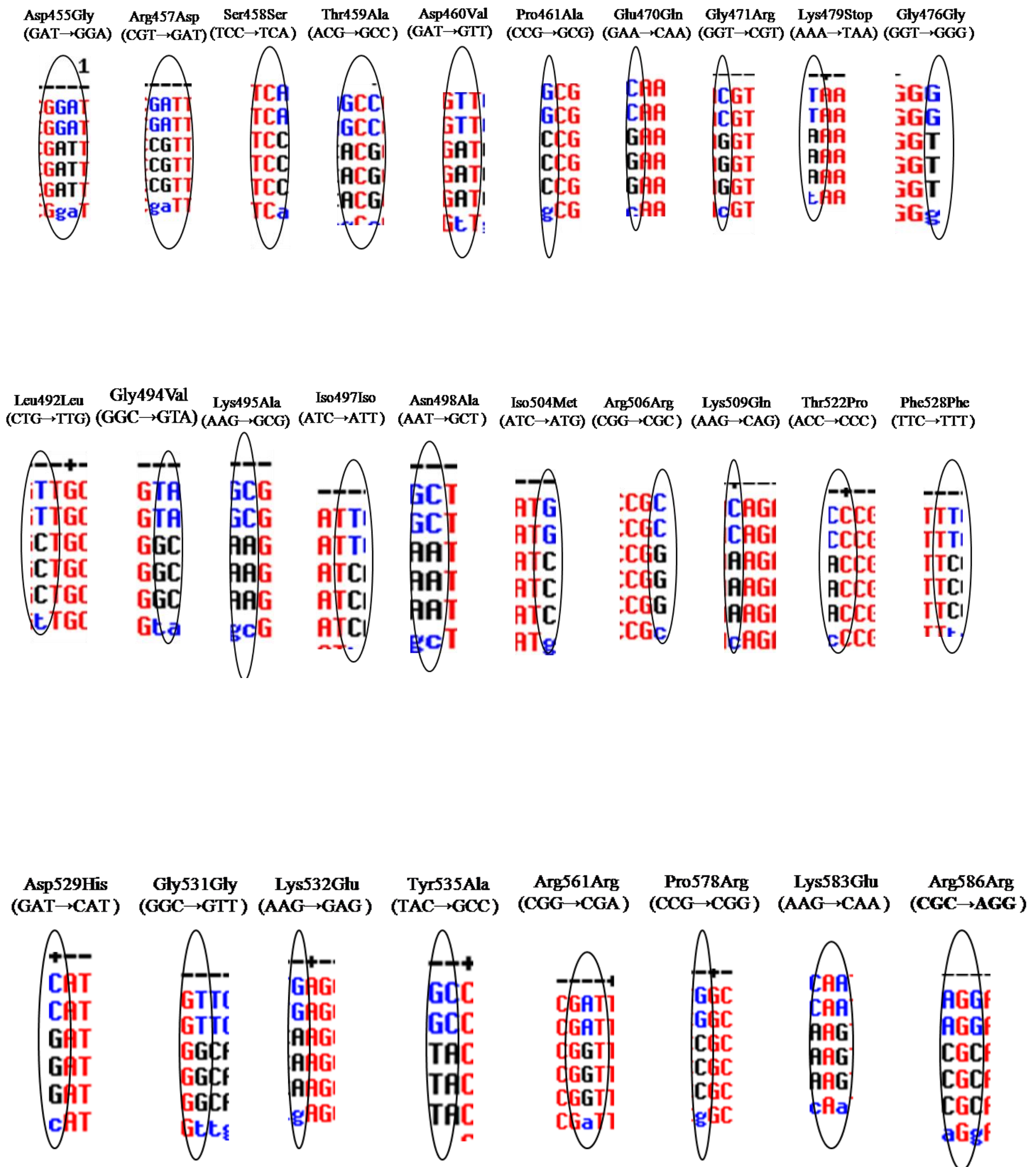
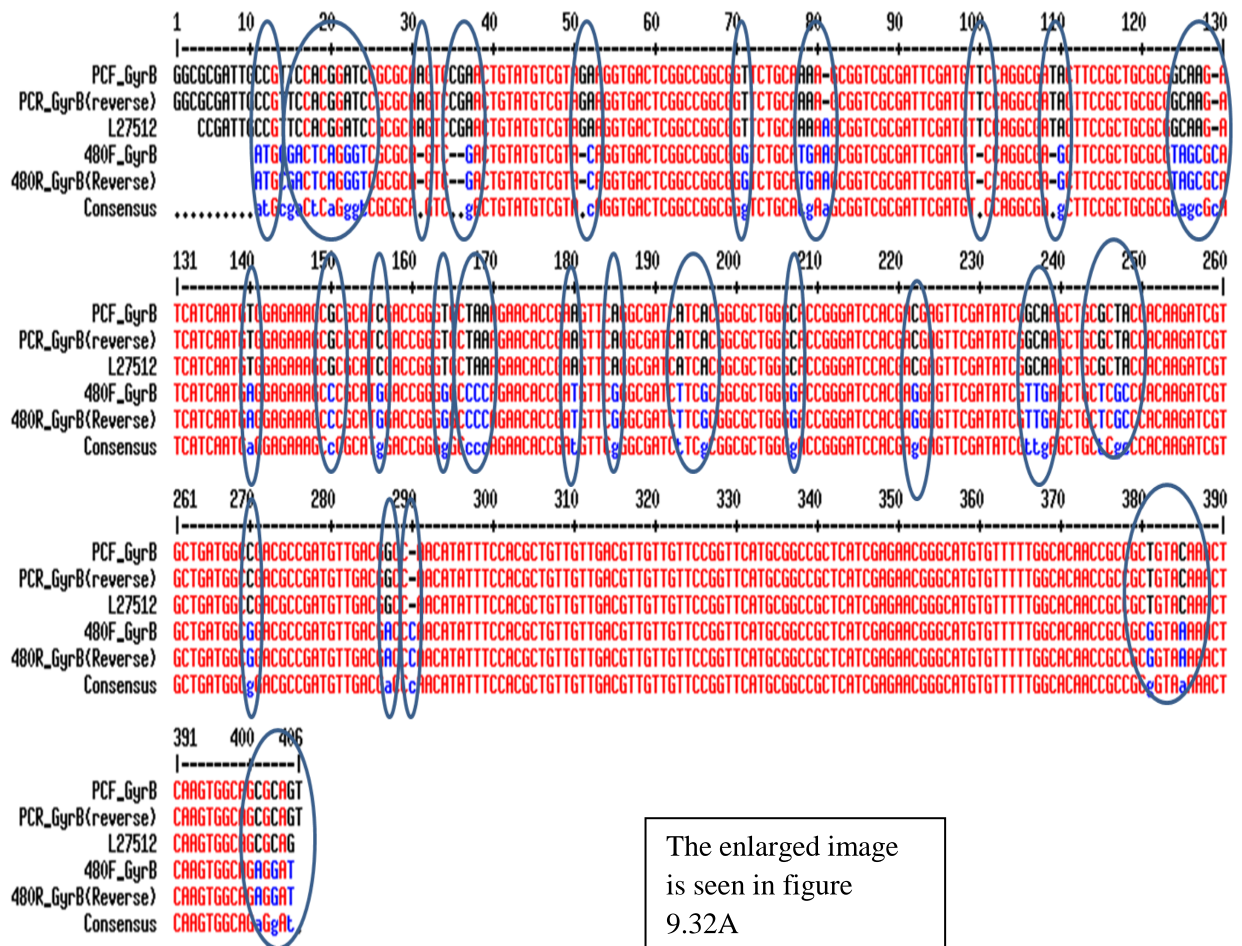


Figure 9.32: Multalin results of LTITM 480- resistant strain targeting *gyr B* gene



Row 1: PC_GYRB- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 2: PC_GYRB (Reverse)- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

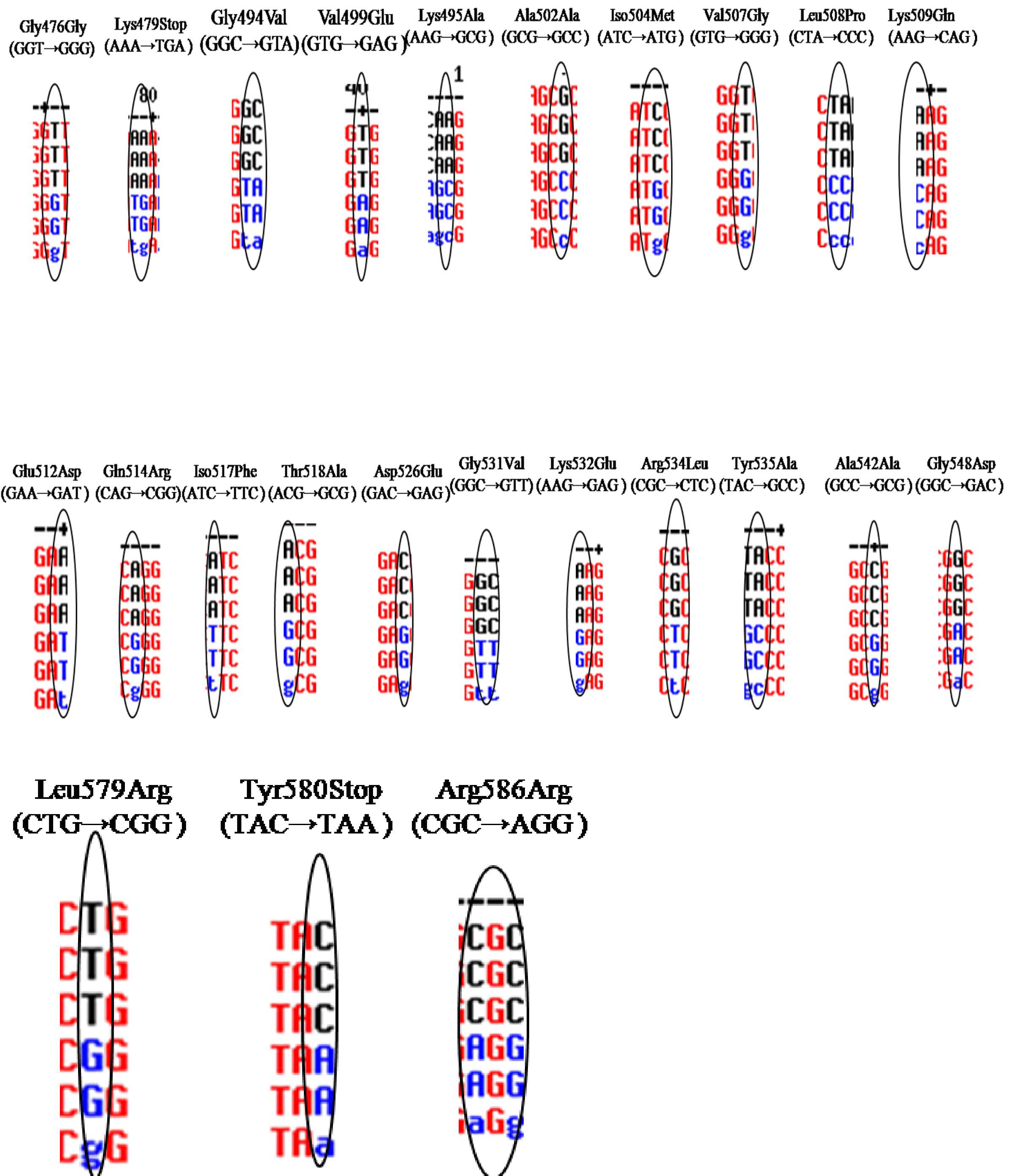
Row 3: L27512- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 4: 480_GYRB - Forward sequence of *M. tuberculosis* isolate showing novel Mutation

Row 5: 480_GYRB (Reverse)- Reverse sequence of *M. tuberculosis* isolate showing novel mutation

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.32A: Multalin results of LTITM 480- resistant strain targeting *gyr B* gene



9.3.6 PCR based DNA sequencing results targeting *tlyA* gene encoding for Aminoglycosides resistance:

Polyresistant strains:

Of 82 polyresistant strains, 14 were phenotypically resistant and 68 were phenotypically sensitive to Aminoglycosides by Micro MGIT BACTEC method. Among 14 resistant strains, none of the isolate showed mutation. Of 68 sensitive strains, 2 sputum isolates showed Novel Silent mutation (Leu11Leu) (Table 9.3, Figure 9.33).

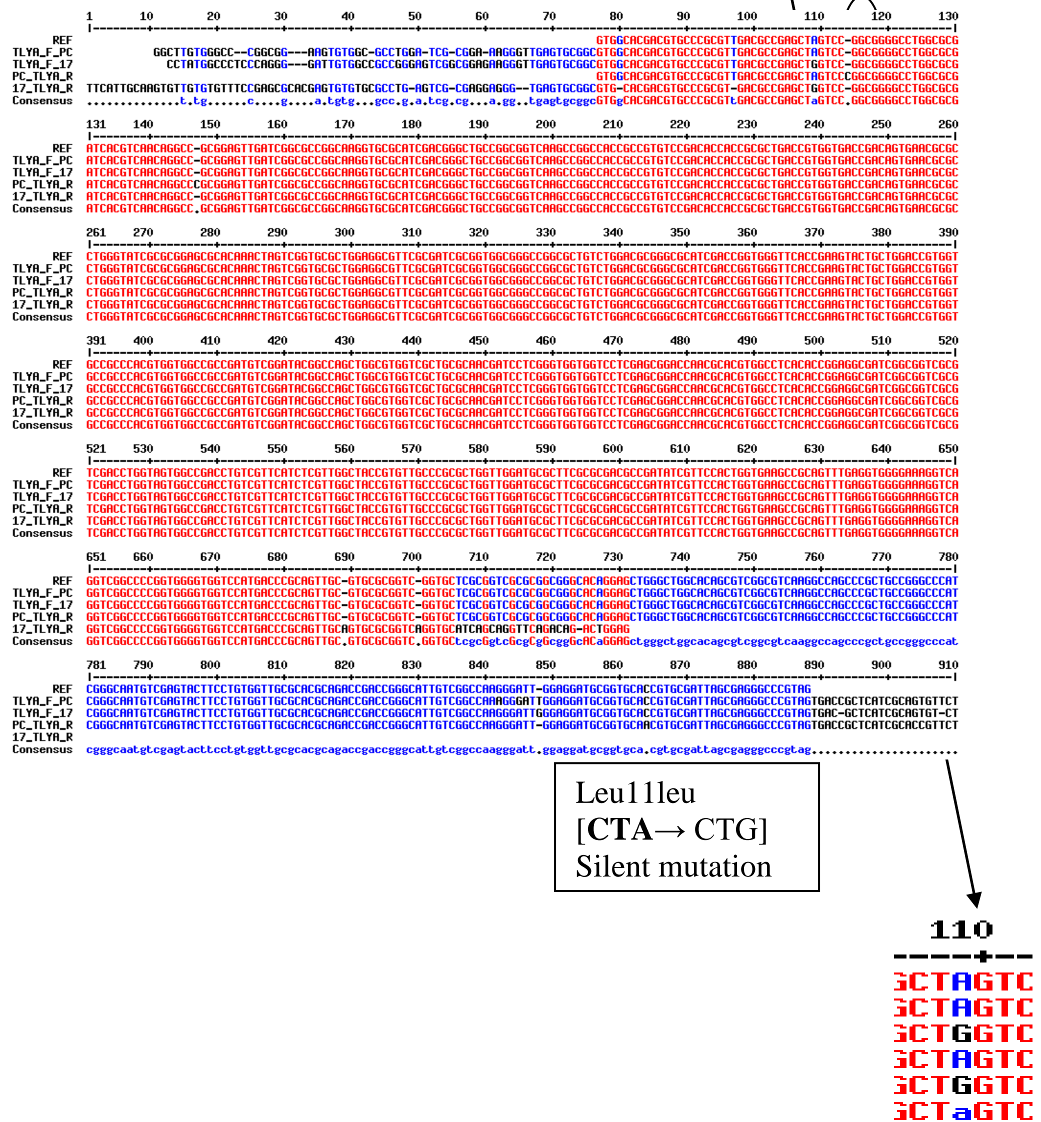
MDR-TB strains:

Of 29 MDR-TB strains, 12 were phenotypically resistant and 17 were phenotypically sensitive to Aminoglycosides by Micro MGIT BACTEC method. Among 12 resistant strains, 3 sputum isolates showed Novel silent mutation (Leu11Leu). 10/17 (9-Sputum, 1-Bronchial wash)sensitive strains showed Novel Silent mutation (Leu11Leu) and Additionally in one sensitive strain 2 novel silent mutation were observed(Leu11Leu, Val55Val).

