Evaluation of Pyrosequencing assay for the rapid detection of resistance to Rifampicin and Second-line drugs in Mycobacterium tuberculosis clinical isolates as compared to the gold standard conventional drug susceptibility testing.

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in April-2017

CERTIFICATE

This is to certify that the dissertation entitled, **"Evaluation of Pyrosequencing assay for the rapid detection of resistance to Rifampicin and Second-line drugs in *Mycobacterium tuberculosis* clinical isolates as compared to the gold standard conventional drug susceptibility testing."** is the bonafide work of Dr. Aishwarya G toward the M.D **(Branch – IV Microbiology)** Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be conducted in **April-2017.**

Dr. Joy Sarojini Michael
Guide,
Professor,
Department of Clinical Microbiology,
Christian Medical College,
Vellore – 632004.

Dr. V. Balaji
Professor and Head,
Department of Clinical Microbiology,
Christian Medical College,
Vellore – 632004.

Principal
Christian Medical College,
Vellore – 632004.

DECLARATION

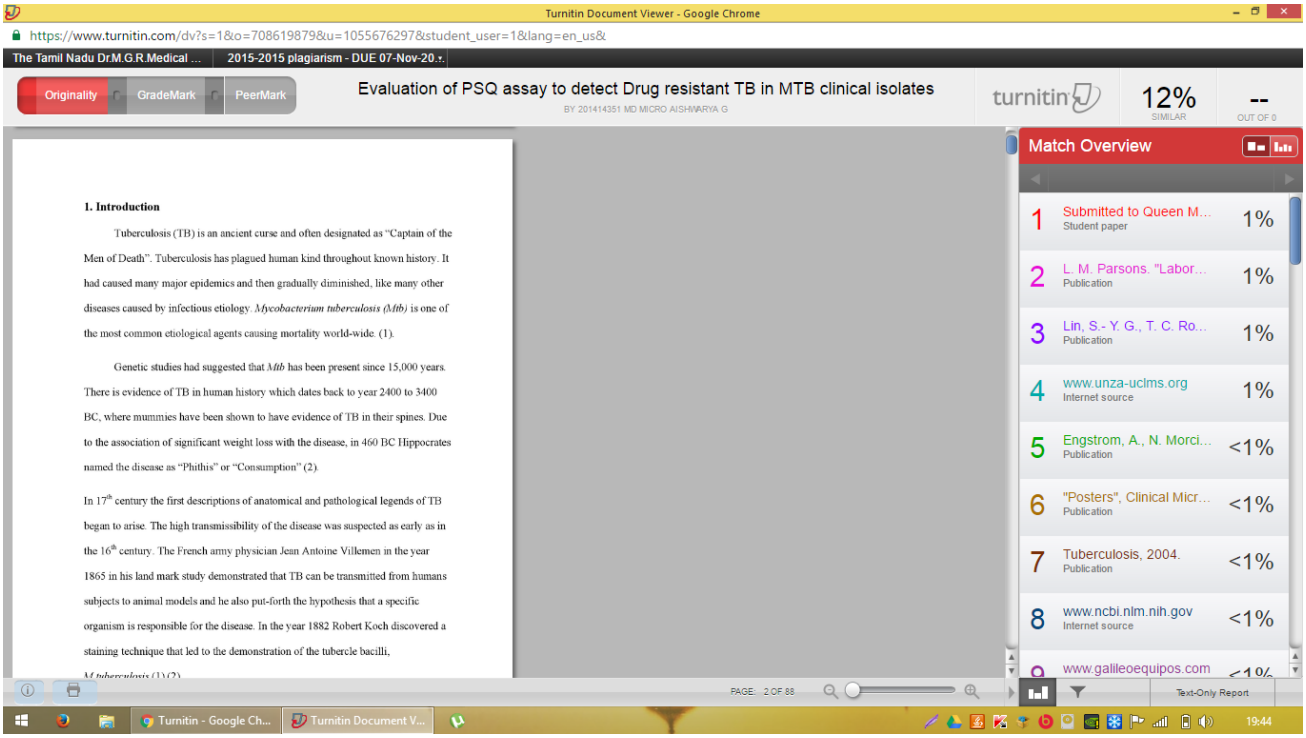
I hereby declare that this M.D Dissertation entitled “Evaluation of Pyrosequencing assay for the rapid detection of resistance to Rifampicin and Second-line drugs in *Mycobacterium tuberculosis* clinical isolates as compared to the gold standard conventional drug susceptibility testing” is the bonafide work done by me under the guidance of Dr. Joy Sarojini Michael, Professor, Department of Clinical Microbiology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

Dr. Aishwarya G

Department of Clinical Microbiology

Christian Medical College

Vellore


Turnitin Document Viewer - Google Chrome
https://www.turnitin.com/dv?s=1&o=708619879&u=1055676297&student_user=1&lang=en_us&
The Tamil Nadu Dr.M.G.R.Medical ...
2015-2015 plagiarism - DUE 07-Nov-20...
Originality
GradeMark
PeerMark
Evaluation of PSQ assay to detect Drug resistant TB in MTB clinical isolates
BY 201414351 MD MICRO AISHWARYA G
turnitin
12%
SIMILAR
--
OUT OF 0
1. Introduction
Tuberculosis (TB) is an ancient curse and often designated as "Captain of the Men of Death". Tuberculosis has plagued human kind throughout known history. It had caused many major epidemics and then gradually diminished, like many other diseases caused by infectious etiology. Mycobacterium tuberculosis (Mtb) is one of the most common etiological agents causing mortality world-wide. (1).
Genetic studies had suggested that Mtb has been present since 15,000 years. There is evidence of TB in human history which dates back to year 2400 to 3400 BC, where mummies have been shown to have evidence of TB in their spines. Due to the association of significant weight loss with the disease, in 460 BC Hippocrates named the disease as "Phithis" or "Consumption" (2).
In 17th century the first descriptions of anatomical and pathological legends of TB began to arise. The high transmissibility of the disease was suspected as early as in the 16th century. The French army physician Jean Antoine Villemen in the year 1865 in his land mark study demonstrated that TB can be transmitted from humans subjects to animal models and he also put-forth the hypothesis that a specific organism is responsible for the disease. In the year 1882 Robert Koch discovered a staining technique that led to the demonstration of the tubercle bacilli,
M tuberculosis (1) (2)
PAGE: 2 OF 88
Match Overview
1 Submitted to Queen M... Student paper 1%
2 L. M. Parsons. "Labor... Publication 1%
3 Lin, S.- Y. G., T. C. Ro... Publication 1%
4 www.unza-uclms.org Internet source 1%
5 Engstrom, A., N. Morci... Publication <1%
6 "Posters", Clinical Micr... Publication <1%
7 Tuberculosis, 2004. Publication <1%
8 www.ncbi.nlm.nih.gov Internet source <1%
www.galileoequipos.com
Text-Only Report
Turnitin - Google Ch...
Turnitin Document V...
19:44

ACKNOWLEDGEMENT

I take great pleasure to express my sincere gratitude and thanks to all the people who have helped me and contributed in making this dissertation possible. First and foremost, I express my profound gratitude to Dr. Joy Sarojini Michael, my guide and mentor for her constant guidance, support and encouragement during the course of the study. She has been a source of inspiration to me throughout and I am grateful to her for the enriching and rewarding experience this dissertation turned out to be. Also, I would like to extend profound thanks to Dr. Rekha Pai for her support, guidance and cheerful encouragement.