Table 9.3: Novel mutations targeting *tly A* gene in polyresistant and MDR-TB strains

S.no	**Lab isolate no.**	**Nucleotide change**	**Aminoacid change**	**Type of mutation**
Polyresistant Strains				
Aminoglycosides sensitive strains				
1	2 sputum strains	**CTA**→ CTG	Leu11leu	Silent
MDR-TB strains				
Aminoglycosides resistant strains				
3	4 sputum strains	**CTA**→ CTG	Leu11leu	Silent
Aminoglycosides sensitive strains				
7	11 sputum strains	**CTA**→ CTG	Leu11leu	Silent

Figure 9.33: Multalin Results Of Dna Sequence Analysis Of MDR-TB Isolate (LTITM 17- Resistant) Targeting *tly A* Gene



Row 1: REF- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: TLYA_F_PC- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: TLYA_F_17- Forward sequence of *M. tuberculosis* isolate showing silent mutation (Leu11leu)

Row 4: PC_TLYA_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: 17_TLYA_R - Reverse sequence of *M. tuberculosis* isolate showing silent mutation (Leu11leu)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

9.3.7 PCR based DNA sequencing results targeting *eis* gene encoding for Aminoglycosides resistance

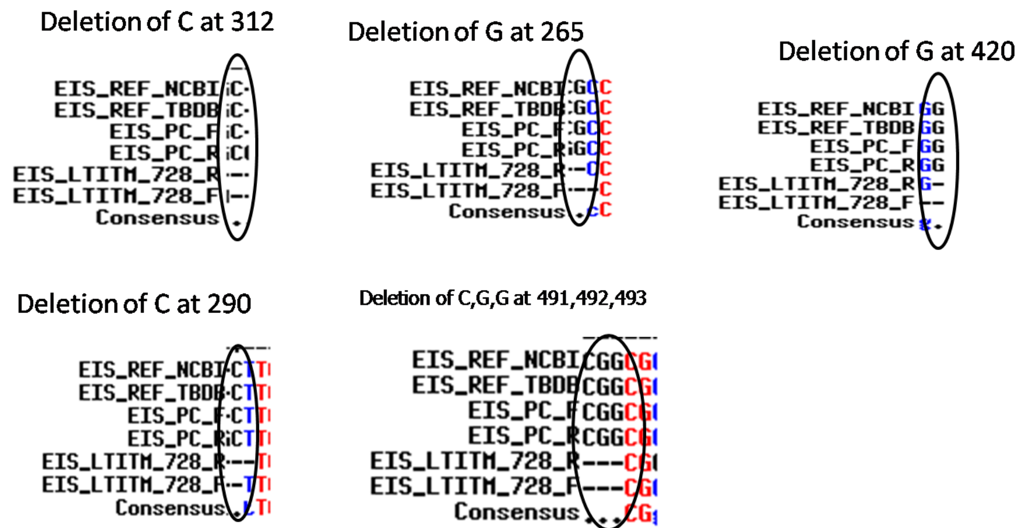
MDR-TB strains:

Of 29 MDR-TB strains, 12 were phenotypically resistant and 17 were phenotypically sensitive to Aminoglycosides by Micro MGIT BACTEC method. Of 12 phenotypically resistant strains, 2 sputum isolate showed 9 Novel deletion mutations at nucleotide positions 239, 265, 290, 312, 420, 491, 492, 493 and 1 insertion mutation at nucleotide position 686. Among 17 sensitive strains, 4 isolates showed 11 Novel mutations (3 deletion mutations at nucleotide positions 685, 870, 894, 5 Insertion mutation at nucleotide positions 555, 595, 636, 755, 776, 2 substitution mutations- Val264Gly and 1 silent mutation - Val303Val) (Table 9.4, Figure 9.34, 9.35, 9.36, and 9.37).

Table 9.4: Novel Mutations targeting e*is* gene in polyresistant and MDR-TB sputum Strains

S.no	Lab isolate no & specimen	Nucleotide change	Type of mutation
MDR-TB STRAINS			
Aminoglycosides resistant strains			
	LTITM 728	Deletion of "C" at pos 239	Frame shift
		Deletion of "G" at pos 265	Frame shift
		Deletion of "C" at pos 290	Frame shift
		Deletion of "C" at pos 312	Frame shift
		Deletion of "G" at pos 420	Frame shift
		Deletion of "C" at pos 491	Frame shift
		Deletion of "G" at pos 492	Frame shift
		Deletion of "G" at pos 493-	Frame shift
	LTITM 232	Insertion of "A" at 686	Frame shift
Aminoglycosides sensitive strains			
	LTITM 17	Insertion of "T" at pos 555	Frame shift
		Insertion of "G" at pos 595	Frame shift
		Insertion of "A" at pos 636	Frame shift
		Insertion of "G" at pos 755	Frame shift
	LTITM 103	GGT→AGT (Gly205Ser)	Subsitution
		Deletion of "T" at pos 685	Frame shift
		Insertion of "A" at pos 776	Frame shift
		GTG→GGG(Val264Gly)	Subsitution
		Deletion of "A" at pos 870	Frame shift
		Deletion of "G" at pos 894	Frame shift

Figure 9.34: Multalin results for LTITM 728 (MDR-TB strain)



Row 1: EIS_REF_NCBI- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: EIS_REF_TBDB- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from tuberculosis Data base.

Row 3: EIS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

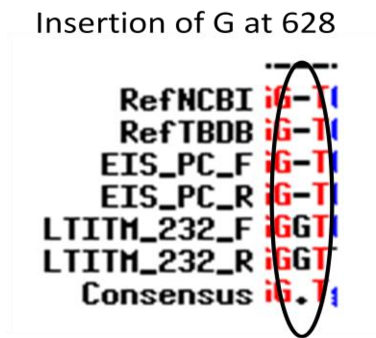
Row 4: EIS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: EIS_LTITM_728_F- Forward sequence of *M. tuberculosis* isolate showing novel Mutation

Row 6: EIS_LTITM_728_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation

Row 7: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.35: Multalin results for LTITM 232 (MDR-TB strain)



Row 1: REF_NCBI- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: REF_TBDB- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from tuberculosis Data base.

Row 3: EIS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

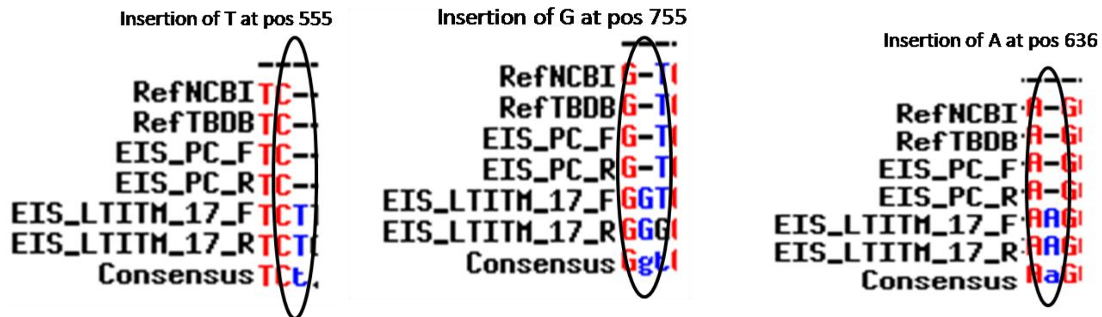
Row 4: EIS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: LTITM_232_F- Forward sequence of *M. tuberculosis* isolate showing novel Mutation

Row 6: LTITM_232_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation

Row 7: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.36: Multalin results for LTITM 17 (MDR-TB strain)



Row 1: REFNCBI- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: REFTBDB- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from tuberculosis Data base.

Row 3: EIS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

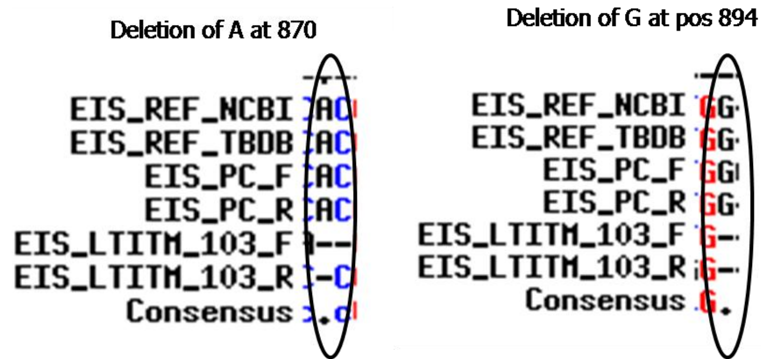
Row 4: EIS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: LTITM_17_F- Forward sequence of *M. tuberculosis* isolate showing novel Mutation

Row 6: LTITM_17_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation

Row 7: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.37: Multalin results for LTITM 103 (MDR-TB strain)



Row 1: EIS_REF_NCBI- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: EIS_REF_TBDB- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from tuberculosis Data base.

Row 3: EIS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: EIS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 5: EIS_LTITM_103_F- Forward sequence of *M. tuberculosis* isolate showing novel Mutation

Row 6: EIS_LTITM_103_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation

Row 7: Consensus- The sequence showing the consenses of the isolate with the reference strain

9.3.8 PCR based DNA sequencing results targeting *ethA1* gene encoding for Ethionamide resistance:

Polyresistant strains:

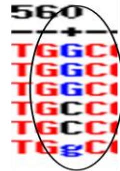
Among 82 polyresistant strains, 5 were phenotypically resistant and 77 were sensitive by Micro MGIT BACTEC method. Out of 5 resistant strains, 2 sputum isolates showed Novel substitution mutation at codon 187 (Gly187Ala) , 1sputum isolate showed 3 novel substitution mutations (Asn190Ser,Ser192Ala, Leu194Arg), 2 Novel Insertion mutation (Insertion of C at position 564 and Insertion of G at pos 565), 1sputum isolate showed novel mutation at codon 194 (Leu194Arg) and 1 sputum isolate showed 8 novel substitution mutation (Ile33Lys, Leu58Pro, Pro62Ala, Val83Gly, Asp99Lys, Val101Gly, Cys122Trp, Met129Lys), 2 novel insertional mutation of "G" at positions 383,335, 1 deletion mutation of "G" at position 375 and 2 novel silent mutations (Gly56Gly, Gly75Gly) (Table 9.5, Figure 9.38, 9.39).

Table 9.5: Novel mutations targeting *ethA1 gene* in polyresistant sputum strains

S.no	Lab isolate no.	Nucleotide change	Aminoacid change	Type of mutation
Polyresistant Strains				
Ethionamide resistant strains				
1	LTITM 283	**GGC**→ GCC	Gly187Ala	Substitution
2	LTITM 735	**GGC**→ GCC	Gly187Ala	Substitution
3	LTITM 978	**AAC**→ AGC	Asn190Ser	Substitution
		TCG→ GCG	Ser192Ala	Substitution
		TTG→ CGT	leu194Arg	Substitution
		Ins of C at pos 564	-	Frame shift
		Ins of G at pos 565	-	Frame shift
4	LTITM 1003	TTG→ CGT	Leu194Arg	Substitution

Figure 9.38: Multalin results for LTITM 283 (Polyresistant strain)

Gly187Ala
(GGC→ GCC)



Row 1: NCBI_ETHA- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: EI_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

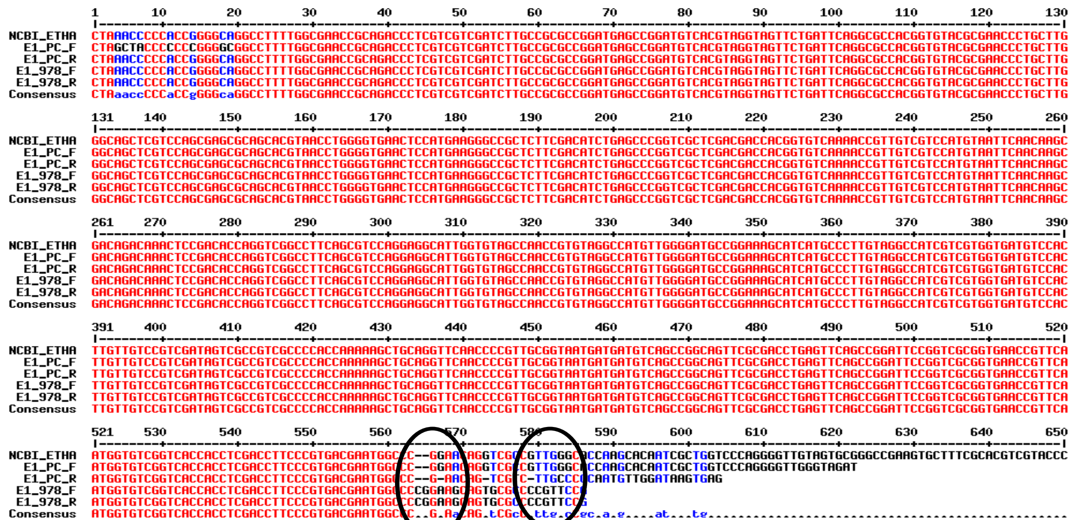
Row 3: EI_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

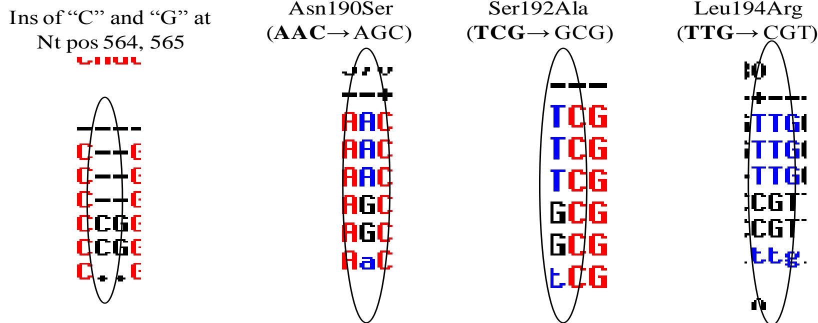
Row 4: EI_283_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Gly187Ala)

Row 5: EI_283_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Gly187Ala)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure9.39: Multalin results for LTITM 978 (Polyresistant strain)





Row 1: NCBI_ETHA- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCCDNA) taken from Genbank.

Row 2: EI_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: EI_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: EI_978_F- Forward sequence of *M. tuberculosis* isolate showing novel mutations (Asn190Ser, Ser192Ala, Leu194Arg)

Row 5: EI_978_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutations (Asn190Ser, Ser192Ala, Leu194Arg)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

9.3.9 PCR based DNA sequencing results targeting *ethA2 and ethA3* gene encoding Ethionamide resistance:

Among 82 polyresistant and 29 MDR-TB strains, none of the isolates showed mutation in *ethA2* and *ethA3* genes.

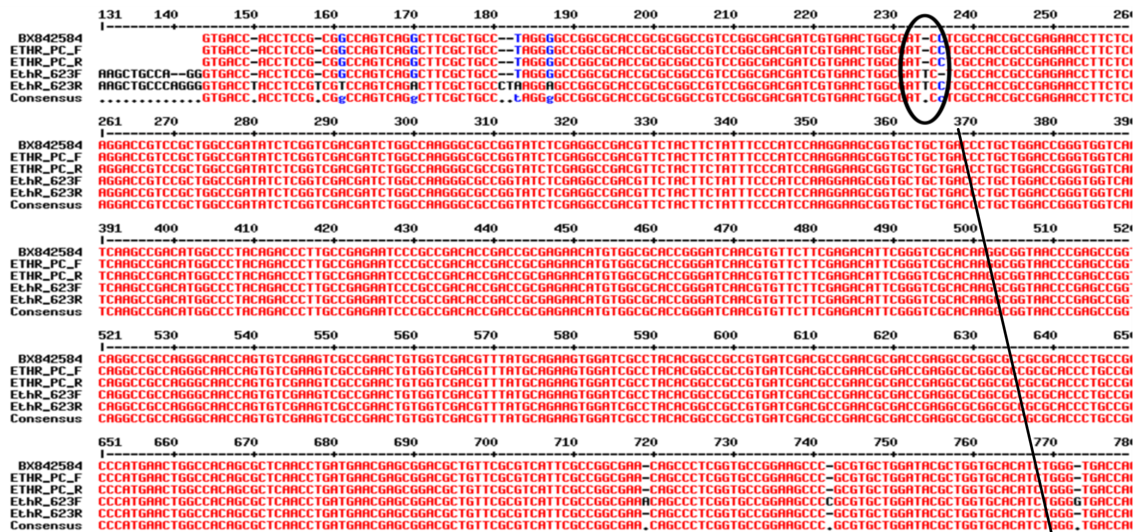
9.5.10 PCR based DNA sequencing results targeting *ethR* gene encoding Ethionamide resistance:

Among 82 polyresistant strains, 4 were phenotypically resistant and 78 were sensitive by Micro MGIT BACTEC method. None of the resistant strains showed mutation. But in 3 of the 78 sensitive strains, 1 novel substitution mutation (Thr102Ile), I silent mutation (Gly49Gly) and 1 Insertion mutation of "T" at nucleotide position 87 has occurred **(Table 9.6, Figure 9.40, 9.41)**.