I sincerely thank the following people who helped me at various stages of this dissertation.

- Mr. Dhananjain S for helping me troubleshoot and having taught me the technical procedure.
- Mrs. B.Sherly Suzana for helping me with collection of clinical isolates.
- Dr. L. Jayaseelan and Ms. M. Thenmozhi for helping me with statistical analysis.
- The entire Department of General Pathology
- Dr V. Balaji and the faculty, Department of Clinical Microbiology for their concern and encouragement.
- The Institutional Review Board (IRB) and the department of Clinical Microbiology for providing financial assistance to carry out the study.

- My friends and colleagues for their cheerful motivation care and support.

- Most importantly, my parents and my siblings for being a constant source of love, concern, and strength.

- Above all, I thank **GOD**, for filling me with strength and wisdom for completing this study.

CONTENTS

1. Introduction

Tuberculosis (TB) is an ancient curse and often designated as “Captain of the Men of Death”. Tuberculosis has plagued human kind throughout known history. It had caused many major epidemics and then gradually diminished, like many other diseases caused by infectious etiology. *Mycobacterium tuberculosis (Mtb)* is one of the most common etiological agents causing mortality world-wide. (1).

Genetic studies had suggested that *Mtb* has been present since 15,000 years. There is evidence of TB in human history which dates back to year 2400 to 3400 BC, where mummies have been shown to have evidence of TB in their spines. Due to the association of significant weight loss with the disease, in 460 BC Hippocrates named the disease as “Phithis” or “Consumption” (2).

In 17th century the first descriptions of anatomical and pathological legends of TB began to arise. The high transmissibility of the disease was suspected as early as in the 16th century. The French army physician Jean Antoine Villemen in the year 1865 in his land mark study demonstrated that TB can be transmitted from humans subjects to animal models and he also put-forth the hypothesis that a specific organism is responsible for the disease. In the year 1882 Robert Koch discovered a staining technique that led to the demonstration of the tubercle bacilli, *M.tuberculosis* (1) (2).

Even before the discovery of the etiological agent of TB, the ancient Greeks pursued the search for the cure of the disease. For around 2000 years no effective medicine was discovered. In 1943, Selman Walksman discovered a compound

called streptomycin that acted against *M.tuberculosis*. After then a rapid succession of anti-TB drugs appeared in the consecutive years (2).

For the treatment of Tuberculosis one requires to take many antibiotics over a prolonged period of six months or more to achieve cure. Since many years no new antibiotics or better anti-TB drugs have been developed. The scenario had worsened with the emergence of drug-resistant *M. tuberculosis*. Resistance in *Mtb* arise due to point mutations in the mycobacterium genome. There are two types of drug resistance in Tuberculosis, primary and acquired. Primary drug resistance is the one in which a person who has not undergone treatment acquires an infection by a resistant strain. Acquired resistance is the one which develops in a person during the course of treatment with anti-tubercular drugs (3).

Drug susceptible tuberculosis can be cured with appropriate and regular treatment within 6 months. However treating drug resistant TB poses major challenge to the treating clinician, with only 50 -70% cure rates, despite a protracted course of treatment over 2 years. Other significant challenges are the cost of treatment, adherence to treatment and a host of different side effects (4) (5).

Smear microscopy and conventional culture methods remains the main stay in the diagnosis in tuberculosis, more so in resource poor settings. However, they have their pitfalls. Smear microscopy has a minimal role in the detection and management of drug resistant tuberculosis, and conventional culture methods are laborious and slow as compared to the newer methods of diagnosis (6) .

The advent of molecular techniques has revolutionized the diagnosis of tuberculosis as well as the diagnosis of drug resistant tuberculosis. Many commercial tests are now available and also new tests are being developed. The selection of test depends on the clinical scenario and on laboratory aspects like the numbers of samples received, availability of molecular diagnostic methods and expertise (7).

In India, where the rates of drug resistant tuberculosis are on the rise, there is an urgent requirement of a rapid, low cost and robust test for the detection of drug resistance and, at the very least, be complementary to existing standards of diagnosis. Hence, there is an acute need for the further development of genotypic and molecular methods, and the evaluation of these methods (8).

However there are various molecular methods with reduced turn-around time which includes line probe assays (LPA), GeneXpert MTB/RIF assay based on molecular beacons and Pyrosequencing (PSQ) assays for rapid identification of drug resistant TB. LPA for the rapid detection of drug resistance to Isoniazid (INH) and Rifampicin (RIF) and the Xpert MTB/RIF test for detection of resistance against Rifampicin are the commercially available systems that are recommended by the World Health Organisation (WHO) for poor resource settings (7). However, there are no rapid molecular tests for the diagnosis of extensively drug resistant TB (XDR-TB) that are currently endorsed by the WHO.

This study aims at evaluating Pyrosequencing assay for the rapid molecular detection of XDR-TB. The clinical isolates of *M. tuberculosis* from laboratory confirmed cases of multi drug resistant (MDR-TB) and XDR-TB will be studied.

These clinical isolates will be subjected to DNA extraction using commercially available DNA extraction kit. The extracted DNA will be subjected to amplification of the target genes by polymerase chain reaction (PCR). This will be done using specific primer sets for each of the genes. Each of the amplicons obtained will be sequenced using Pyrosequencing technology to look for point mutations which are known to confer drug resistance against Rifampicin and second line anti-tubercular drugs. The results will be correlated with the conventional gold standard drug susceptibility testing.

2. Aim and Objectives

Aim:

This study aims to evaluate Pyrosequencing (PSQ) assay for the rapid detection of resistance to Rifampicin and Second-line drugs in *Mycobacterium tuberculosis* clinical isolates as compared to the gold standard conventional drug susceptibility testing.

Objectives:

i) To evaluate the validity of Pyrosequencing assay for rapid diagnosis of resistance to Rifampicin and Second-line drugs in *Mtb* clinical isolates as against conventional drug susceptibility testing.

ii) To estimate the turnaround time as compared to drug susceptibility testing by 1% agar proportion method on Lowenstein Jensen media which is the current gold standard method.

iii) To compare the performance of PSQ assay with Xpert MTB/Rif assay and Line probe assays (GenoTypeMTBDR*plus* and GenoType MTBDR*sl*)

3. Review of literature

3.1 Epidemiology:

3.1.1 Global Burden:

Tuberculosis is one of the oldest known diseases affecting humans and a leading cause of death among millions of people each year worldwide. In the year 2014, there were about 96 lakh new TB cases, among which 54 lakh were males, 32 lakh were females and 10 lakh were children. Among the 96 lakh new TB cases, 58% were in the Western Pacific regions and South-East Asia and 28% in African regions. India (23%), China (10%), and Indonesia (10%) had the largest number of cases among the global total. TB deaths are around 15 lakhs (11 lakh among HIV-negative persons and 4 lakhs among HIV-positive patients). TB ranks along side HIV as a major cause of death worldwide (9).

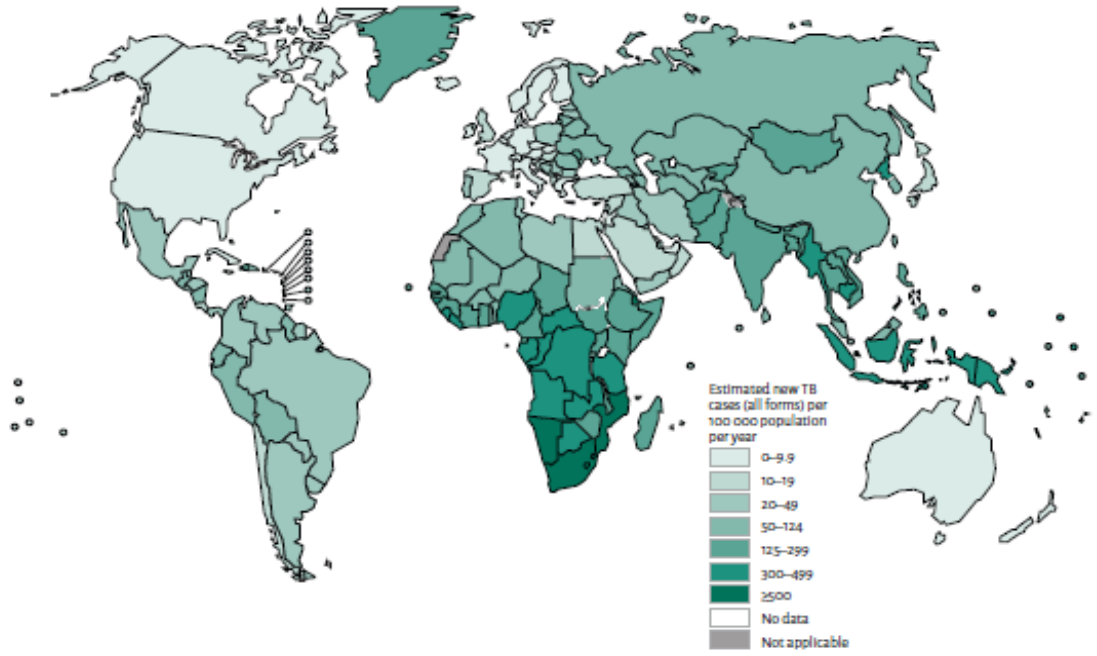


Figure 1: Estimated TB Incidence in 2014 (9)

3.1.2 Drug Resistant TB:

TB incidence rates are decreasing and improvements are being made in international tuberculosis control. TB mortality has been reduced by 45% worldwide (10). The emergence of drug-resistant tuberculosis threatens this progress. "Multidrug-resistant TB (MDR TB) is caused by an organism that is resistant to at least Isoniazid and Rifampicin, the two most potent TB drugs" (11). Recent World Health Organization's (WHO) global estimates suggest that 20.5% of previously treated cases and 3.5% of new cases have multidrug-resistant tuberculosis (9). Rates of multidrug-resistant tuberculosis vary widely between countries and regions with 27 high burden countries accounting for more than 85% of cases. Worldwide, China, India, and Russia contribute most to the total number of cases. The highest proportions are found in Central Asia and Eastern Europe, with around 20% of new TB cases and about 50% of previously treated cases having multidrug-resistant tuberculosis (10).

In the year 2006, the term "Extensively drug-resistant tuberculosis" was coined by Centre for Disease Control and Prevention (CDC), USA, based on WHO guidelines for management of drug-resistant tuberculosis (12).

"Extensively drug resistant TB (XDR TB) is resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin)" (13). More than 105 countries have reported XDR-TB has been reported by 105 countries. It is estimated that on an average about 9.7% of patients with MDR-TB have XDR-TB (9) (14).

Figure 2: By the end of 2013 Countries noted to have at least 1 case of XDR-TB (14).

3.1.3 Burden in India:

India ranks second among the most populous countries in the world with 1/4th of the global new TB cases occurring in India annually (15). Among the high-burden countries India ranks first with 21 lakh new cases of TB every year. This contributes about 24% to the estimated worldwide new TB cases and 1/5th of global tuberculosis related mortality in the year 2013. The prevalence of MDR-TB in India is estimated to be low. The prevalence of MDR-TB is about 15% among retreatment cases and 2.2% among new cases. Among the 27 high burden MDR-TB countries worldwide India still ranks first despite its low prevalence probably because of the number of TB cases annually reported and the overall population size. Among the notified cases of MDR-TB globally, India contributes about 21% (16). Of these, 598 cases of XDR-TB have been reported from 9 different cities in India between the years 2000-2008 (17).

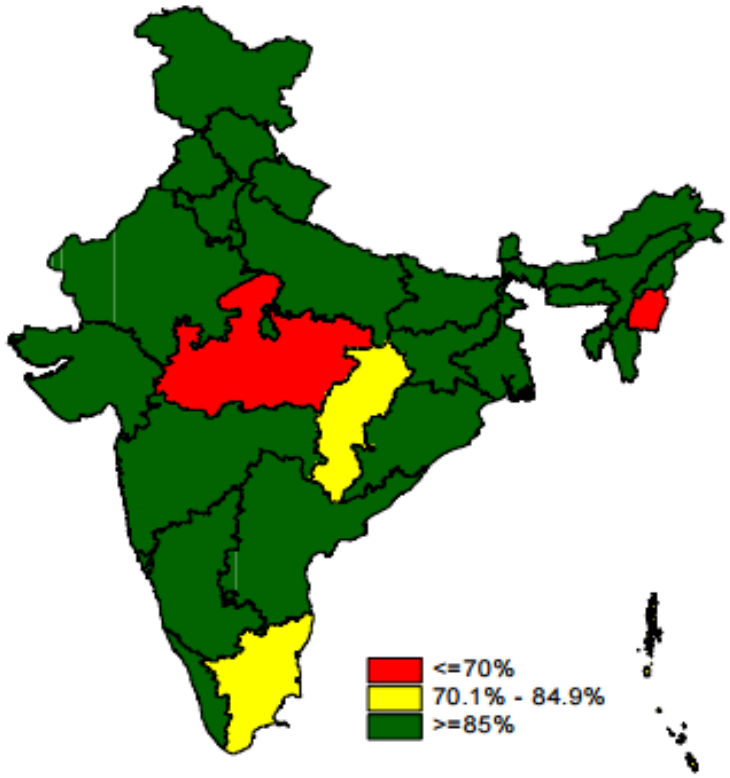


Figure 3: Proportion of MDR-TB diagnosed in India and initiated on treatment (15).