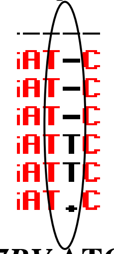
Table 9.6 : Novel mutations targeting *ethR gene* in polyresistant strains

S.no	lab isolate no.	Nucleotide change	Aminoacid change	Type of mutation
Polyresistant Strains				
Ethionamide Sensitive strains				
1	LTITM 623 (Sputum)	Insertion of "T" at 87th position	-	Frame shift
2	LTITM 238 (Sputum)	**GGC**→ GGG	Gly49Gly	silent
3	LTITM 447 (Sputum)	**ACA**→ ATA	Thr102Ile	Substitution

Figure 9.40: Multalin results for LTITM 623 (Polyresistant strain)



Insertion of "T" at 87th position



Row 1: BX842584- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: EI_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

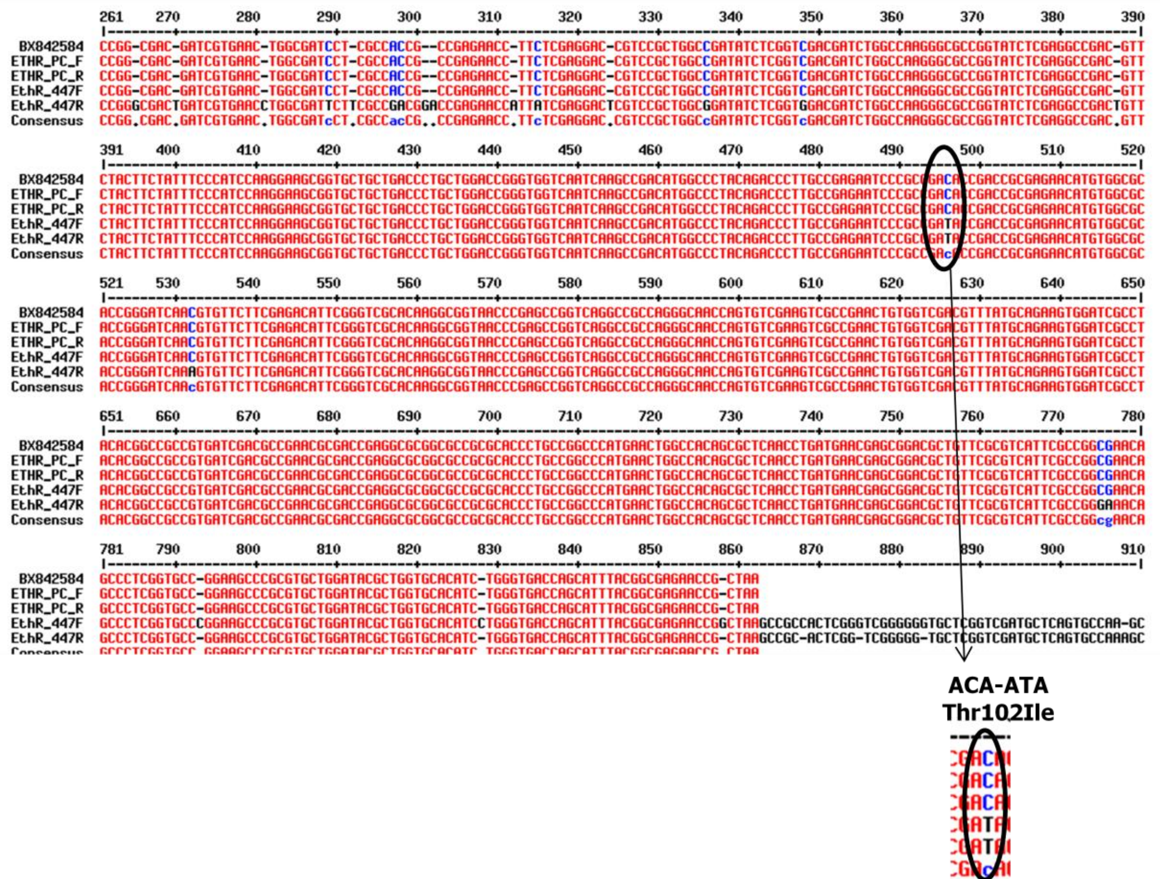
Row 3: EI_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: EI_623_F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Ins of "T" at 87th position)

Row 5: EI_623_R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Ins of "T" at 87th position)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.41: Multalin results for LTITM 447 (Polyresistant strain)



Row 1: BX842584- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: ETHR_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: ETHR_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 4: ETHR_447F- Forward sequence of *M. tuberculosis* isolate showing novel mutation (Thr102Ile)

Row 5: ETHR_447R - Reverse sequence of *M. tuberculosis* isolate showing novel mutation (Thr102Ile)

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

9.3.10 PCR based DNA sequencing results targeting *thyA* gene encoding for Para-amino salicylic acid resistance:

Polyresistant strains:

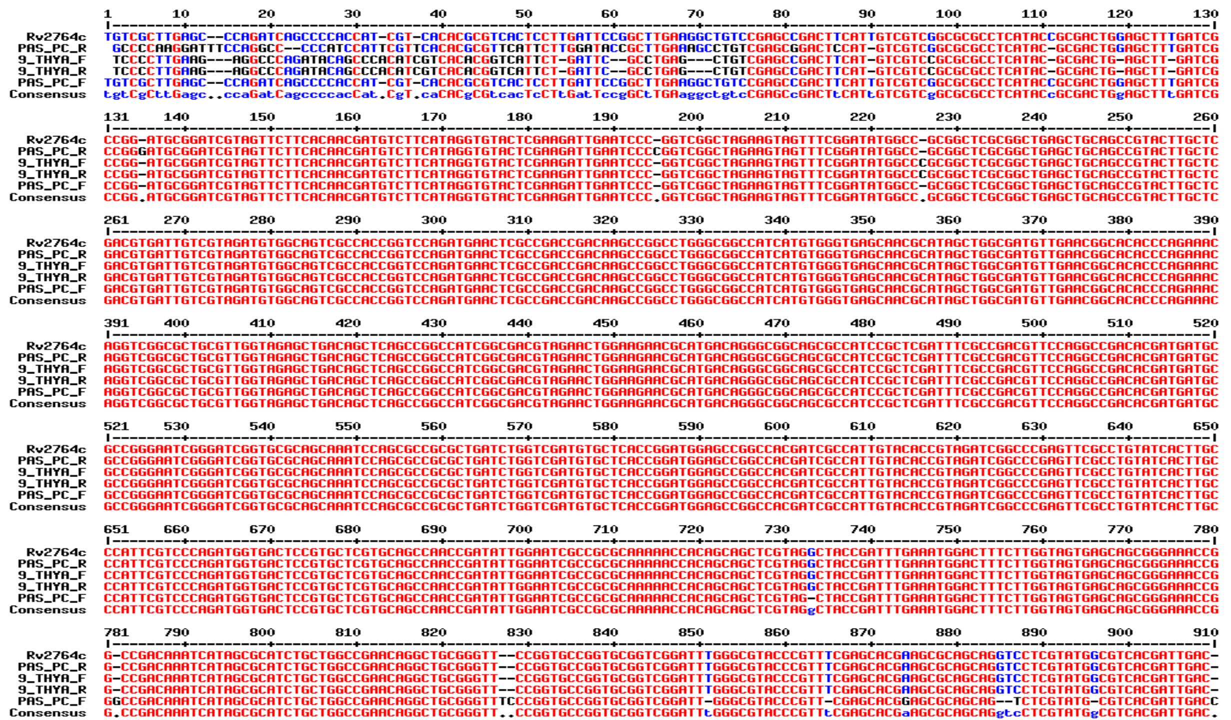
Among 82 polyresistant strains, 6 were phenotypically resistant and 76 were sensitive by Micro MGIT BACTEC method. Of 6 resistant strains, 3 sputum isolates showed 14 Novel substitution mutations (Gln2Arg, Arg12His, Val15Thr, Leu22Pro, Gly98Arg, Arg269Pro, Gly281Glu, Arg44Leu,Ser49Lys,Stop50Lys,Asp125-Tyr, Gly126Cys, Arg146Trp, Thr148Arg, Ileu265Phe, Ala270Lys, Lys285Asp), 8 Novel Insertion mutations (Insertion of "G" at nucleotide positions 67,104,126,348,441,537, Insertion of "C" at position 552 and Insertion of "A" at position 561), 5 Novel deletion mutations (Deletion of "G" at positions-119, 47, Deletion of "T" at position 125, Deletion of "c" at positions- 226,110) and 3 novel silent mutations (Gly144Gly, Val149Val, Ser289Ser). None of the sensitive strains and MDR-TB strains showed any mutations (Table 9.7, Figure 9.42, 9.43, 9.44).

Table 9.7: Novel mutations targeting *thyA gene* in polyresistant strains

S.no	lab isolate no.	Nucleotide change	Aminoacid change	Type of mutation
POLYRESISTANT STRAINS				
Para aminosalicylic acid resistant strains				
1	LTITM 9 (Sputum)	CAG→ AGG	Gln2Arg	Substitution
		CGT→ CAT	Arg12His	Substitution
		GCG→ ACG	Val15Thr	Substitution
		TTG→ CTG	Leu22Pro	Substitution
		GGC→ CGC	Gly98Arg	Substitution
		Del of "G" at pos 119	-	Frame shift
		Del of "T" at pos 125	-	Frame shift
		Ins of "C" at pos 226	-	Frame shift
2	LTITM 238 (Sputum)	Insertion of "G" at pos 67,104,126	-	Frame shift
		Del of "c" at pos110	-	Frame shift
		Del of "G" at pos 47	-	Frame shift
3	LTITM 565 (Sputum)	CGC→CTC	Arg44Leu	Substitution
		TCG→AAG	Ser49Lys	Substitution
		TAG→AAG	Stop50Lys	Substitution

		Insertion of "G"AT pos 348	-	Frame shift
		AAC→TAC	Asp125Tyr	Substitution
		GGC→TGT	Gly126Cys	Substitution
		GGC→GGT	Gly144Gly	Silent
		CGG→TGG	Arg146Trp	Substitution
		Insertion of "G" at pos 441	-	Frame shift
		ACG→AGG	Thr148Arg	Substitution
		GTA→GTG	Val149Val	Silent
		Insertion of "G" at pos 537	-	Frame shift
		Insertion of "C" at pos 552	-	Frame shift
		Insertion of "A" at pos 561	-	Frame shift
		ATT→TTT	Ile265-Phe	Substitution

Figure 9.42: Multalin results for LTITM 9 (Polyresistant strain)



Row 1: RV2764C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PAS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: 9_THYA_F- Forward sequence of *M. tuberculosis* isolate showing novel mutations

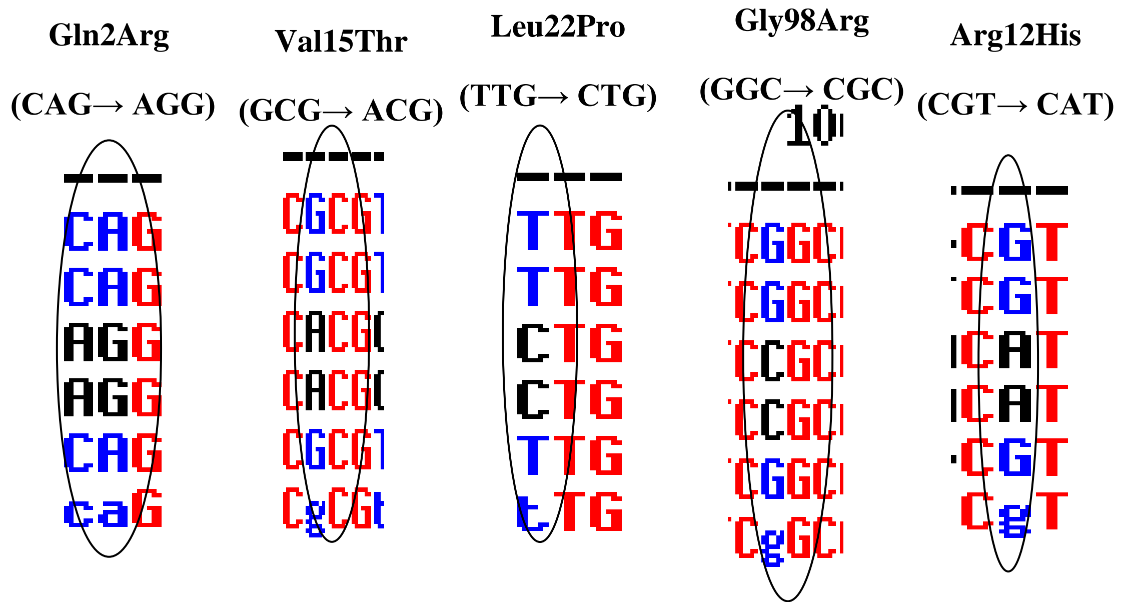
Row 4: 9_THYA_F - Reverse sequence of *M. tuberculosis* isolate showing novel Mutations

Row 5: PAS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

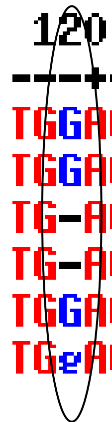
Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.42A: Multalin results for LTITM 9 (Polyresistant strain)

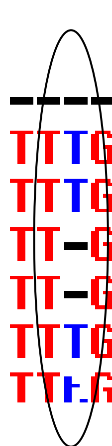
Enlarged image of Figure 9.42



Del of "G" at
Nt pos 119



Del of “T" at
Nt pos 125



Del of “C" at
Nt pos 226

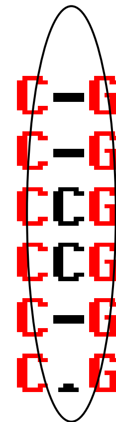
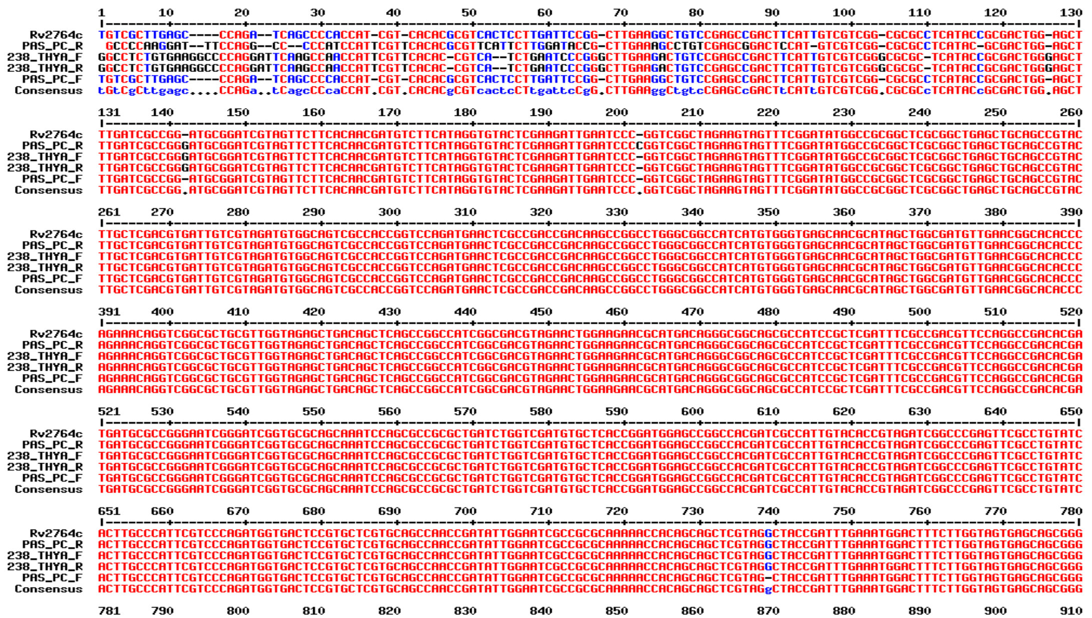


Figure 9.43: Multalin results for LTITM 238 (Polyresistant strain)



Row 1: RV2764C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PAS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: 238_THYA_F- Forward sequence of *M. tuberculosis* isolate showing novel mutations

Row 4: 238_THYA_F - Reverse sequence of *M. tuberculosis* isolate showing novel mutations

Row 5: PAS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.43A: Multalin results for LTITM 238 (Polyresistant strain)

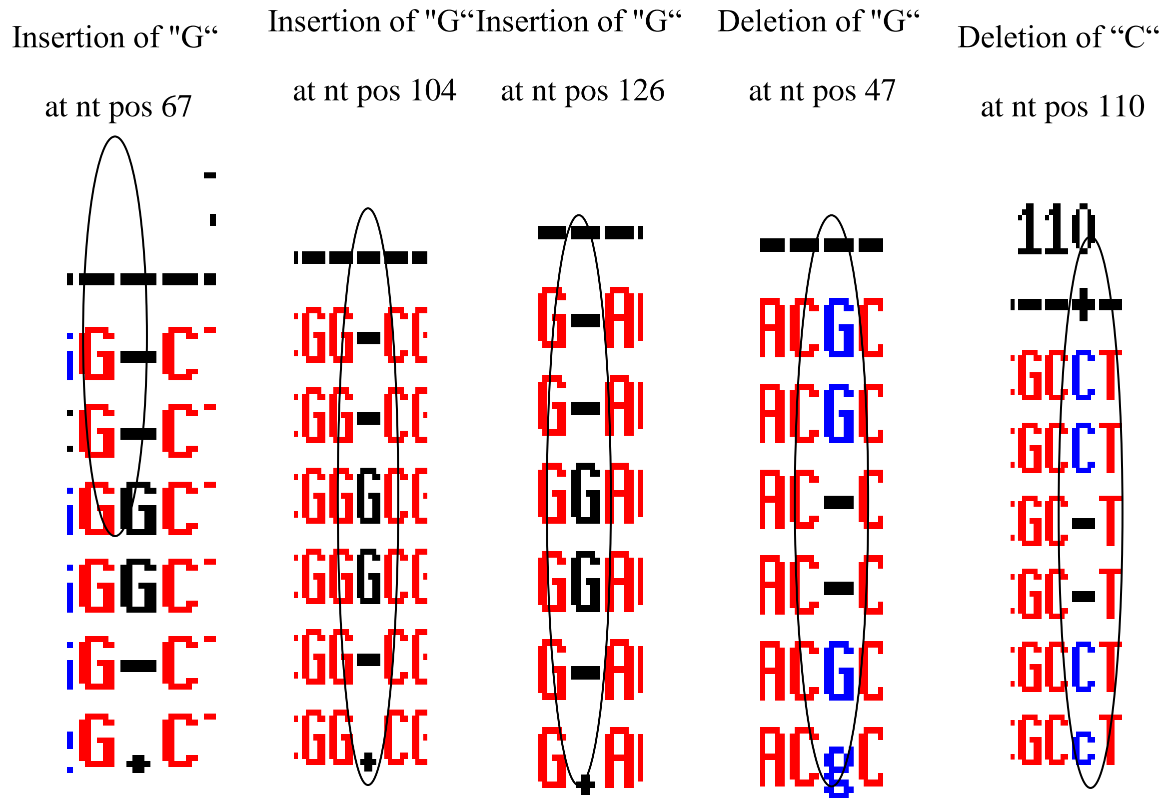
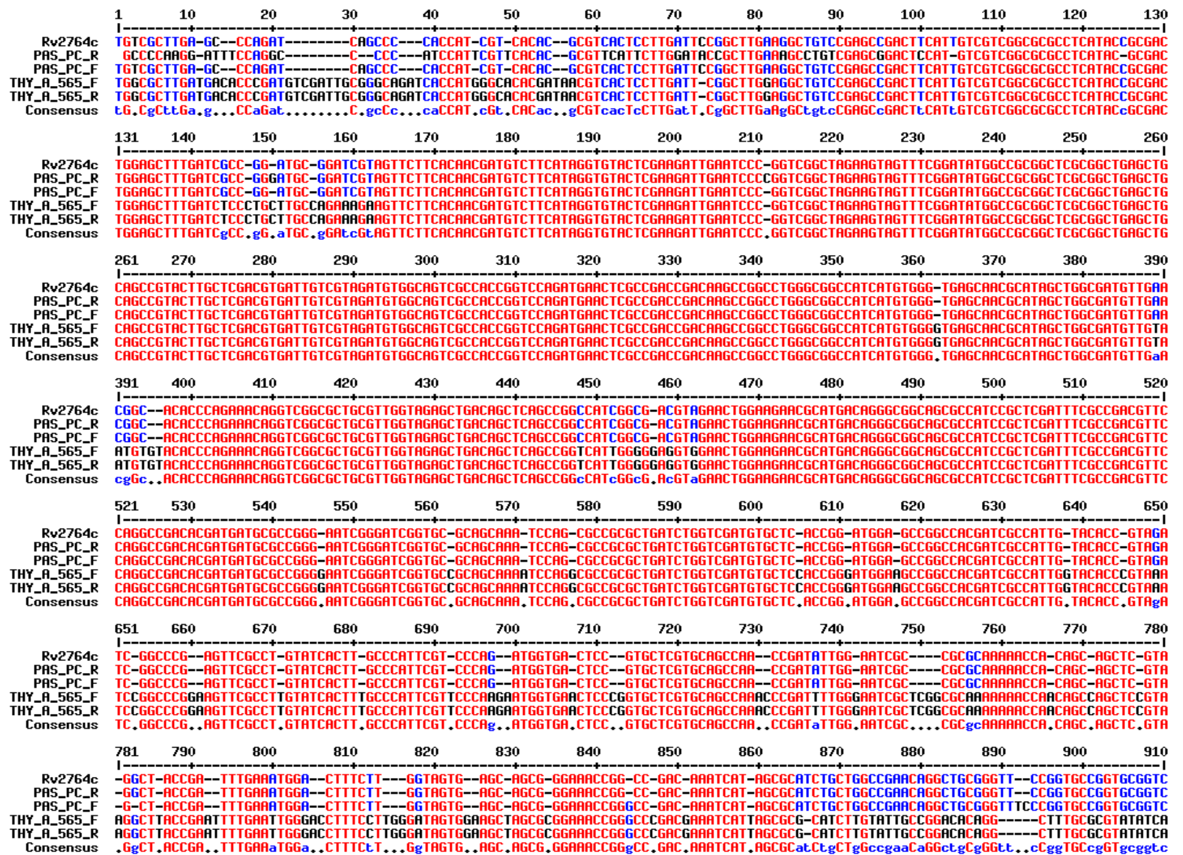


Figure 9.44: Multalin results for LTITM 565 (Polyresistant strain)



The enlarged view is shown in Figure 9.44A

Row 1: RV2764C- Reference sequence of standard strain (*M. tuberculosis H37RV* ATCC DNA) taken from Genbank.

Row 2: PAS_PC_R- Reverse sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

Row 3: THYA_565_F- Forward sequence of *M. tuberculosis* isolate showing novel mutations

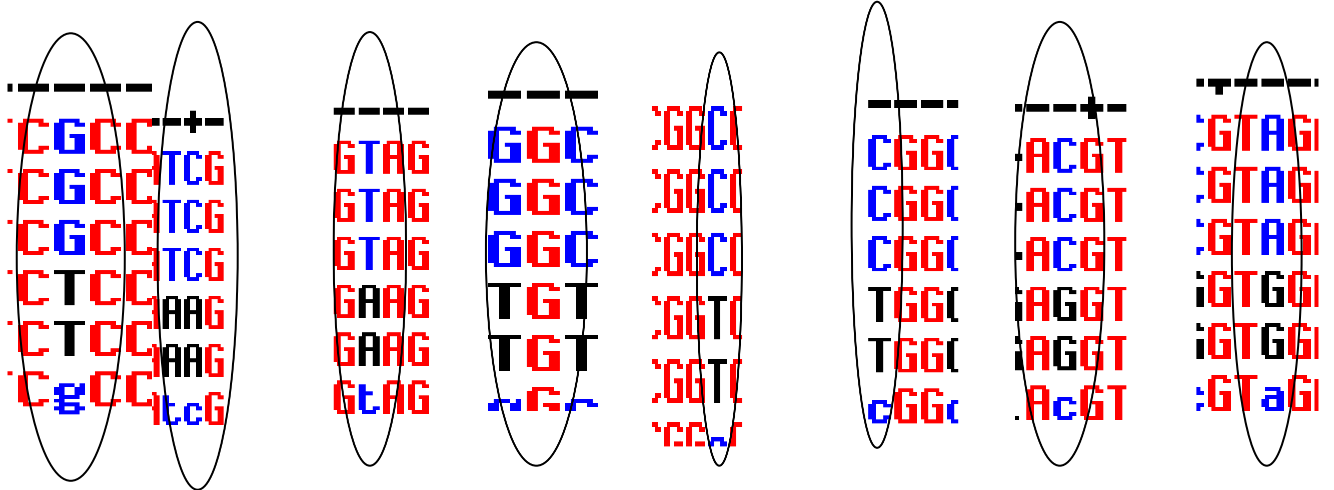
Row 4: THYA_565_F - Reverse sequence of *M. tuberculosis* isolate showing novel mutations

Row 5: PAS_PC_F- Forward sequence of positive control (*M. tuberculosis H37RV* ATCC DNA) Sequence

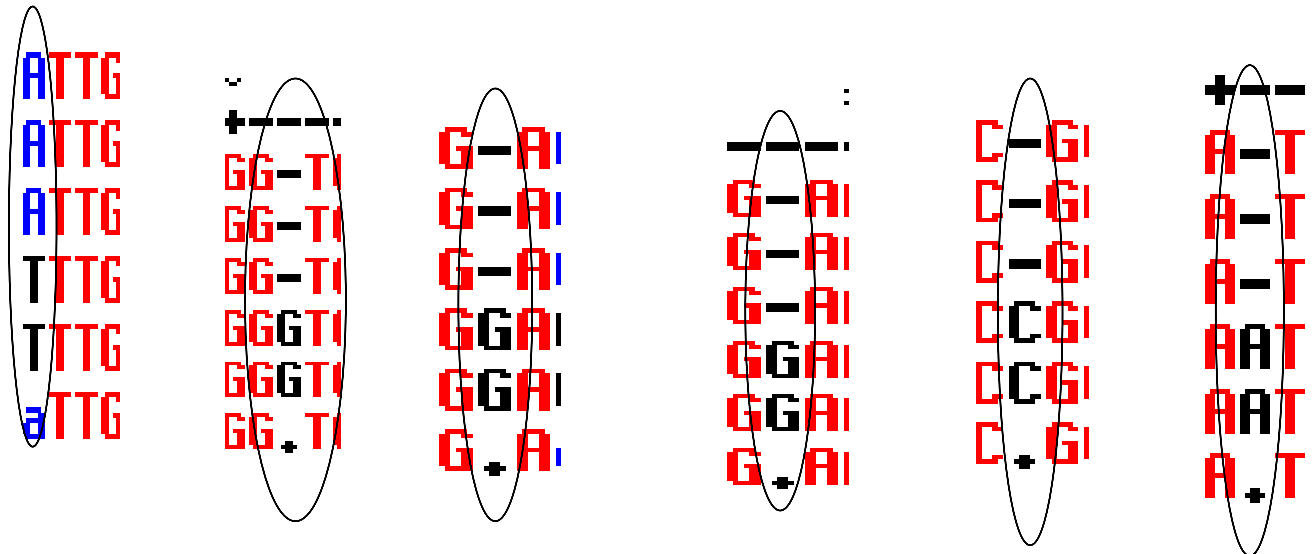
Row 6: Consensus- The sequence showing the consenses of the isolate with the reference strain

Figure 9.44A: Multalin results for LTITM 565 (Polyresistant strain)

Arg44Leu (CGC→CTC) | Ser49Lys (TCG→AAG) | Stop50Lys (TAG→AAG) | Gly126Cys (GGC→TGT) | Gly144Gly (GGC→GGT) | Arg146Trp (CGG→TGG) | Thr148Arg (ACG→AGG)



Ile265-Phe (ATT→TTT) | Ins of “G”at nt pos 348 | Ins of “G”at nt pos 441 | Ins of “G”at nt pos 537 | Ins of “C” at nt pos 552 | Ins of “A”at nt pos 561



9.4 DISCUSSION:

Tuberculosis is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually. Although the initial diagnosis of *M. tuberculosis* often is based on clinical data, definitive diagnosis usually involves the isolation and identification of *M. tuberculosis* in the laboratory. The usual laboratory procedure for clinical specimens involves decontamination and digestion of the specimen, microscopic examination for the presence of acid-fast bacilli (AFB), isolation of the organism by culture, and identification and drug susceptibility testing of the recovered organism. Because of the slow growth rate of Mycobacteria, isolation, identification, and drug susceptibility testing can take several weeks or longer. The rise of multidrug-resistant (MDR) and extensively drug resistant (XDR) TB has drawn attention to the need for rapid diagnosis of drug-resistant TB. During the last decade, major advances in understanding the genetic structure of *M. tuberculosis* have been made. Based on this newer knowledge about the specific gene sequences, several gene probes/gene amplification systems for tuberculosis have been developed.

Drug-resistant *M. tuberculosis* isolates are a serious threat to TB control because only a few effective drugs are available for treatment of this disease. *M. tuberculosis* acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to emergence of MDR-TB and XDR-TB. The present study mainly focuses on the detection of mutations by genotypic method by PCR based DNA sequencing on target genes of second line anti-tuberculous drugs which confers resistance to XDR-TB.

The molecular mechanism of resistance to FQs is the most completely understood of the resistance mechanisms of all the second line drugs used in the treatment of MDR-TB and it has been well established that mutations within the QRDR of *gyrA* and *gyrB* genes occur in 95% or more of FQs-resistant isolates [Campbell et al, 2011]. The most common single nucleotide mutation sites were codons 94, 91, and 90 in *gyrA* gene and, in total, the relative frequencies of these codons were 56.8%, 6.3% and 25.3% respectively [Zhenling et al.,

2009]. In a study from china, [Cheng et al, 2003] reported in all 138 isolates point mutation at codon 95 and Lau et al found in one strain a novel mutation at codon 95 [Lau et al., 2009]. In this study, Ser95Thr polymorphism was observed in *gyrA* gene from both FQs resistant and sensitive strains. In *gyrB* gene the most common mutation was observed at codon 485 and 500 with 11.6% frequency in ofloxacin resistant strains [Feuerriegel et al., 2010]. In the present study, two FQs resistant isolates showed 26 novel substitution mutations, 10 novel silent mutations and 2 novel deletion mutations, whereas in one FQs sensitive strain, there were 19 substitution mutaions, 5 silent mutations and one reported substitution mutations were observed in *gyrB* gene confering resistance to FQs.

It is clear from the literature reviewed that *M. tuberculosis* mutations associated with injectable drug resistance are understudied compared to the mutations associated with resistance to first-line drugs [Johnson et al., 2009]. Many reports suggest that the *tlyA* gene, that is not essential for survival but plays a role in CAP resistance in *M. tuberculosis.* Complementation of a capreomycin-resistant tlyA transposon mutant with *tlyA* gene expressed from an inducible promoter restored capreomycin susceptibility and confirmed the role of the *tlyA* gene product in capreomycin resistance. Many studies also suggested that the upregulation of *eis* gene is found in Kanamycin resistant isolates [Zaunbrecher et al., 2009].Understanding the mechanisms by which increasing levels of Eis affect intracellular growth requires further invest- igation, but the differences in expression levels may have clinical significance.

In the present study, Leu11leu polymorphism was observed in 14 sensitive and 4 resistant strains when targeted *tlyA* gene. Mutations within *tlyA* are uncommon, possibly due to limited and local usage of CAP to treat *M. tuberculosis* infections. The *eis* gene, alternative target for aminoglycoside drug resistance showed 8 novel deletion and 1 insertion mutation in resistant isolates and 5 novel insertion mutation, 3 deletion mutation and 2 substitution mutation in sensitive strains. However, *eis*-mediated resistance is due to promoter mutations that alter *eis* expression levels, and the impact of open reading frame mutations on KAN resistance would potentially require a separate mechanism.

Recently, the *thyA* gene of the folate pathway was shown to be associated with PAS resistance in *M. tuberculosis* [Rengarajan,et al., 2004]. In this study, 3/10 (3%) PAS resistant strains showed 12 novel substitution, 9 insertion, 4 deletion, 2 silent mutations and no mutation was observed in any of the sensitive strains. These results clearly picturises the correlation of PAS resistance with the mutation in the *thyA* gene.

Our finding of 20 different mutations (Substitution-14, Insertion- 4, Deletion- 1, Silent- 1) among 5 ETH resistant isolates combined with the 34 previously described mutations suggests that a high degree of genetic diversity occurs within the *ethA* genes of ETH-resistant *M. tuberculosis* isolates. The fact that no such well-adapted *ethA* mutation has emerged in the ETH-resistant *M. tuberculosis* investigated suggests the existence of one or more enzymes with functional redundancy to ETH. Clearly further study is needed to substantiate the association between *ethA* mutations and ETH resistance and to establish the extent of genetic diversity in this gene. *ethR* gene mutation was observed in 3/10 (3%) ETH sensitive strains.