3.2 Etiological agent:

Tuberculosis is caused by a bacteria of *M. tuberculosis* complex (MTBC) which comprises 7 species and subspecies, among which *Mycobacterium tuberculosis is* the most common and important agent (18). The MTBC is formed by several closely related sub-species which infect human and animal populations. The human-adapted species, which cause tuberculosis, include *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. canettii*. Species infecting animals include *M. microti* (voles), *M. pinnipedii* (seals), *M. caprae* (goats) and *M. mungi* (mongooses) and rarely cause human disease (19).

M. tuberculosis complex species share 99.9% sequence identity and are likely evolved from a single clonal ancestor (20).

3.2.1 Taxonomy and Genus characteristics:

Mycobacteria belong to

Family Mycobacteriaceae

Order Actinomycetales

Genus *Mycobacterium*

Genus characteristics are as follows:

1) Acid –alcohol fastness (resists decolorisation by an acidified alcohol after staining with a primary stain

2) The presence of mycolic acids which can been cleaved into fatty acid methyl esters by pyrolysis

3) 61-71 % G+C content of the DNA, *M.leprae* being the only exception (51%)

3.2.2 Morphology & Identification:

Mycobacteria are gram positive organisms, even though mycobacterial cell wall has features similar to gram-positive bacteria and gram-negative bacteria (21). They are aerobic, nonmotile, non–spore forming, very thin, slightly curved and straight rods which measure around 0.2 to 0.6$\times$ 1 to 10 μm. Rarely some species may display a branching morphology (22) . Growth is slow because of their hydrophobic cell surface, the generation time being 15 to 20 hours (23), compared to bacterial pathogens with less than one hour of generation time, and it takes 3 to 8 weeks for visible growth on solid media. The organism tends to grow in parallel groups, thereby producing the characteristic serpentine cording (24).

3.3 Structure of *M.tuberculosis*:

3.3.1 Mycobacterial Cell envelope:

Mycobacterium tuberculosis has a complex cell wall structure required for growth of the cell, antibiotic resistance and virulence mechanisms (25) (26) (27) . The cell envelope is made up of 3 major macromolecules – "arabinogalactan, peptidoglycan and mycolic acids", enclosed by a noncovalently linked capsule made up of polysaccharides and proteins (28). This is called the cell wall core the "mycolyl arabinogalactan peptidoglycan (MAGP) complex". The high density of lipids creates a hydrophobhic permeability barrier in the cell wall thereby preventing accuracy of Gram stain. Ziehl–Neelsen stain utilizes acid fast dyes to stain the Mycobacteria as they are acid fast (28). The cell wall of mycobacteria is one of the important targets of anti-TB drugs, and various compounds that are being used in clinical practice for treating TB (28).

Peptidoglycan:

The peptidoglycan layer is surrounded by the plasma membrane and it contains long polymers of *N* acetyl muramic acid and *N* acetyl glucosamine, that is linked via peptide bridges. Instead of the traditional 4–3 crosslinks, majority (80%) of the peptidoglycan layer contains the 3–3 peptide crosslinks (29) (25). It also has modifications, like amidation of the D-Glu and *meso* diaminopimelic acid residues of the peptide side chain and glycolylation of *N* acetyl muramic acid residues. Recognition by innate immune receptor nucleotide binding oligomerization domain containing 1 NOD1 is masked by amidation, but NOD2

can recognize glycolylated NAM thereby inducing production of inflammatory mediators by the macrophages infected (30).

Arabinogalactan:

Arabinogalactan layer surrounds the peptidoglycan layer. Galactan is a modification of long arabinin polymers produced by galacto furanosyl transferases (26). Arabinan chain terminus are branched and modified by non-*N* acetylated galactosamine (GalN) and succinyl moieties (26). The modifications of GalN usually seen in mycobacteria which are pathogenic, thereby promoting active infection.

Mycolic acids:

The thick waxy lipid coat of mycobacteria is made up of long carbon chain mycolic acids ligated with arabinin. They contribute majorly to virulence mechanisms and cell wall impermeability. Mycolic acids are composed of two types of fatty acids, a shorter saturated α-branch (C_{20}–C_{26}) connecting to a meromycolate branch (C_{60}–$_{90}$). Mycolic acid is processed by cascade of enzymes resulting in 3 different variants which are methoxy-meroacids, α-meroacids and keto-meroacids. Virulence of the infecting Mycobacteria is determined by these three variants (31).