9.5 SUMMARY

In the present study, detection of mutations by genotypic method by PCR based DNA sequencing on target genes of second line anti-tuberculous drugs which confers resistance to XDR-TB was standardized and applied on 82 polyresistant and 29 MDR-TB strains. Ser95Thr polymorphism was observed in *gyrA* gene from both FQs resistant and sensitive strains, two FQs resistant isolates showed 26 novel substitution mutations, 10 novel silent mutations and 2 novel deletion mutations, whereas in one FQs sensitive strain, there were 19 substitution mutaions, 5 silent mutations and one reported substitution mutations were observed in *gyrB* gene confering resistance to FQs. Leu11leu polymorphism was observed in 14 sensitive and 4 resistant strains when targeted *tlyA* gene. The *eis* gene showed 8 novel deletion and 1 insertion mutation in resistant isolates and 5 novel insertion mutation, 3 deletion mutation and 2 substitution mutation in sensitive strains. 3 PAS resistant strains showed 12 novel substitution, 9 insertion, 4 deletion, 2 silent mutations and no mutation was observed in any of the sensitive strains. Our finding of 20 different mutations (Substitution-14, Insertion- 4, Deletion- 1, Silent- 1) among 5 ETH resistant isolates combined with the 34

previously described mutations suggests that a high degree of genetic diversity occurs within the *ethA* genes of ETH-resistant *M. tuberculosis* isolates.

9.7 CONCLUSION

Genotypic drug resistance by PCR based DNA sequencing targeting drug resistant genes for second line anti-tuberculous drugs were standardized and applied on 111 *M. tuberculosis* isolates. The genotypic results obtained from this study demonstrate the utility of detection of mutations associated with drug resistance to rapidly and accurately determine the drug susceptibility of *M. tuberculosis* isolates. Moreover, the mutations obtained from this study will serve as an epidemiological marker in the local population.

9.8 FUTURE ASPECTS

The significance and characterization of mutations obtained from this study this should be validated by applying on more number of MDR-TB isolates. Further the functional and structural analysis has to be done on the drug resistant *M. tuberculosis* isolates using Bioinformatics tools.

9 CONCLUSIONS

- In the first objective, nRT-PCRs targeting mRNA of *icl_2, hspx and rRNAP1* genes were optimized and applied on 233 (203- from clinically suspected tuberculosis patients and 30- control) clinical specimens. applied on the same 203 sputum specimens, 111 (54.6%) were positive for *icl_2* gene, followed by 107 (52.1%) for *hspx* gene and 80 (39.1%) for *rRNAP1* genes. When comparing the 3 nRT-PCRs results with that of the BACTEC culture, *icl_2*, *hspx* and *rRNAP1* genes were able to pick up 63.9%, 67.2% and 58.75% more culture negative sputum specimens respectively. All the 30 control specimens negative for both culture and 3 nRT-PCRs included in the study. Thus combinations of indigenously standardized nRT-PCRs is definitely a useful contribution for development of a rapid molecular diagnostic test for specific and rapid detection of actively multiplying *M. tuberculosis* genome directly from clinical specimens.

- The second objective was detection of drug resistance for first line anti-tuberculous drugs by phenotypic micro MGIT BACTECculture method. Out of 326 *M. tuberculosis* isolates from new cases of clinically suspected tuberculosis patients included in this study, 18(5.5%) were MDR-TB strains, 37 (11.3%) were polyresistant strains and 53 (16.2%) were monoresistant strains to first line anti-tuberculosis drugs.

- The third objectine was application of PCR based DNA sequencing targeting drug resistance genes for the first line antituberculous drugs on 242 *M. tuberculosis* isolates. Among 326 isolates analysed, no mutation in *rpoB* gene were observed in RIF resistant strains. In case of INH resistant strains, 1 strain showed novel mutation at codon 149 in *oxyR-aphC* gene. Regarding *embB* gene for EMB resistance, no novel mutations were observed in resistant strains. In 2 PZA resistant strains, Pro54Ser novel substitution mutation was observed in *pncA* gene. In case of STR resistant strains,, 5 novel substitution mutations (Pro75Leu, Leu59Arg, Trp33Cys, Thr93Pro, Ile114Thr) and 1 deletion of nucleotide "C" at position 105 were observed. There were also 3 reported mutations detected (Val110Val, Arg20Arg 2 and 1 substitution mutation - Leu16Arg) in 24 STR resistant strains.

- The fourth objective of the study was focused on the bioinformatics analysis of one single strain with the novel mutation at Ala102Pro detected in the putative active site (Lys96-

Tyr103) region of PZase. The SNP anlaysis by I-mutant server showed that the stability of the protein was affected with negative Gibbs free energy value -1.10 at pH 5.5 and 37ºC. In addition, the level of activity displayed by the *PncA* mutants seems to be well correlated with the location of the mutated residues and the structural modifications they can cause in the vicinity of the putative active-site cysteine residue found at position 138.

- The fifth objective was to study the prevalence of XDR-TB circulating in the study population by phenotypic drug susceptibility testing for second line drugs by micro MGIT BACTEC system. In this study, 10 (2.4%) of *M. tuberculosis* strains were detected to be XDR-TB strains, among 402 *M. tuberculosis* isolates by phenotypic method.

- The sixth objective was to detect mutation in second line drug resistance genes which leads to emergence of XDR-TB PCR based DNA sequencing. Two XDR-TB strains showed Ser95Thr polymorphism in *gyrA* gene. In case of *gyrB* gene, 21 novel substitution mutations were observed in 1 XDR-TB strain. There were no novel mutations observed in *tlyA* gene. The *eis* gene showed 8 novel deletion and 1 insertion mutation in 2 resistant isolates. In case of *thyA* gene, 3 PAS resistant strains showed 12 novel substitution, 9 insertion, 4 deletion mutations. In case of 2 ETH resistant strains, 20 different novel mutations (Substitutions-14, Insertions-4, Deletions- 1) were observed in *ethA1* gene, but there were no mutations observed in *ethA2* and *ethA3* genes.

10. SPECIFIC CONTRIBUTIONS OF THE PRESENT STUDY

- The Nested Reverse transcriptase PCRs (nRT-PCRs) targeting mRNA of *icl*$_{2}$, *hspx* and *rRNAP1* genes were optimized with indigenously designed primers using Primer Blast Software and successfully applied for the rapid detection of actively multiplying (viable) *M. tuberculosis* directly from clinical specimens. This is the first study in India, reporting on rapid and sensitive molecular diagnostic tests (Nested Reverse transcriptase PCR targeting 3 gene of *M. tuberculosis*). It will be an excellent new addition to the armamentarium of molecular diagnostic test to detect actively multiplying *M. tuberculosis* directly from clinical specimens**.**
- The Phenotypic drug susceptibility testing for the second line anti-tuberculous drugs by Micro MGIT BACTEC method to detect XDR-TB was optimized for the first time in a private laboratory (L&T Microbiology Research Centre) in Chennai. The prevalence of XDR-TB was 2.4% in this study in Chennai population. PCR based DNA sequencing targeting *gidB* gene for the detection of Streptomycin resistance was standardized and 5 novel mutations were detected.

- PCR based DNA sequencing targeting the drug resistant genes for the 8 second line anti-tuberculous drugs were optimized for the first time in L&T Microbiology Research Centre and applied on 111 *M. tuberculosis* isolates. Novel mutations targeting *gyrA*, *gyrB* genes for Fluoroquinolones, *eis* gene using *indigenously* designed primers for Aminoglycosides, *thyA* gene for Para aminosalicylic acid and *ethA* gene for Ethionamide were detected. The mutations detected could be used as a molecular markers for the early detection of XDR-TB strains circulating in Chennai population. This is the first report from Chennai reporting on the genotypic DST method for the second line anti-tuberculous drugs.

- An important contribution in this study is the standardization and application of bioinformatic tool to study the functional variation and to understand the effect of novel mutation by studying the crystal structure of pyrazinamidase enzyme in *M. tuberculosis* at molecular level in a PZA resistant strain. The study represents a valuable tool to decipher the structure-function relationships and investigate the molecular mechanisms of resistance to PZA.

11. FUTURE SCOPE OF WORK

- The optimized nRT-PCRs targeting mRNA of *icl*$_2$, *hspx* and *rRNAP1* genes developed in the present study along with mRNA of *85B* gene (optimized earlier in L&T Microbiology Research centre) can be utilized to develop a rapid diagnostic tool in a diagnostic kit format for the early detection of actively multiplying *M. tuberculosis* directly from clinical specimens

- The significance and characterization of novel mutations obtained by PCR based DNA sequencing technique should be validated by including more number of MDR-TB/XDR-TB isolates. Further the functional and structural analysis of the mutations need to be carried out on the drug resistant *M. tuberculosis* isolates using Bioinformatics tools.

- The signature sequences obtained in this study will result in generating the useful data to develop suitable diagnostic kit for detection of drug resistant *M. tuberculosis* strains (directly from clinical specimens). This study can be further extended to larger population covering different geographical locations in India to develop a kit for the detection of drug resistant TB rapidly in Indian population.

- The whole genome sequencing of the drug resistant *M. tuberculosis* strains will aid in better understanding of the mutation patterns conferring drug resistance and provides an insights towards exploring the molecular epidemiology of MDR-TB/XDR-TB in a particular geographical location and in developing newer drug targets.

REFERENCES:

- Ananthanarayanan R and Jayaram Paniker CK. Textbook of Microbiology, Orient Lomgman, 6th edition, 2000, 324-326.
- Adolf K, Edna D , Oti K . Evaluation of cost-effective total nucleic acids extraction protocols for cultured *Mycobacterium tuberculosis*; a comparison by PCR amplification of genes associated with drug resistance. *BMC Research Notes*.2010; 3:48.
- Angeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J Clin Microbiol* 2002; 40: 553–555.
- Almeida D, Rodrigues C, Udwadia F, Lalvani A, Gothi GD et al. Incidence of Multidrug tuberculosis strains from urban and rural India and Implications for prevention. *Clin Infect Dis*. 2003; 36 (12): 152-154.
- Aleyamma T, Rajeswari Ramachandran, Fathima Rehaman, K. Jaggarajamma, T. Santha , N. Selvakumar, Nalini Krishnan, Nalini Sunder Mohan, V. Sundaram, Fraser Wares and P.R. Narayanan. Management of multi drug resistance tuberculosis in the field:Tuberculosis research centre experience. *Indian J Tuberc.* 2007: 54: 117-124.
- Albert H, Trollip A, Seaman T, Mole RJ. Simple, phage based (FASTPplaque) technology to determine rifampicin resistance of *Mycobacterium tuberculosis* directly from sputum. *Int J Tuberc Lung Dis* 2004; 8: 1114–1119.
- Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL, Manavathu, EK, & Lerner SA. Mechanisms of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*. 1998; 42(5):1295-7.
- Al-Sharrah YA. The Arab Tradition of Medical Education and its Relationship with the European Tradition. *Springer* 2003; 33(4):413-25.
- Accelrys Software Inc., Discovery Studio Modeling Environment, Release 3.1, San Diego: Accelrys Software Inc.2011.
- Almeida Da Silva PE, & Palomino JC. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *The Journal of Antimicrobial Chemotherapy* 2011; 66(7), 1417-30.
- Agarwal M, Selami Gunal, Riza Durmaz and Zhenhua Yang. Integration of *Mycobacterium tuberculosis* Drug Susceptibility Testing and Genotyping with Epidemiological Data Analysis to Gain Insight into the Epidemiology of Drug-Resistant tuberculosis in Malatya, Turkey. *Journal of Clinical Microbiology*. 2010; 48 (9): 3301–3305

- Braslavsky , Hebert B, Kartalov E. & Quake SR. Sequence information can be obtained from single DNA molecules. *Proc. Natl. Acad. Sci. USA.*2003; 100: 3960–3964.
- Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, & De Lisle G. Jacobs WR. Jr. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science.*1994; 263(5144): 227-30.
- Balaji V, Daley P, Anand A, Sudarsanam T, Michael JS, et al. Risk Factors for MDR and XDR-TB in a Tertiary Referral Hospital in India. *PLoS ONE* .2010; 5 (3), e9527.
- Bwanga F, Hoffner S, Haile M, Joloba ML. Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. *BMC Infect Dis*. 2009; 9:67.
- Banerjee R, Schecter GF, Flood J, Porco TC. Extensively drug-resistant tuberculosis: new strains, new challenges. *Exp. Rev. Anti- Infect. Ther*.2008; 6:713–724.
- BD Diagnostics. BACTEC ™ micro MGIT system product and procedure manual.
- Benkert P, Künzli M, Schwede. QMEAN server for protein model quality estimation. *Nucleic Acids Res* 37(Web Server issue):W510-4. 2009; PubMed PMID: 19429685; PubMed Central PMCID: PMC2703985.
- Brzostek A, Sajduda A *et al*. “Molecular characterisation of streptomycin-resistant *Mycobacterium tuberculosis* strains isolated in Poland”. *Int J Tuberc Lung Dis*. 2004; 8: 1032–1035.
- Bergmann JS & Woods GL .Clinical evaluation of the BDProbe-Tec strand displacement amplification assay for rapid diagnosis of tuberculosis. *J Clin Microbiol*. ;1998 36: 2766–2768.
- Bonah C *et al*. The experimental stable of the BCG vaccine: safety, efficacy, proof, and standards, 1921–1933. *Stud Hist Philos Biol Biomed Sci* 2005; 6(4):696-721.
- Boehme CC, Nabeta P, Henostroza G et al. Operational feasibility of using loop-mediated isothermal amplification (LAMP) for the diagnosis of pulmonary TB in microscopy centers of developing countries. *J Clin Microbiol.* 2007; 45: 1936–1940.
- Bermudez LE and Goodman J. *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. *Infect. Immun*. 1996; 64:1400–1406.
- Beauty Makamure, Jesca Mhaka, Salome Makumbirofa, Reggie Mutetwa, Lucy Mupfumi, Peter Mason John Z. Metcalfe. Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of Drug-Resistant Tuberculosis in Harare, Zimbabwe. Plos one. 2013; 8(5): 55872.
- Bodnar KA, Serbina NV, and Flynn JL. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect. Immun*. 2001; 69:800–809.

- Benjamin WH, Waites KB, Beverly A, Gibbs L, Waller M, Nix S, Moser SA. et al.. Comparison of the MB/BacT system with a revised antibiotic supplement kit to the BACTEC 460 system for detection of mycobacteria in clinical specimens. *J. Clin. Microbiol*. 1998; 36:3234–3238.
- Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, & De Lisle G. Jacobs WR Jr. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science.* 1994; 263(5144): 227-30.
- Behera D. Issues in the management of drug resistant tuberculosis in India. *Lung India*.2013; 30(4): 269-272.
- Chang KC, Yew WW, Chan RCY. Rapid assays for fluoroquinolone resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *The Journal of Antimicrobial Chemotherapy* .2010; 65(8), 1551-61.
- Collee JG, Marmion BP, Fraser AG, Simmons A, Mackie and Mc.Cartney. Medical Microbiology, 2000; 14th edition; 328-333
- Chaisson MJ, Brinza D & Pevzner PA. *De novo* fragment assembly with short mate-paired reads: does the read length matter? *Genome Res.*2009; 1: 336–346.
- Collee JG, Marmion BP, Fraser AG, Simmons A. Mackie and Mc.Cartney Medical Microbiology, 14th edition; 2002; 328-333.
- Crevel R, Ottenhoff TH, and. van der Meer JW. Innate immunity to *Mycobacterium tuberculosis. Clin. Microbiol. Rev*. 2002; 15:294–309.
- Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucl Acids Res* 1988; 16:10881-10890.
- Cheng AF et al. Multiplex PCR amplimer conformation analysis for rapid detection of gyrA mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob. Agents Chemother*. 2004; 48:596– 601.
- Chandrasekaran S, Jagota P, Chaudhuri K. Initial drug resistance to anti - tuberculosis drugs in urban and rural district tuberculosis programme. *Indian J Tuberc* 1992; 39: 171-5.
- Cooksey RC, Morlock GP, Mcqueen A, Glickman SE, & Crawford JT. Characterization of streptomycin resistance mechanisms among *Mycobacterium tuberculosis* isolates from patients in New York City. *Antimicrobial Agents and Chemotherapy.*1996; 40(5): 1186-8.
- Dietze R, Teixeira L, Rocha LM, Palaci M, Johnson JL, Wells C *et al*. Safety and bactericidal activity of rifalazil in patients with pulmonary tuberculosis. *Antimicrob. Agents Chemother*.2001; 45:1972–1976.

❖ Du X, Wang W, Kim R, Yakota H, Nguyen H, et al. Crystal structure and mechanism of catalysis of a pyrazinamidase from *Pyrococcus horikoshii*. *Biochemistry* .2001; 40:14166–141672.

❖ Da Silva CH, Campo VL, Carvalho I, Taft CA. Molecular modeling, docking and ADMET studies applied to the design of a novel hybrid for treatment of Alzheimer's disease. *J Mol Graph Model.*2006; 25: 169-175.

❖ Daum LT et al. Genetic analysis of *Mycobacterium tuberculosis* drug-resistance genes from samples shipped in Prime Store MTM and sequenced using the next-generation Ion Torrent. *Eur. Soc. Paediatr. Infect. Dis.* 2012; 8:1-12.

❖ Dannenberg AM, Rook JA. Pathogenesis of pulmonary tuberculosis: interplay of tissue-damaging and macrophage-activating immune responses. Dual mechanisms that control bacillary multiplication, *American Society for Microbiology*, Washington, D.C. 1994; 459–483.