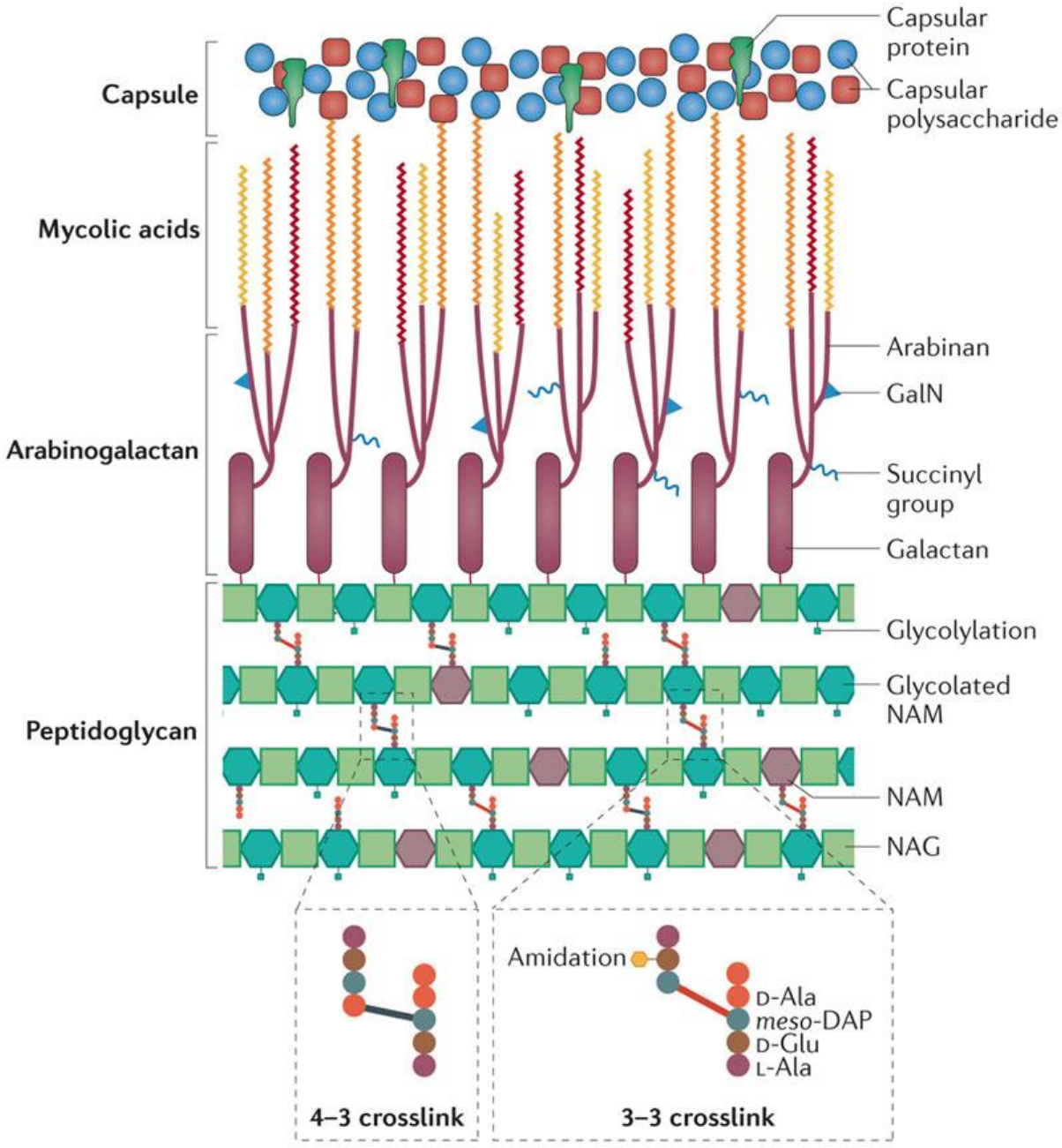


Figure 4: The Cell Envelope of Mycobacteria (31).

3.4 Transmission of Tuberculosis:

The airborne particles which carry *Mtb*, are called the "droplet nuclei". They measure 1– 5 microns in diameter (32). When a patient having laryngeal TB or pulmonary TB disease coughs, shouts, sneezes, speaks, or sings there occurs generation of these infectious droplet nuclei. The droplet nuclei can remain

suspended in the air for several minutes to hours depending on the environment. It is not transmitted by surface contact rather it is transmitted through air (33). When a person inhales the droplet nuclei containing tubercle bacilli, transmission occurs, and it traverses oropharyngeal cavity, the upper respiratory tract, the bronchi and thereby reaches the alveoli of the lungs (19).

3.4.1 Factors determining probability of disease transmission:

1) Susceptibility – Immunity of the exposed individual

2) Infectiousness is related directly to the number of tubercle bacilli that is expelled in the air by the patients infected with TB. Patients who expel many tubercle bacilli are more infectious and vice versa.

3) Environment factors – Closed space, number of infectious droplet nuclei, poor aeration contributing to recirculation of droplet nuclei (34), inappropriate disinfection of equipment used for medical practice, improper handling of patient specimens etc.

4) Duration, Frequency and Proximity of exposure – Longer duration, frequent exposures, close proximity lead to a higher risk of TB transmission (33,34).

3.5 Risk factors for Tuberculosis:

The risk factors can be divided into social, environmental and biological determinants (35).

The risk factors include the following:

a) Genetic factors – Certain genes contribute to increased risk of developing TB namely interferon gamma, natural resistance associated macrophage protein-1, Vitamin D receptor, mannan binding lectin, nitric oxide synthase 2a some Toll like receptors

b) Physiological factors – pregnancy, postpartum, aging

c) Undernutrition – Poverty, homelessness, urbanisation, overcrowding, housing conditions, migration, economic trends

d) Immunological disease affecting CMI – HIV infection and AIDS

e) Organ transplantation

f) Malignancies – Hodgkin's and non-Hodgkin's lymphoma, head and neck carcinoma, Carcinoma of lungs, stomach and intestines

g) Intravenous drug abuse – addiction to heroin

h) Alcohol consumption, Tobacco smoking

i) Silicosis

j) Chronic disease – Chronic kidney disease, Chronic liver disease, long term haemodialysis.k) Iatrogenic factors - Post-gastrectomy, ureteral catheterization, cardiac valve homograft replacement extra-corporeal shockwave lithotripsy, intravesical BCG therapy for bladder cancer

l) Drugs – long term steroid therapy, immunomodulators like anti-tumour necrosis factor

m) Others – Diabetes, Connective tissue disorders, Indoor air pollution

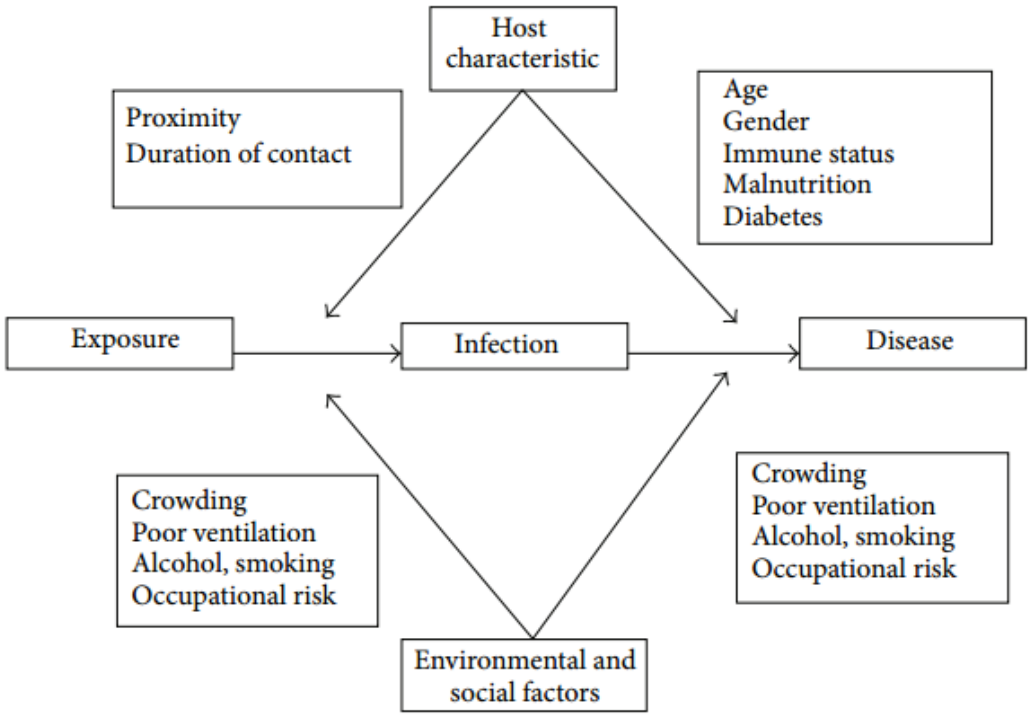


Figure 5: Risk factors for TB infection and Disease (36).