❖ De Beenhouwer H, Lhiang Z, Jannes G, Mijs W, Machtelinckx L, Rossau R, et al. Rapid detection of rifampin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. *Tuber Lung Dis* 1995; 76:425–30.

❖ Dalmer O, Walter E. Verfahren zur Herstellung von Derivaten der Pyrazinmonocarbonsaure. *DRP*. 1936; 623: 257.

❖ Douglass J & Steyn LM. A ribosomal gene mutation in streptomycin-resistant *Mycobacterium tuberculosis* isolates. *The Journal of Infectious Diseases* 1993; 167(6): 1505-6.

❖ Doustdar F, Khosravi AD, Farnia P. *Mycobacterium tuberculosis* genotypic diversity in pyrazinamide-resistant isolates of Iran. *Microb Drug Resist*. 2009; 15: 251–256.

❖ Datta BS, Hassan G, Kadri SM, Quereshi W, Kamili MA, Singh H, et al. Multidrug-Resistant and extensively drug resistant tuberculosis in Kashmir, India. *J. Infect. Dev.Ctries.* 2010; 4(1), 019-023.

❖ Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 2004; 14:1394–1403.

❖ Deepak Almeida, Camilla Rodrigues, Tester F Ashavaid, Ajit Lalvani, Zarir F Udwadia. High incidence of the Beijing genotype among multidrug-resistant isolates of *Mycobacterium tuberculosis* in a tertiary care center in Mumbai, India. *Clinical infectious disease*. 2005; 40, 881-886.

❖ Davies J and Wright GD. Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol*, 1997; 5:234–240.

❖ Debarber AE, Mdluli K, Bosman M, Bekker LG, & Barry CE. Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America.* 2000; 97(17): 9677-82.

❖ Eid J. *et al.* Real-time DNA sequencing from single polymerase molecules. *Science*.2009: 32: 133–138.

❖ Erlich Y, Mitra P, delaBastide M, McCombie W R. & Hannon, G. J. Alta-Cyclic: a self-optimizing base caller for next-generation sequencing. *Nature Methods.* 2008: 5: 679–682.

❖ Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, et al . Comparative protein structure modeling using MODELLER. *Curr Protoc Protein Sci.* Chapter 2007; 2: Unit 2.9. doi: 10.1002/0471140864.ps0209s50. PubMed PMID: 18429317.

❖ Flusberg BA, Webster DR. Lee JH. et al., "Direct detection of DNA methylation during single-molecule, realtime sequencing," *Nature Methods*. 2010; 7:461–465.

❖ Fleischmann RD, Alland D, Eisen JA et al. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J Bacteriol* 2002; 184: 5479–5490.

❖ Fenton MJ and Vermeulen MW. Immunopathology of tuberculosis: roles of macrophages and monocytes. *Infect. Immun*. 1996; 64:683–690.

❖ Frieden TR, Khatri GR. Multi-drug resistant tuberculosis.In: Narain JP, editor. Tuberculosis epidemiology and control. WHO/SEA/TB/248.New Delhi: World Health Organization Regional Office for South-East Asia; 2002 p.105-15.

❖ Freeman WM, Walker SJ, Vrana KE. "Quantitative RT-PCR: pitfalls and potential". *BioTechniques*. 1999; 26 (1): 112–22, 124–5.

❖ Freeman WM, Vrana SL, Vrana KE. Use of elevated reverse transcription reaction temperatures in RT-PCR. *Biotechniques*. 1996; 20: 782-783.

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

❖ Fehniger TA, Wylie TE, Germino et al., "Next-generation sequencing identifies the natural killer cell microRNA transcriptome," *Genome Research*.2010; 2:1590–1604.

❖ Feuerriegel S, Cox HS, Zarkua N, Karimovich HA, Braker K, et al. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-

resistant tuberculosis patients undergoing treatment. *Antimicrob Agents Chemother*. 2009; 53: 3353–3356.

- Fedurco M, Romie, A, Williams S, Lawrence I. & Turcatti G. BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies. *Nucleic Acids Res.*2006; 34: e22.
- Gingeras et al. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Mycobacterium DNA arrays. *Genome Research*. 1998; 8:435-448.
- Goto M, Oka S, Okuzumi K, Kimura S, Shimada K. Evaluation of acridinium-ester-labeled DNA probes for identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* complex in culture. J Clin Microbiol 1991;29:2473–6.
- Ginsburg AS et al. Fluoroquinolone resistance in patients with newly diagnosed tuberculosis. *Clin. Infect. Dis*. 2003; 37:1448–1452.
- Gonzalez-Juarrero M, and. Orme IM. Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis. Infect. Immun*. 2001; 69:1127–1133.
- Global tuberculosis control 2013. Geneva, World Health Organization, 2013 (WHO/HTM/TB/2010.7).
- Gale EF, Cundliffe E, Reynolds PE Richmond MH, & Waring MJ. The molecular basis of antibiotic action. John Wiley and Sons, Inc., *New York.* 1981.
- Gunisha P, Madhavan HN, Jayanthi U et al. Polymerase chain reaction using IS-6110 Primer to detect *M tuberculosis* in clinical samples. Indian *J Pathol Microbiol*. 2001; 44:97-102.
- Hickman SP, Chan J, and Salgame P. *Mycobacterium tuberculosis* induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization. *J. Immunol*. 2002; 168:4636–4642.
- Hu Y, Movahedzadeh F, Stoker NG, and Coates AR. Deletion of the *Mycobacterium tuberculosis* alpha-crystallin-like *hspX* gene causes increased bacterial growth in vivo. *Infect. Immun*.2006; 74:861–868.
- Hutchison CA. III. DNA sequencing: bench to bedside and beyond. *Nucleic Acids Res.* 2006; 35: 6227–6237.
- Hopewell PC, Pai M, Maher D, et al International standards for Tuberculosis care; *Lancet Infect. Dis.* 2006; 6:710–725.

- Hellyer TJ, DesJardin LE, Hehman GL, Cave MD, and Eisenach KD. Quantitative analysis of mRNA as a marker for viability of *Mycobacterium tuberculosis*. *J. Clin. Microbiol*. 1999; 37:290–295.
- Heydenrych A, Brookes R et al. Performance of a rapid phage-based test, FASTPlaqueTB, to diagnose pulmonary tuberculosis from sputum specimens in South Africa. *Int J Tuberc Lung Dis* 2002; 6: 529–537.
- Heym B, Alzari PM, Honore N, & Cole ST. Missense mutations in the catalase peroxidase gene, *kat*G, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Molecular Microbiology*. 1995; 15(2):235-45.
- Hirano K, Abe C, Takahashi M. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J. *Clin. Microbiol*. 1999; 37: 2663–2666
- Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. 1999; 41:95-98.
- Hess B, Kutzner C, van der Spoel D, Lindahl E. GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J Chem Theory Comp*.2008; 4:435–447.
- Huse SM, Huber JA, Morrison HG *etal*, “Accuracy and quality of massively parallel DNA pyrosequencing,” *Genome Biology.* 2007; 8: 143.
- Hazbón MH, Brimacombe, M, Bobadilla del Valle, M, Cavatore, M, Guerrero, M. I, Varma-Basil, M, Billman-Jacobe, H, Lavender, C, Fyfe, J, García-García, L, León, C. I, Bose, M, Chaves, F, Murray, M, Eisenach, K. D, Sifuentes-Osornio, J, Cave, M. D, Ponce de León, A, & Alland, D. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy.* 2006; 50(8): 2640-9.
- Indian Council of Medical Research. Prevalence of drug resistance in patients with pulm onary tuberculosis presenting for the first time with symptoms at chest clinics in India. I: Findings in urban clinics among patients giving no history of previous chemotherapy. *Indian J Med Res 1968;* 56: 1617-30.
- Joseph P, Vijaya Bhaskara Rao Desai, Nalini Sunder Mohan, Jemima Sheila Fredrick, Rajeswari Ramachandran, Balambal Raman et al. Outcome of standardized treatment for patients with MDR-TB from Tamil Nadu, India. *Indian J Med Res,* 2011; 133: 529-534.
- Jain NK, Agnihotri, krishan. Sharma, et al. Extensively drug resistance (XDR- TB) is not always fatal. *Indian j tuberc*. 2009; 56, 48-50.

- Jackett PS, Bothamley GH, Batra HV, Mistry A, Young DB, and J. Ivanyi J. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. *J. Clin. Microbiol.* 1988; 26:2313–2318
- Kempsell KE, Ji Y, Estrada G, Colston CE, Cox RA. The nucleotide sequence of the promoter, 16s rRNA and spacer region of the ribosomal RNA operon of *Mycobacterium tuberculosis* and comparison with *Mycobacterium leprae* precursor rRNA. *J Gen Microbiol*. 1992; 138: 1717-1 727.
- Katoch VM, Sharma VD.Advances in the diagnosis of mycobacterial diseases. *Indian J Med Microbiol*. 1997; 15:49-55.
- Kapur V, Li L-L, Hamrick MR, Plikaytis BB, Shinnick TM, Telenti A, et al. Rapid Mycobacterium species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. *Arch Pathol Lab Med* 1995; 119:131–8.
- Katoch VM, Sharma VD. Advances in the diagnosis of mycobacterial diseases. *Indian J Med Microbiol.* 1997; 15:49-55.
- Kent TK, Kubica GP. Public Health Mycobacteriology. A guide for the level III laboratory Atlanta, center for diseases control, 1985.
- Krishnaswamy KV, Rahim MA. Primary drug resistance in pulmonary tuberculosis. *Indian J Chest Dis* 1976; 28: 233-7.
- Laura E, Sang Nae Cho. *et al*. “Polymorphisma associated with resistance and cross-resistance to aminoglycosides and capreomycin in *Mycobacterium tuberculosis* isolates from South Korean patients with drug resistant tuberculosis*”. Journal of clinical microbiology*.2010;48(2):402-411
- Lee BY, Hefta SA, and Brennan PJ. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis. Infect. Im mun.* 1992; 60:2066–2074.
- Lipscomb MF and Masten BJ. Dendritic cells: immune regulators in health and disease. *Physiol. Rev.* 2002; 82:97–130.
- Li L, Mahan CS, Palaci M, Horter L, Loeffelholz L, Johnson JL, Dietze R *et al*. Sputum *Mycobacterium tuberculosis* mRNA as a Marker of Bacteriologic Clearance in Response to Antituberculosis Therapy. *Journal of Clinical Microbiology*.2010; 46–51.
- Leung KL, Yip CW, Yeung YL, Wong KL, Chan WY, Chan MY, & Kam KM. Usefulness of resistant gene markers for predicting treatment outcome on second-line anti-tuberculosis drugs. *Journal of Applied Microbiology* .2010; 109(6): 2087–94.

❖ Leung ET, Ho PL, Yuen KY, Woo WL, Lam TH, Kao RY, Seto WH, & Yam WC. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. *Antimicrobial Agents and Chemotherapy* 2006; 50(3): 1075-8.

❖ Lemaitre N, Sougakoff W, Pernot CT, Jarlier V. Characterization of New Mutations in Pyrazinamide-Resistant Strains of *Mycobacterium tuberculosis* and Identification of Conserved Regions Important for the Catalytic Activity of the Pyrazinamidase *pncA*. *J Antimicrob. Chemother.* 1999; 43:1761–1763.

❖ Lemaitre N, Callebaut I, Frenois FD, Jarlier F, Sougakoff W. Study of the structure–activity relationships for the pyrazinamidase (*pncA*) from *Mycobacterium tuberculosis. Biochem. J*. 2001; 353: 453-458.

❖ Landegren U, Kaiser R, Sanders J & Hood L. A ligase-mediated gene detection technique. *Science*. 1998; 24: 1077–1080.

❖ Levene MJ. et al. Zero-mode waveguides for singlemolecule analysis at high concentrations. *Science*. 2003; 29: 682–686.

❖ Lau WT, Pak-Leung Ho, Richard Y. T. Kao Wing-Wai Yew, Terrence C. K. Lau, Vincent C. C. Cheng. Molecular Characterization of Fluoroquinolone Resistance in *Mycobacterium tuberculosis*: Functional Analysis of *gyrA* Mutation at Position 74. *antimicrobial agents and chemotherapy*. 2011; 55(2): 608–614.

❖ Mehta PK, King CH, White EH, Murtagh JJ, and Quinn FD. Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. *Infect. Immun*. 1996; 64:2673–2679.

❖ McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, *et al.* Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000; 406: 735-738.

❖ Muñoz-Elías EJ, McKinney JD. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nat Med* 2005; 11: 638-644.

❖ McFadden JJ, Kunze Z, Seechum P. DNA probes for detection and identification. In: McFadden J editor, Molecular Biology of the Mycobacteria, Surrey niversity Press, UK. 1990; 139-72

❖ Mardis ER. “The impact of next-generation sequencing technology on genetics,” *Trends in Genetics*.2008; 2:133–141.

❖ Michos AG, Daikos GL, Tzanetou K, Theodoridou M, Moschovi M, Nicolaidou P, Petrikkos G, Syriopoulos T, Kanavaki S & Syriopoulou VP (2006) First detection of *Mycobacterium*

tuberculosis DNA in respiratory and nonrespiratory specimens by the Amplicor MTB PCR. *Diagn Micr Infec Dis* 54: 121–126.

- Maurya AK, AK Singh AK, Kumar M, Umrao J, S Kant S, VL Nag VL, RAS Kushwaha RAS, TN Dhole TN. Changing patterns and trends of multidrug-resistant tuberculosis at referral centre in Northern India: A 4-year experience. *Ind J Med Res.* 2013; 31(1):40-46.
- Morlock GP, Crawford JT, Butler WR, Brim SE, Sikes D, et al. Phenotypic characterization of *pncA* mutants of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*.2000; 44:2291-5.
- Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, et al. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem.* 1998; 19:1639–62.
- Metzker ML. Emerging technologies in DNA sequencing. *Genome Res.* 2005; 15:1767–1776.
- Masjedi MR, Farnia P, Sorooch S, et al. Extensively drug-resistant TB—two years of surveillance in Iran. *Clin. Infect. Dis*. 2006; 43, 841-847.
- Morgan M, Kalantri S, Flores L, Pai M. A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: A systematic review and meta-analysis. *BMC Infect Dis*. 2005; 5, 62.
- Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* .1985; 66(3): 219-25.
- Maus CE, Plikaytis BB, and Shinnick TM. Mutation of *tlyA* confers Capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*, 2005; 49:571-577.
- Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT e tal. Toll-like receptor 2-dependentinhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J. Immunol*. 2001;167:910–918.
- Neufert C, Pai RK, Noss EH, Berger M, Boom WH, and Harding CV. *Mycobacterium tuberculosis* 19-kDa lipoprotein promotes neutrophil activation. *J. Immunol.* 2001; 167:1542–1549.
- Nathanson E, Lambregts-van Wezenbeek C, Rich ML, et al. Multidrug-resistant tuberculosis management in resourcelimited settings*; Emerg. Infect. Dis.*2006; 12:1389–1397.
- Narang P, Nayyar S, Mendiratta DK, Tyagi NK, Jajoo UN. Smear and culture positive cases of tuberculosis found among symptomatics surveyed in Wardha district. *Indian J Tuberc 1992;* 39: 159-63.

- Pfyffer GE, Welscher HM, Kissling P, et al. Comparison of the Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid culture for recovery of acid-fast bacilli. *J Clin Microbiol* 1997; 35: 364–368.
- Perdigao J, Macedo R, Ribeiro A, Brum L, & Portugal I. Genetic characterisation of the ethambutol resistance-determining region in *Mycobacterium tuberculosis*: prevalence and significance of *embB306* mutations. *International Journal of Antimicrobial Agents*. 2009; 33(4): 334-8.
- Paramasivan CN, Venkataraman P , ChandrasekaranV,Bhat S, Narayanan PR. Surveil lance of drug resistance tuberculosis in two districts of south India. *Int J Tuberc Lung Dis* 2002*;* 6: 479-84.
- Petrella S, Ziental NG, Maudry A, Laurans C, Boudjelloul R, et al. Crystal Structure of the Pyrazinamidase of *Mycobacterium tuberculosis*: Insights into Natural and Acquired Resistance to Pyrazinamide. *PLoS ONE*.2011; 6:e15785. doi:10.1371/journal.pone.0015785, 2011.
- Pooja Singla., Rajvir Singh., Madhu Sharma., Aparna and Uma Chaudhary., 2014. Extensively Drug Resistant Tuberculosis: A Mini Review. *Int.J.Curr.Microbiol.App.Sci*. 2014; 3(1), 219-234.
- Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, et al. Mutations in the *gyrA* and *gyrB* genes of Fluoroquinolone-resistant *Mycobacterium tuberculosis* from TB patients in Thailand. *Southeast Asian J Trop Med Public Health*. 2005; 36(4): 228–237.
- Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, & Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Molecular Microbiology* .2007; 63(4): 1096-106.
- Ryan KJ, Ray CG Sherris Medical Microbiology. McGraw Hill. 2004.
- Rodrigues CS, Shenai SV, Almeida DVG, Sadani MA, Goyal N, Vadher C, Mehta AP. Use of BACTEC 460 TB System in the Diagnosis of Tuberculosis. *Indian J Med Microbiology*. 2007; 25: 32-36.
- Ramachandran R, Paramsivan CN. What is new in the diagnosis of tuberculosis? Part II-Techniques for drug susceptibility testing. *ICMR Bulletin*, 2002; 32:81-85.
- Rodrigues CS, Shenai SV, Almeida DVG, Sadani MA, Goyal N, Vadher C, Mehta AP. Use of BACTEC 460 TB System in the Diagnosis of Tuberculosis. *Indian J. Med. Microbiology*, 2007, 25: 32-36.