3.6 Pathogenesis:

Patients with active pulmonary disease are the source of *Mycobacterium tuberculosis* (37). A person is infected when he inhales the *Mtb* containing droplet nuclei, that finally reaches the alveoli of the lungs. The alveolar macrophages ingest and destroy tubercle bacilli, so no actual infection occurs (33). However, at times *Mtb* will not be killed instantly, thereby developing into a small infiltrate called the "Primary complex" along with a draining lymph node, which is called the "Ghon's focus" (38). At this point infection is stabilised in most the cases. But few cases develop active disease "primary tuberculosis", infecting the lungs or elsewhere after haematogenous spread of tubercle bacilli. After several months or years due to conditions like waning immune response, infection which was latent might get reactivated resulting in "post primary TB". The disease is typically restricted to the

upper lobes of the lung without any evidence of infection in any other parts of the body (39).

3.7 Clinical presentation:

3.7.1 Primary Tuberculosis:

The initial infection by the tubercle bacilli results in Primary pulmonary TB. Patient may be asymptomatic or may present with pleuritic chest pain and fever. The lower and middle lobes of the lung are most commonly. This lesion is described as Ghon's focus and usually accompanied by transient paratracheal or hilar lymphadenopathy (Ghon's complex). In most cases, the lesion heals spontaneously and becomes a calcified nodule. Few patients may develop erythema nodosum in the legs or phlyctenular conjunctivitis (18).

3.7.2 Reactivation or Secondary Tuberculosis:

It is also called as post primary (adult - type) TB or recrudescent TB. It results from endogenous reactivation recent infection or latent infection. It is usually localized to the apical and posterior segments of the upper lobes of the lung (18). Symptoms include malaise, low-grade fever, wasting, progressive weight loss along with night sweats and chills. The extensive disease results in dyspnoea, orthopnoea and haemoptysis (40).

3.7.3 Extrapulmonary tuberculosis:

The most common extrapulmonary sites involved are the lymph nodes followed by pleura of the lung, bones and joints, the genitourinary tract, meninges, pericardium & peritoneum (18). Haematogenous dissemination of tubercle bacilli results in

miliary TB, more commonly seen in settings of advanced HIV infection and long-term immunosuppression (41).

3.8 Diagnosis of Tuberculosis:

The current tests for the diagnosis of TB are aimed at,

a) Early diagnosis and

b) Drug susceptibility testing (DST)

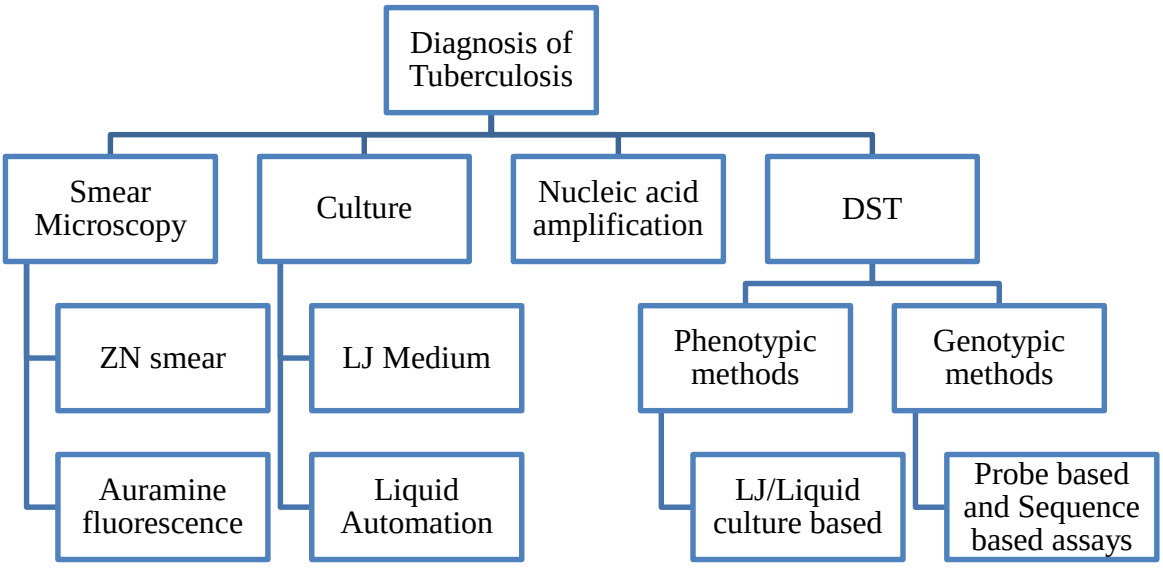


Figure 6: Laboratory diagnosis of Tuberculosis (18). ZN-Ziehl Neelsen, LJ- Lowenstein Jensen, DST – Drug susceptibility testing

3.8.1 Smear Microscopy:

Worldwide, the most common method used for diagnosis of TB is Sputum smear microscopy. The sputum specimens are examined in a microscope (42). It is the primary method for diagnosis of pulmonary TB in low and middle-income

countries (43). It is a simple, inexpensive technique and in areas with very high prevalence of tuberculosis, it is very specific. (43).

Identification of the acid-fast bacilli (AFB) by microscopy is vital due to the following reasons:

a) Rapid method for diagnosing if a person has TB

b) Identifies high risk group persons who are at increased risk of death due to the disease

c) Identifies most likely transmitters of infection

Thus, it has been an integral part of the global strategy for TB control and applicable widely in different populations with varied socio-economic levels (43) (44) (45). It has been shown that the vast majority of cases are diagnosed by the analysis of two sputum samples (spot and early morning). That is, initial specimen was positive in 83%-87%, while 10%-12% in the second specimen. The examination of a third sputum sample further improves the yield by only 3-5 % (46). Thus, present WHO guidelines state that two sputum samples will suffice where work-load is very high, human resources are limited and a good external quality assurance system is in play (5). Further, the WHO, enforces that the countries which have implemented the recent policy for a two-specimen strategy also consider switching to a "same-day diagnosis", mainly in countries with more number of defaulters, though significant organizational changes might be required for the maximum benefit of this strategy (47).

Disadvantages:

Ziehl-Neelsen (ZN) staining has a low sensitivity of 22–43% for a single smear. Maximum sensitivity is up to 60% when compared with that of cultures (48). The threshold of detection of AFB in sputum is 10^4 to 10^5 bacilli per ml. This yield is often decreased due to technical and operational constraints (49). The sensitivity is even lower in paediatric and AIDS patients who usually present a pauci bacillary picture usually. (50) (51).

To overcome the above-mentioned disadvantages of conventional light microscope, "Light-Emitting Diode fluorescence microscope" (LED-FM) was introduced in 1930. It uses an auramine-rhodamine or auramine O dye (acid-fast fluorochrome), weaker acid as a decoloriser , and a counter stain with a quencher such as potassium permanganate (52). The fluorochrome staining is simple as compared to ZN staining (53). Fluorescence Microscopy was recommended by the WHO for detection of AFB in countries with high TB burden. LEDs are inexpensive relatively, can be powered using batteries and mains, with thousands of hours as effective lifespan (54). It's sensitivity is 8–10% higher than ZN staining and specificity similar to ZN staining (55). The increased sensitivity is attributed to the following reasons:

i) The absorbable nature of mycolic acid for carbol-auramine is stronger than for carbol-fuchsin, thus large number of AFB's can be stained with FM in comparison to ZN (56).

ii) Under high power field, larger field areas can be examined in FM as compared to by ZN which uses oil immersion fields (OIF)

iii) Fine contrast between the background and the bacilli, thus enables identification easier (55).