- Rinder H, Thomschke A, Rüsch-gerdes S, Bretzel G, Feldmann K, Rifai M, & Löscher T. Significance of *ahpC* promoter mutations for the prediction of isoniazid resistance in *Mycobacterium tuberculosis*. *European Journal of Clinical Microbiology and Infectious Diseases* .1998; 17(7): 508-11.
- Rattan A, Kalia A and Ahmad N. Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular Perspectives. *Indian J Tuberc.* 1999; 46: 51-68.
- Ramaswamy SV, Amin AG, Göksel S, Stager CE, Dou SJ, Sahly EI, Moghazeh H, Kreiswirth SL. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*. 2000; 44(2): 326-36.
- Rosenkrands I, King A, Weldingh K, Moniatte M, Moertz E, and Andersen P. Towards the proteome of *Mycobacterium tuberculosis*. *Electrophoresis*. 2000; 21:3740–3756.
- Ramaswamy S and Musser, J.M. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: update. *Tuber Lung Dis* 1998; 79:3 –29.
- Ronaghi M, Uhlén M. & Nyrén P. A sequencing method based on real-time pyrophosphate. *Science.* 1998; 281: 363–365.
- Ronaghi M, Karamohamed S, Pettersson B, Uhlé, M. & Nyrén P. Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 1996; 24: 84–89.
- Rengarajan J, Sassetti, CM, Naroditskaya V, Sloutsky A, Bloom BR, & Rubin EJ. The folate pathway is a target for resistance to the drug *para*-aminosalicylic acid (PAS) in mycobacteria. *Molecular Microbiology* .2004; 53(1), 275–82.
- Raviglione, MC, Smith, IM. XDR tuberculosis—implications for global public health. *N. Engl. J. Med*. 2007; 356, 656–659.
- Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, & Moghazeh SL. J. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. *Antimicrobial Agents and Chemotherapy*. 1997; 41(8): 1677-81.
- Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B & Alland D. Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*. 2010; 54(1): 103-8.
- Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, and Mounier J. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocyticvacuole and plamid-mediated contact haemolysis. *Infect. Immun.* 1986; 51:461–469.

- Sun Z et al. Comparison of *gyrA* gene mutations between laboratory- selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. *Int. J. Antimicrob. Agents*. 2008; 31:115–121.
- Sassetti CM, Boyd DH, and Rubin EJ. Genes required for Mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol*, 2003; 48:77-84.
- Scorpio A, Zhang Y. Mutations in *pnc*A, a gene encoding pyrazinamidase or nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med.* 1996; 2: 662–667.
- Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N, Prachee, *et al*. Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. *Antimicrob Agents Chemother* 2002; 46:443- 50.
- Soini H, Bo¨ttger EC, Viljanen MK. Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. *J Clin Microbiol* 1994; 32:2944–7.
- Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. *Antimicrob Agents Chemother*. 1997; 41:636-640.
- Sharma SK and Mohan A. Multidrug-resistant tuberculosis: a menace that threatens to destabilize tuberculosis control; *Chest.* 2006; 130: 261–272.
- Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, et al . Characterization of *pncA* mutations in pyrazinamide- resistant *Mycobacterium tuberculosis* . *Antimicrob. Agents Chemother*.1997; 41:540 –543.
- Somoskovi A, Parsons LM, Salfinger M. The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*. *Respiratory Research.* 2001; 2:164–168.
- Sharma K, Sanjeev Kumar, P.K. Saha *et al.* Prevalence of multidrug-resistant tuberculosis among Category II pulmonary tuberculosis patients. Departments of Medicine & Microbiology, All India Institute of Medical Sciences. New Delhi, *Indian J Med Res* 2011; 133:312-315.
- Sharma S K and Mohan A Multidrug-resistant tuberculosis: a menace that threatens to destabilize tuberculosis control; *Chest.* 2006; 130: 261–272
- Shankar P, Manjunath N, Mihan KK. 1991. Rapid diagnosis of tuberculosis meningitis by polymerase chain reaction. *Lancet*.1997; 337: 3-7
- Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible

involvement of efflux mechanism. *Antimicrobial Agents and Chemotherapy.*2008; 52(8): 2947-9.

- Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, et al. Characterization of *pncA* mutations in pyrazinamide- resistant *Mycobacterium tuberculosis* . *Antimicrob. Agents Chemother.* 1997; 41:540 –543.
- Somoskovi A**,** Parsons LM, Salfinger M**.** The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*. *Respiratory Research.* 2001; 2:164–168.
- Scorpio A, Zhang Y. Mutations in *pnc*A, a gene encoding pyrazinamidase or nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med*. 1996; 2:662–667.
- Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. *Antimicrob Agents Chemother.* 1997; 41:636-640
- Salman H. Siddiqui. BACTEC 460TB System, Product and Procedure Manual MA0029, Becton Dickinson and Company, Maryland, USA.
- Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopeæ E, Zwolska Z, Kirikae F, et al. Detection of Multidrug Resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2007; 45:179-192.
- Stoermer MJ. Current status of virtual screening as analysed by target class. *Med Chem* .2006; 2: 89-11
- Shah NS, Wright A, Bai GH, et al. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg. Infect. Dis.*2007; 13,380–387.
- Shi R, Zhang J, Li C, Kazumi Y, Sugawara I. Emergence of ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates from China as determined by *gyrA* mutation analysis using denaturing high-pressure liquid chromatography and DNA sequencing. *J Clin Microbiol* . 2006; 44: 4566–4568.
- Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE 3rd, & Stover CK. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science*.1996; 272(5268): 1641-3.
- Shaw KJ, Rather PN, Hare RS, and Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev*, 1993; 57:138–163.

❖ Scarpellini P, Braglia S, Brambilla AM, Dalessandro M, Cichero P, Gori A, et al. Detection of rifampin resistance by single-strand conformation polymorphism analysis of cerebrospinal fluid of patients with tuberculosis of the central nervous system. *J Clin Microbiol.* 1997; 35: 2802-6.

❖ Shendure J. *et al.* Accurate multiplex polony sequencing of an evolved bacterial genome. *Science.*2005*;* 309: 1728–1732.

❖ Stauffer F, Mutschlechner R, Hasenberger P, Stadlbauer S, Schinko H. Detection of *Mycobacterium tuberculosis* complex in clinical specimens by a commercial polymerase chain reaction kit. *Eur J Clin Microbiol Infect Dis* 1995; 14:1046–51.

❖ Scarparo C, Piccoli P, Rigon A, Ruggiero G, Scagnelli M, Piersimoni C. Comparison of enhanced *Mycobacterium tuberculosis* amplified direct test with Cobas Amplicor *Mycobacterium tuberculosis* assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory and extrapulmonary specimens. *J Clin Microbiol* 2000; 38:1559–62.

❖ Singla R, Sarin R, Khalid UK, Mathuria K, Singla N, Jaiswal 3. A, *et al*. Seven-year DOTS-Plus pilot experience in India: Results, constraints and issues. *Int J Tuberc Lung Dis* 2009; *13: 976-81*

❖ Tateishi Y, Aki Tamaru, Yoshitoshi Ogura, Mamiko Niki, Takayuki Wada, Taro Yamamoto, Kazuto Hirata, Tetsuya Hayashi, Sohkichi Matsumotoa. Whole-Genome Sequence of the Potentially Hypertransmissible Multidrug-Resistant *Mycobacterium tuberculosis* Beijing Strain OM-V02_005. *Genome Announcements*.2013;1: 00608-13.

❖ Trias J, Jarlier V and Benz R. Porins in the cell wall of mycobacteria. *Science* 1992; 258: 1479-81.

❖ Trias J and Benz R. Permeability of the cell wall of *Mycobacterium smegmatis*. *Mol. Microbiol.* 1994; 14: 283-290.

❖ Tripathy SP, Mitchison DA, Nair NGK, Radhakrishna S, and Subbammal SA comparison of various measures of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *Tubercle.* 1970; 51:375–388.

❖ Tascon RE, Soares CS, Ragno S, Stavropoulos E, Hirst EM, and Colston MJ. *Mycobacterium tuberculosis*-activated dendritic cells induce protective immunity in mice. *Immunology.* 2000; 99:473–480.

❖ Tripathy SP, DA. Mitchison NG. Nair K, Radhakrishna S, and Subbammal S. A comparison of various measures of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *Tubercle*, 1970; 51:375–388.

❖ Tevere VJ, Hewitt PL, Dare A, Hocknell P, Keen A, Spadoro JP, Young KKY. Detection of Mycobacterium tuberculosis by PCR amplification with pan-Mycobacterium primers and hybridization to an *M. tuberculosis*-specific probe. *J Clin Microbiol* 1996; 34:918–23.

❖ Troesch A, Nguyen H, Miyada CG, Desvarenne S, Gingeras TR, Kaplan PM, et al. Mycobacterium species identification and rifampin resistance testing with high-density DNA probe arrays. *J Clin Microbiol* 1999; 37:49–55.

❖ Torres MJ, Criado A, Ruiz M, Llanos AC, Palomares JC, Aznar J. 2003. Improved real-time PCR for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates. *Diagn Microbiol Infect Dis;* 45: 207-12.

❖ Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicentre study. *J Clin Microbiol* 1999; 37: 3578–3582.

❖ Tholcken CA, Huang S, and Woods GL. Evaluation of the ESP Culture System II for recovery of mycobacteria from blood specimens collected in Isolator tubes. *J. Clin. Microbiol*. 1997; 35:2681–2682

❖ Trapnell C & Salzberg SL. How to map billions of short reads onto genomes. *Nature Biotech.*2009; 2: 455–457.

❖ Tomkinson AE., Vijayakumar S, Pascal JM. & Ellenberger T. DNA ligases: structure, reaction mechanism, and function. *Chem. Rev.*2006; 10: 687–699.

❖ Udwadia ZF, Jain S, Rodrigues C, Mehta A. XDR tuberculosis in India: what's in a name? *Lancet.* 2007;7:441-2

❖ Udwadia F, Rohit A. Amale, Kanchan K. Ajbani, and Camilla Rodrigues. Totally Drug-Resistant Tuberculosis in India. *Clin Infect Dis.*2011*;* 67(4): 286-288.

❖ Vareldzis BP, Grosset J, de Kantor I, Crofton J, Laszlo A, Felten M et al. Drug-resistant tuberculosis: laboratory issues. World Health Organization recommendations. *Tubercle and Lung Diseases.* 1994; 75:1-7.

❖ Vetrivel U, Pilla K. Open discovery: An integrated live Linux platform of bioinformatics tools. *Bioinformation* .2008; 3:144-6.

❖ Via LE, Cho SN, Hwang S, Bang H, Park SK, and Kang SH, et.al. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and Capreomycin in *Mycobacterium tuberculosis* isolates from South Korean patients with drug-resistant tuberculosis. *Journal of Clinical Microbiology*, 2010; 48:402–411.

- World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis. Geneva, WHO, 2008 (WHO/HTM/TB/2008.402).
- WHO**.** Anti-tuberculosis drug resistance in the world. Report No.2: Prevalence and trends. The WHO/IUATLD Global project on Anti tuberculosis drug resistance surveillance (WHO/CDC/TB/2000.278). Geneva, World Health Organisation.document, 2000.
- World Health Organization. 2013. Global tuberculosis control: WHO report 2013 World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/global_report/2013/gtbr11_full.pdf.
- Wolucka BA, Mcneil MR, De Hoffmann E, Chojnacki T, & Brennan PJ. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. *The Journal of Biological Chemistry*. 1994; 269(37): 23328-35.
- Wang JY, Hsueh PR, Jan IS, Lee LN, Liaw YS, Yang PC & Luh KT. Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. *Thorax.* 2006; 61(10): 903-8.
- Wold B. & Myers RM. Sequence census methods for functional genomics. *Nature Methods.*2008; 5: 19–21.
- Winder F. Mode of Action of the Antimycobacterial Agents and Associated Aspects of the Molecular Biology of Mycobacteria. In: Ratledge C, Stanford J, eds. The Biology of Mycobacteria. Vol I. New York, NY, USA: Academic Press.1982; 354-438.
- Wenjing Chen, Tapan Biswas, Vanessa R. Porter, Oleg V. Tsodikov, and Sylvie Garneau-Tsodikova. “Unusual regioversatility of acetyltransferase Eis,a cause of drug resistance in XDR-TB”, *Proceedings of national academy of sciences.* 2011; 108(24): 9804–9808.
- Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR, and Barry CE 3rd. The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc. Natl. Acad. Sci*. USA. 1998; 95:9578–9583.
- Yajko DM, Madej JJ, Lancaster MV, et al. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *J Clin Microbiol* 1995; 33: 2324–2327.
- Yeager RL, Munroe WG, Dessau FI. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. *Am Rev Tuberc*. 1952; 65: 523–534.
- Zhang Y and Telenti A. Genetics of drug resistance in *Mycobacterium tuberculosis*, *ASM Press*. 2000; 235–251.

- Zarir, F. Udwadia, Rohit, A., Amale., Kanchan, K. Ajbani., Camilla Rodrigues., 2011 Totally Drug-Resistant Tuberculosis in India. *Clin Infect Dis*.67 (4), 286-288.
- Zumia, A., Grange, JM., 2001. Multidrug-resistant tuberculosis—can the tide be turned? *Lancet Infect. Dis*.1, 199–202.
- Zaunbrecher MA, Sikes RD, Metchock B, Shinnick TM, and Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A*. 2009; 106:20004–20009.
- Zhang Y, Chen C, Liu J, Deng H, Pan A, Zhang L, Zhao X, Huang M, Lu B, Dong H, Du P, Chen W, Wan K. Complete genome sequences of *Mycobacterium tuberculosis* strains CCDC5079 and CCDC5080, which belong to the Beijing family. *J. Bacteriol.* 2011; 193:5591–5592.

APPENDIX I

ZIEHL-NEELSEN STAIN

PRINCIPLE: Organisms such as Mycobacterium are extremely difficult to stain by ordinary methods because of the high lipid contents of the cell wall. Initial staining is done with Carbol fuchsin combined with phenol and heated. The stain binds to the mycolic acid of the cell wall. After staining an acid decolourising solution is applied. This removes the red dye from the background cell tissues and other organisms except ***Mycobacteria*** which retain the dye. Following decolourisation, the smear is counter stained with malachite green or methylene blue, which stains the background material appearing blue in colour, providing a contrast colour where as the Mycobacteria retains the primary stain and appears pink in colour against the blue back ground.

MATERIALS REQUIRED:

Clean, dry glass slides, glass marker, Carbol fuchsin stain, Decoloriser - 20% H_2So_4, Methylene blue, Distilled water for washing steps.

PROCEDURE:

- A thin smear of the specimen was prepared within the marked area before and after decontamination step, air died and fixed by gentle heating over a Bunsen burner flame for 2 minutes.
- The smear was flooded with carbol fuchsin and heated till fumes arise and left for 5 -7 minutes .
- The stain was removed and washed with distilled water
- The smear was covered with 20 % sulphuric acid till faint pink colour persists and washed well with distilled water
- The smear was covered with Methylene blue for 3 minutes and washed well with distilled water, dried and examined under low power objective and then under oil immersion objective.

INTERPRETATION OF RESULTS:

If any definite bacilli (pink rods) are seen, the smear was reported as AFB positive and an indication of the number of bacteria present was reported as follows:

Table showing grading Chart for ZN Microscopy (1000X)

ZN staining grading (RNTCP)	**Reporting /Grading**
>10 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	Positive, 2+
10-99 AFB/100 field	Positive, 1+
1-9 AFB/100 field	Positive, Scanty
No AFB per 100 fields	Negative

APPENDIX II

REAGENT PREPARATION FOR BACTEC CULTURE

PREPARATION OF STERILE 4% SODIUM HYDROXIDE (NaOH) SOLUTION:

- 4g of Sodium hydroxide crystals (SRL grade) were weighed and dissolved in 100 ml distilled water
- After dissolving completely, NaOH was distributed in 10 ml amounts in bottles and sealed with rubber cork and aluminium cap and autoclaved at 121°C for 15 min at 15lbs pressure.
- Stored at room temperature for a maximum of 2 months

PREPARATION OF 2.8% SODIUM CITRATE:

- 2.8g of Sodium citrate (SRL grade) were weighed and dissolved in 100 ml distilled water
- After dissolving completely, sodium citrate was distributed in 10 ml amounts in 15ml glass bottles and sealed with rubber cork and aluminium capped and autoclaved at 121°C for 15 min at 15lbs pressure.
- Stored at room temperature for a maximum of 2 months

PREPARATION OF 0.5% N-acetyl L-cysteine (NALC) (Himedia, India)-NaOH

- 0.025g of NALC was weighed in sterile brown bottles following aseptic precautions.
- Equal volume 2.5ml of 4%NaOH and 2.8% sodium citrate were added to NALC and mixed well.
- It was left at room temperature for 2 hours before use.
- NALC-NaOH should be prepared freshly every day or it can be used within 24 hours of preparation.