The shortcomings of FM is that the fluorochrome dyes are incorporated by inorganic objects resulting in a possibility of false-positive results (53). It cannot replace culture when it comes to the diagnosis of drug resistance (42).

3.8.2 Culture methods:

Definitive diagnosis is based on identification and isolation of *Mtb* from the clinical specimens (18). Cultures require only 10 to 100 organisms to detect *Mtb* thus, the sensitivity is 80% to 93% and specificity is as high as 98%. The sensitivity for the diagnosis of *M. tuberculosis* is increased by cultures and it also allows drug-susceptibility testing, speciation, and genotyping for epidemiologic purposes if needed (57). *M. tuberculosis*, grows slowly in solid media. It takes around 4–8 weeks for visible growth to be detected. Over the last decades, many new methodologies for diagnosis have been introduced, involving liquid culture, which are faster, with the turnaround time of 10 days though they run the risk of contamination.

a) Solid media:

Egg-based media:

The "Lowenstein-Jensen (LJ) media" is the most commonly used medium. It contains whole egg yolk or whole egg, salts, potato flour and glycerol. It is solidified by inspissation. The characteristics of the media are its good buffering capacity, several months of long shelf life if refrigerated, and supporting good growth of most mycobacteria. Disadvantages of this media are depending on the

quality of the eggs used there can be batch to batch variability, difficulties in discerning colonies from debris, and the inability to achieve consistent and accurate drug concentrations for susceptibility testing (58).

Agar-based media:

The Synthetic Middle brook media -7H10 and 7H11 are the most commonly used agar based media. Compared to egg-containing media, agar-based media are better defined chemically. They do not readily support the growth of contaminants, however, the plates are expensive to prepare and their shelf life is relatively short (1 month in the refrigerator). Agar-based media are transparent and provide a ready means of detecting the early growth of microscopic colonies and can be easily distinguished from inoculum debris (58).

b) Liquid media:

Cultures based on liquid media yield significantly more rapid results (around 8 to 10 days) than solid-medium-based cultures. The rate of isolation for mycobacteria are higher. Middle brook 7H9 and Dubos Tween-albumin broths are commonly used liquid media for both sub culturing stock strains of mycobacteria and preparing the inoculum for drug susceptibility tests and other in vitro tests (58). Currently, for the isolation of mycobacteria, commercially available culture systems in the market ranges from simple tubes, like the MGIT (Becton, Dickinson Microbiology Systems), to the fully automated systems like Bactec MGIT 960 (Becton, Dickinson), VersaTREK culture system II (Trek Diagnostic Systems, Cleveland) or the ESP culture system II and MB/BacT Alert 3D system (bioMérieux) (58). These nonradiometric techniques have been tested in various

studies which showed good concordance against solid media and the radiometric Bactec 460 technique, which was for long considered the gold standard but no longer used now because of concerns of handling radiolabelled products, its disposal and cost (59)(60).

3.9 Drug susceptibility testing (DST):

3.9.1 Need for DST:

Effective TB control mainly aims at preventing drug resistant TB resulting from multifactorial causes like patient-related factors, health care provider, health-care facility. Poor quality and irregular supply of drugs, errors while prescribing the drugs and poor adherence to treatment are known to contribute to drug resistance of *Mtb*. Definitive diagnosis of MDR-TB and XDR-TB requires *Mtb* isolation and identifion followed by a drug-susceptibility testing (DST) using either a solid or liquid media or by doing a molecular test approved by the WHO for the detection of *Mtb* DNA and resistance conferring mutations. (61).

With increasing incidence of drug resistant tuberculosis, the need for a reliable DST report also increases following which an appropriate treatment regimen can be initiated to the patient. There occurs selective multiplication of resistant mutants within the lesions due to inadequate or improper treatment leading to drug resistance. The resistance level of these mutants and its frequency of occurrence vary depending on the sites of action of the drug and the mutated genes (62).

3.9.2 Methods for Drug susceptibility testing:

The techniques available for DST can be divided as follows

Phenotypic methods:

Phenotypic DST involves culturing the *Mtb* in the presence of anti-TB antibiotics to detect the presence of growth (indicates resistance to the drug) or inhibition of growth (indicates susceptibility to the drug).

Genotypic methods:

Genotypic method target unique molecular mutations that can identify resistance of *Mtb* to anti-TB drugs (63).