RECONSTITUION OF PANTA (POLYMYXIN, AMPHOTERICIN B, NLDIXIC ACID, TRIMETHOPRIM, AZLOCILLIN LYOPHILIZED ANTIBIOTIC POWDER)

- The freeze dried powder of PANTA (BD diagnositics) was reconstituted with 3.5 ml of sterile distilled water
- Aliquotted in 0.5ml amounts, stored at 4°C and used within 6 months of reconstitution.

APPENDIX III

PCR AND DNA SEQUENCING

PREPARATION OF 0.01% DIETHYL PYROCARBONATE (DEPC) COATED COLLECTON CONAINERS FOR RT-PCR

- To 1000ml of MilliQ water, 100µl of DEPC was added and mixed well.
- 1.5ml new PCR vials were put in a broad beaker container and soaked in DEPC added MilliQ water and left undisturbed overnight.
- The next day water was drained and the vials autoclaved at 121°C for 15 min at 15lbs pressure.
- After autoclaving, the vials were dried well before using for RT-PCR

AGAROSE GEL ELECTROPHORESIS- REAGENT PREPARATION

10X TBE buffer: (pH 8)

Tris- Hcl – 54.1g
EDTA – 3.65g
Boric acid – 27.8g
Milli Q water – 500ml

Added Tris- HCl to 200ml of Milli Q water and dissolved completely. Then EDTA and boric acid was added. Mixed well and the volume was then made up to 500 ml with Milli Q water and then pH was checked. The stock 10X TBE buffer was diluted to 1X concentration before use.

GEL LOADING DYE:

Solution A - 0.1% Bromophenol blue in 1x TBE buffer

Solution B - 40% sucrose in 1x TBE buffer

Prepare solution A and B separately and equal volume was mixed and used for gel loading.

PREPARATION OF GEL AND VISUALIZATION OF PCR PRODUCTS:

Agarose- 0.5 g was weighed and dissolved in 25ml of 1X TBE buffer, by heating in microwave oven until no granules were seen. Then added 8µl (12.5µg) of ethidium bromide mixed well and poured to the gel tray with appropriate combs and allowed to solidify. The gel was then placed in the tank with 1X TBE buffer. 10µl amplified products were mixed with 2 - 3µl loading gel and loaded onto wells along with a molecular weight marker in each run.

The electrophoresis was run at 100 Volts for 30 minutes, and the results were read and documented in the gel documentation system (Vilber Lourmat, Marne La Valle, Cedex, France).

PREPARATION OF PURIFICATION REAGENTS

- **500mM EDTA:** 18.6 g of EDTA in 100 ml Milli Q water
- **125mM EDTA:**1µl of 500mM EDTA + 3 µl Milli Q water
- **3 M sodium acetate: (pH 4.6) :** 2.46g of sodium acetate was first dissolved in 5 ml of water and then pH was adjusted to 4.6 with acetic acid. Then the volume was made upto 10ml with water.

APPENDIX-IV

LIST OF CONSUMMABLES

CONSUMMABLE	**COMPANY**
Agarose	SRL, India
AST SIRE kit	Vivek Enterprises, Chennai, India
Big Dye Terminator ready reaction kit	Applied Biosystems, India
Boric acid, Molecular Grade	SRL, India
Bromophenol blue	SRL, India
cDNA conversion kit	Applied biosystems
Chloroform (molecular grade)	Hi Media, Mumbai, India
Desalted Oligonucleotide bases	Bangalore Genei Pvt, Ld, India
dNTPs	Bangalore genei Pvt, Ltd, India.
EDTA, Molecular grade	SRL,India
Ethanol	SD fine chemicals Pvt Ltd, India
Ethidium Bromide	Hi Media, Mumbai, India
Isopropanol (molecular grade)	Hi Media, Mumbai, India
Molecular weight marker- 100 bp ladder	Bangalore genei Pvt, Ltd, India.
MGIT culture media (4ml, 7ml)	Vivek Enterprises, Chennai, India
OADC supplement	Vivek Enterprises, Chennai, India
PANTA	Vivek Enterprises, Chennai, India
PZA supplement	Vivek Enterprises, Chennai, India
PZA test media	Vivek Enterprises, Chennai, India
Primers	Bangalore genei Pvt, Ltd, India.
Qiamp DNA Blood mini Kit	Qiagen, Germany:Genetix Pvt Ltd
Reconstituting fluid - PZA testing	Vivek Enterprises, Chennai, India
Taq DNA Polymerase	Bangalore genei Pvt, Ltd, India.
Turbo DNase kit	Applied Biosystems, India
Tri Reagent	Applied Biosystems, India
Tris-HCl, Molecular biology grade	SRL, India
Whole genome sequencing reagents	Invitrogen, Singapore

LIST OF PUBLICATIONS

ARTICLES PUBLISHED

1. Lily Therese Irudayam, **Dhanurekha Lakshmipathy**, Gayathri Ramasubban,Umashankar Vetrivel, H. N. Madhavan, R. Sridhar, N. Meenakshi. Whole-Genome Sequencing and Mutation Analysis of Two Extensively Drug-Resistant Sputum Isolates of *Mycobacterium tuberculosis* (VRFCWCF XDRTB 232 and VRFCWCF XDRTB 1028) from Chennai, India. *Genome announc*. 2014; 2(6): e01173-14.

2. Gayathri Ramasubban, **Dhanurekha Lakshmipathy**, Umashankar Vetrivel, Lily Therese Irudayam, H. N. Madhavan, R. Sridhar, N. Meenakshi. Draft Genome Sequence of Streptomycin resistant resistant *Mycobacterium tuberculosis* CWCFVRF MRTB 180 strain from Chennai. *Genome announc* .2014;2(5): e00919-14.

3. **Dhanurekha Lakshmipathy**, Umashankar Vetrivel, Lily Therese Irudayam, Gayathri Ramasubban, H. N. Madhavan, R. Sridhar, N. Meenakshi. Draft Genome Sequence of Polyresistant resistant sputum isolate of *Mycobacterium tuberculosis* CWCFVRF PRTB 19 strain from Chennai, India closely clustering with East African India 5 Genogroup.- Genome annuouncement.

4. **Dhanurekha Lakshmipathy**, Umashankar Vetrivel, Lily Therese Irudayam, Gayathri Ramasubban, H. N. Madhavan, R. Sridhar, N. Meenakshi. Draft Genome Sequence of Multidrug-Resistant *Mycobacterium tuberculosis* Strain CWCFVRF MDRTB 670, Isolated from the Sputum of a Patient from Chennai, India, with Clinically Suspected Tuberculosis. *Genome announc.* 2014; 2(3): e00475-14.

5. Lily Therese kulandai, **Dhanurekha Lakshmipathy**, Gayathri Ramasubban, and Madhavan Hajib Narahari Rao. “First report on isolation of Mycobacterium massiliense from corneal biopsy in India”. JMM Case Reports. 2014; doi: 10.1099:1-3.

6. **Dhanurekha Lakshmipathy**, Gayathri Ramasubban, Lily Therese, Umashankar Vetrivel, Muthukumaran Sivashanmugam, Sunitha Rajendiran, Sridhar R, Madhavan HN, and Meenakshi N. In silico Analysis of Novel Mutation ala102pro Targeting pncA Gene of M. Tuberculosis .*J Comput Sci Syst Biol* 2013; 6: 083-087.

7. **Dhanurekha Lakshmipathy**, Gayathri Ramasubban, Lily Therese Kulandai, R.Sridhar, Madhavan Hajib Narahari, N.Meenakshi. Extensively Drug Resistant Tuberculosis

(XDR-TB) by Phenotypic Drug Susceptibility Using BACTEC Micro MGIT Culture System A Pilot study in Hospital Based Population in Chennai, India. *Int.J.Curr.Microbiol.App.Sci* .2014; 3(5): 129-135.

8. Therese KL, Gayathri R, **Dhanurekha L**, Sridhar R, Meenakshi N, Madhavan, HN " Diagnostic appraisal of simultaneous application of two nested PCRs targeting MPB64 gene and IS6110 region for rapid detection of M. tuberculosis genome in culture proven clinical specimens" *Indian Journal of Medical Microbiology*, (2013) 31(4), 366-369.
9. Therese KL, Gayathri R, **Dhanurekha L**, Sridhar R, Meenakshi N, Madhavan HN, Edwin Manoj S, Kamala Vinayagam A. Detection of viable *Mycobacterium tuberculosis* directly from sputum specimens of suspected tuberculosis patients and drug resistance pattern by phenotypic methods using BACTEC MicroMGIT culture system - a pilot study in Chennai. *Indian Journal of Medical Research*. 2012. 135: 778-782.

LIST OF PRESENTATIONS

NATIONAL-ORAL PRESENTATION

1. Presented paper entitled "Evaluation of nested PCR targeting *rpoB* gene for the detection of *Mycobacterium tuberculosis* genome in clinical specimens" on January 2011 in the 65th Annual Meeting of Indian Tuberculosis Association held in Bangalore.

2. Presented paper entitled "Detection of novel mutations by PCR based DNA sequencing to pyrazinamide targeting *pncA* gene in *M. tuberculosis* isolates from chennai population –The first report" in 1st Annual conference of IAMM Tamil nadu and Puducherry chapter held on 2nd Feb 2013 at Salem

NATIONAL POSTER PRESENTATIONS

1. Presented poster entitled "*in vitro* antifungal susceptibility testing by disk diffusion method against Amphotericin B and voriconazole on ocular isolates of non sporulating moulds"in IERG conference held on 30th - 31st July 2011 at Hyderabad.
2. Presented poster entitled "Optimisation and Application of PCR based DNA sequencing targeting "*gidb* gene"for the detection of mutations in *Mycobacterium tuberculosis* isolates in Chennai population" in XXXVII National Conference of the Indian Association of Medical Microbiologists (IAMM) on 22nd – 23rd November 2013 at Hyderabad.

AWARDS WON

1. Bangalore Genei Pvt. Ltd. Endowment award for the Best performance in Clinical Genetics
2. The Young women scientist award in memory of Smt. Sreerangamma N. Narayan and Smt. Radhamani N. Anantha for the year 2013 for working in the field of Infectious diseases. She is at present working as a Junior scientist in the L&T Microbiology Research Centre.

BIOGRAPHY OF THE CANDIDATE

Ms. L. Dhanurekha completed her under graduation in B.Sc Microbiology from Valliammal College of Arts & Science, Anna nagar, Chennai in the year 2007. She joined MS Medical Laboratory Technology conducted by BITS, Pilani and graduated in the year 2010. She worked as a Junior Research Fellow for 2 years and Senior Research Fellow for 18 months in the project entitled "A Pilot study on rapid detection of viable *Mycobacterium tuberculosis* and multidrug resistant (MDR) tuberculosis from clinically suspected tuberculosis patients in Chennai – A Major Public Health Problem in India" funded by Chennai Willingdon Corporate Foundation, Chennai and currently she is working as a Junior Scientist in the L&T Microbiology Research Centre in April 2014. She registered for PhD in BITS, Pilani in August 2010 under the guidance Dr. K. Lily Therese. She has made 2 oral and 2 poster presentations in National conferences. She has 9 publications and 2 articles are under Review. She had conducted practical and theory classes in microbiology for the under graduate (BS Optometry) and post graduate (MSMLT) students registered under off campus courses of BITS Pilani. She have also involved in conducting Antimicrobial surveillance program conducted by WHO-WHONET 2011& 2013 at L&T Microbiology research center. She was also involved in training the post graduate students (Short term and summer training) from other institutes in various molecular biological techniques. She was awarded Bangalore Genei Pvt. Ltd. Endowment award for the Best performance in Clinical Genetics, the Young women scientist award in memory of Smt. Sreerangamma N. Narayan and Smt. Radhamani N. Anantha for the year 2013 for working in the field of Infectious diseases. At present, she is working as a Junior scientist in the L&T Microbiology Research Centre.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. K. Lily Therese completed her PhD from Delhi University in 1993 and joined Vision Reseacrh Foundation in 1994 as Post doctoral fellow. At present she is the Senior Professor and HOD of Department of Microbiology, L & T Microbiology Research Centre, Vision Research Foundation, KNBIRVO building, Chennai. She has 26 years of rich experience in Medical Microbiology with 15 year postgraduate teaching in Medical microbiology. She is a reviewer for 12 Peer reviewed Journals. She is a recognized Ph. D guide in BITS-Pilani, The Tamilnadu Dr. MGR Medical University and SASTRA and Ph.D examiner, Doctoral Committee member for Ph.D in Madras University, She is the co-ordinator for BITS Ph.D programme conducted at Medical Research Foundation, Sankara Nethralaya in collaboration with Birla Institute of Science and Technology, Pilani. She is in-charge of Vidya Sagar Institute of Biomedical Sciences technology (VIBS), a unit of Medical Research Foundation. She has conducted National/State level CME programmes and hands on training programmes in Molecular techniques for post graduates from different research and academic institutions. She had initiated and involved in development of nucleic acid–based molecular biological methods in L&T Microbiology Research Centre, Vision Research Foundation, Chennai. She was Principal Investigator of 5 and Co- Principal Investigator of 10 Research projects sanctioned by ICMR, DST and CWCF. She was awarded the Best Research Scientist Award Vision Research Foundation, Sankara Nethralaya in 2002, Research Fellowship Award by Alcon Labs TX, USA in 2006. She was honoured for the contribution in “Molecular Microbiology” on the “International Women’s day” in March 2010. Her areas of special interest are development of rapid diagnostic tests based on molecular techniques for rapid detection of infectious agents and diagnostic DNA chip for infectious diseases and to study the Molecular epidemiology of specific infectious diseases particularly related to ophthalmic infections. She has guided 3 Ph.D students from BITS pilani and one from Dr. MGR university. She has 90 publications in peer reviewed National and International journals,3 patents and 5 chapters in book. She had done 55 presentations in National & International conferences, Invited academic lectures, National & International Symposia; CME programmes. She is a Life Member of Indian Association of Medical Microbiologists, Life Member of Indian Virological Society and Annual member of American society of Microbiology.

BRIEF BIOGRAPHY OF THE CO-SUPERVISOR

Prof.Lalita Gupta received her Ph.D. in Molecular Biology from the Banaras Hindu University in 1999. During her Post-doc at Colorado State University, Colorado, USA from 2000-2003, she was working on molecular interaction of mosquitoes with Plasmodium or Dengue virus infection. At NIH, USA (2003-2008), she was investigating the effect of mosquito immune system on malaria disease transmission. She Joined BITS-Pilani in 2008 and continue her research on mosquito immunity and disease control program. At present she is the Associate professor in department of Biological science, BITS-Pilani. She is involved in interactive teaching of first degree (M.Sc. Hons.), higher degree (M. E. Biotech) and Master of Public Health courses. Her research intrest in parasite. The courses taught by her were General Biologyand physiology, Recombinant DNA Technology, Molecular Mechanism of Gene expression, Instrumental Method of Analysis, Biological Project laboratory, Epidemic and Disaster Management, Preventive Nutrition and Health Promotion, Biological Laboratory, Microbiology, Molecular Parasitology and Vector Biology, Integrated Biology. She was Principal Investigator of 2 and Co- Principal Investigator of 1 Research projects sanctioned by SERB, ABG and DST. She also got three projects approved [Vector Science Forum, ICMR (2)] She was awarded the Outstanding achievement award in International Congress of Entomology 2012, Employee special performance achievement award by National Institute of Health USA for 4 years (2004-2008), Outstanding achievement Post-doc category poster awards, Colorado State University, Fort Collins, CO, USA (2002). Her areas of special interest on understand the molecular mechanism of mosquito immune system in the defense against She has guided 2 Ph.D students from BITS pilani. She has 12 publications in peer reviewed National and International journals. She had done 50 presentations in National & International conferences, Invited academic lectures, National & International Symposiums. Following are the fellowships got by her: Post-doctoral fellowship by Colorado State University (2000-2003), Post-doctoral fellowship (March 2000 to Nov. 2000), MENRT, France, Senior research fellowship by University Grants Commission (UGC), Government of India (1997-2000) and Junior research fellowship, UGC, Government of India (1995-1997).