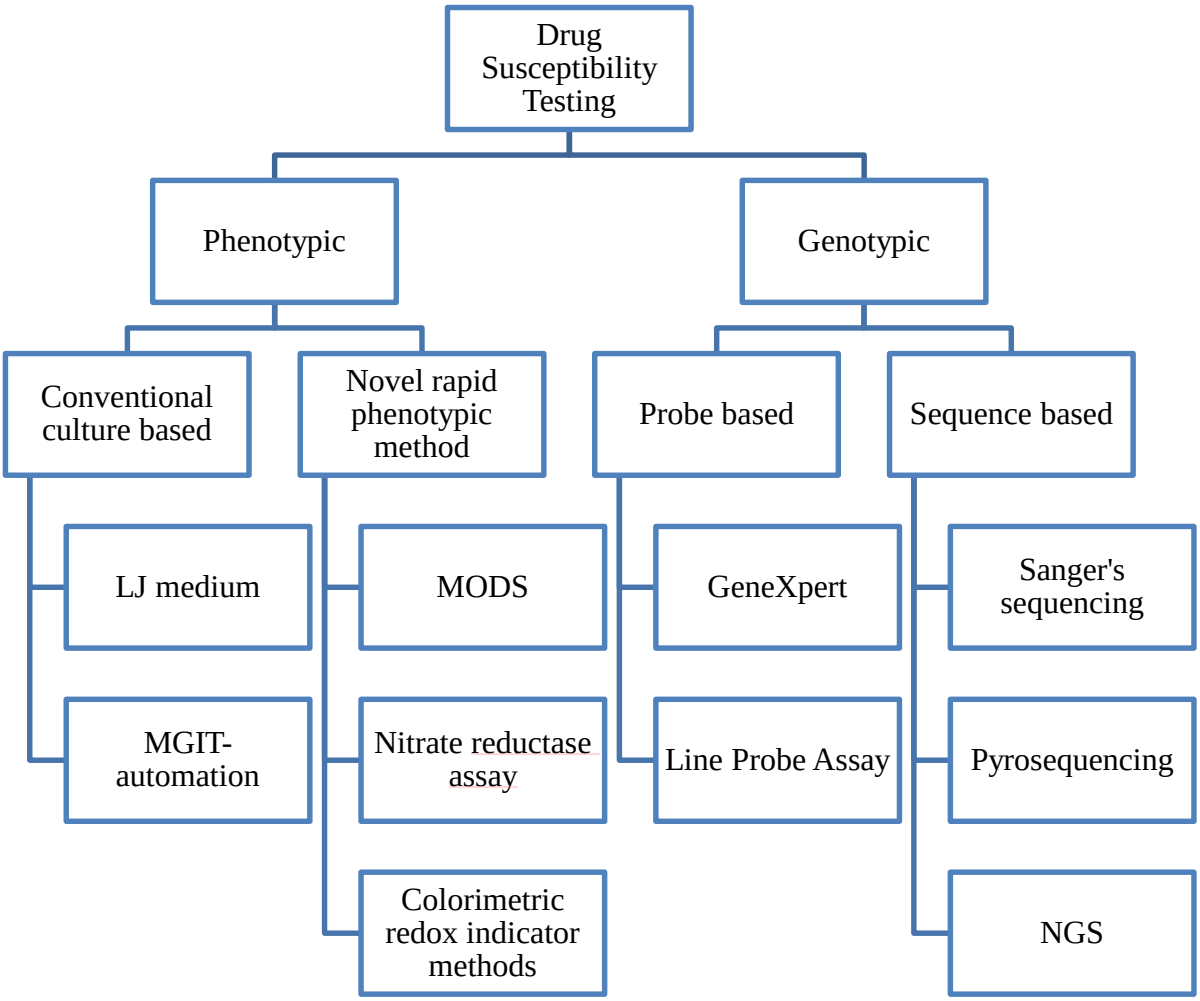


Figure 7: Current Laboratory methods for diagnosis of drug resistant TB (61). MODS – Microscopically Observed Drug Susceptibility, NGS – Next Generation Sequencing

3.10 Phenotypic methods:

Phenotypic methods can be performed either directly or indirectly, on a solid or liquid media.

a) Direct testing:

In direct testing the concentrated specimen is inoculated directly in a medium which is drug-free and and in a medium with drugs. This test is performed only on those specimens that contain adequate numbers (at least one bacillus in ten HPF) of acid-fast bacilli in direct smear examination, because the results may not be reliable when there is scanty growth in culture.

Direct sensitivity testing is not performed in many laboratories, because it is not practical as a routine to examine smears before inoculation of the culture medium and, direct sensitivity tests are not as reliable as the indirect methods. When the direct smear examination is negative or scanty, indirect sensitivity testing may be required (64).

b) Indirect testing:

This method involves inoculation of a pure culture grown from the original specimen in a drug-containing medium. Indirect sensitivity testing is usually used on smear-negative samples or if the direct test results are invalid in case of the drug-free quadrants with insufficient numbers of colonies, contamination or poor growth following three weeks incubation (58). Indirect phenotypic testing methods have been extensively validated and are considered as the current gold standard method. The commonly used methods for solid media are the resistance ratio methods, absolute concentration and the proportion method for solid media and the

proportion method for liquid culture systems. The results obtained with the three methods have shown good concordance to DST for first-line anti-tubercular drugs (63).

3.10.1 Solid culture based methods:

a) Absolute concentration method:

The method employs an inoculum which is standardized, and tested by growing it in media with drugs with graded concentration and in media without drugs. Different concentrations of each drug are tested and resistance is expressed in terms of minimum inhibitory concentration (MIC) (65). The MIC is defined as "the lowest concentration of anti-TB drugs that inhibits more than 99% of the growth of the mycobacterial culture". The MIC is estimated by comparing the control well with growth. The MIC interpretation, is mainly based on breakpoint concentrations for each drug. The reading is interpreted as susceptible if growth occurs at the breakpoint concentration, and interpreted as resistant if growth occurs at higher concentration of the drug (66). Satisfactory results can be obtained only when the inoculum employed is adequately standardized, the critical concentration of the drug should also be standardized for the laboratory and for the reference method to an adequate sample of wild strains (64).

The disadvantage of this method is that it is a phenotypic based method which involves the actual growth of slow growing mycobacteria as an indicator of resistance (66). The viability of organisms may also affect this method (65).

b) Resistance ratio method:

This method involves the comparison of the growth of unknown strains of tubercle bacilli with the standard laboratory reference strain (H37Rv). Media with two- fold dilutions of the drug in parallel are inoculated with the standard reference strains H37Rv. Resistance is calculated as "the ratio of the MIC of the test strain to the MIC of the reference strain in the same set" (65). The test strain is interpreted as resistant if growth occurs on the media containing a given drug, in a given concentration in which control strain is susceptible (67).

The disadvantage of this method is that it is greatly affected by the inoculum size and the strain viability and variations in susceptibility of the reference strain tested may affect the resistance ratio of test strain (65).

c) Agar proportion method:

It employs the following principle - Every wild strain of tubercle bacilli contains some mutants resistant to antibacterial drugs. The difference between a resistant strain and a susceptible strain is that the proportion of bacteria that is resistant among the entire number of bacteria making up the strain is much higher in a resistant strain than in a susceptible one (64).

It uses the seeding of drug-free (control quadrant) and solid media containing drugs with equal quantities of two dilutions of a standardized inoculum. To compare the growths on different media accurately, care must be taken to ensure that a suspension of cells is homogeneous. For the test to be valid, at least one of the dilutions on drug-free media should have isolated countable colonies (50-100 colonies) by 3 weeks (68). If there are less than 50 colonies it indicates insufficient

growth, and the test is invalid and it should be repeated. In this method, the percent resistance is obtained by dividing the colony count on the drug containing media by the colony count on the drug-free media. When the percent resistance is greater than 1%, the strain is considered to be resistant to the drug (58). The proportion method is current phenotypic solid culture based method of choice for detecting drug resistance in MTBC (65) .

3.10.2 Liquid culture based methods:

a) BACTEC 460TB system:

In the developed nations, the radiometric, semi-automated BACTEC 460 TB system (Becton Dickinson, Sparks, MD, USA) is accepted as the reference standard. The growth of mycobacteria occurs in BACTEC 12B medium which contains Middle brook 7H12 broth along with PANTA (Amphotericin B, Azlocillin, Nalidixic acid and Polymyxin B and Trimethoprim) and palmitic acid that is C^{14} labeled. The detection of mycobacterial growth is carried out by measuring $^{14}CO_2$ quantitatively, produced by the metabolism of C^{14}-labelled palmitic acid in the medium and reported as “Growth index” (GI). It gives culture and DST results in 10-14 days, but in India there are only 37 installations of BACTEC 460 TB system, suggesting that it’s not commonly used in India (59). The major disadvantage of this system is the production of radioactive waste, and the consequent dilemma of radioactive waste disposal. Another drawback is the cumbersome handling of vials during re-incubation (59) (60). This system is no longer available commercially (58).

b) MB/BacT System:

MB/BacT ALERT 3D system (Organon Teknika Corp. Boxtel, Netherlands), is a continuously monitored closed nonradiometric fully automated system with a computerized database management. This method employs a middle brook 7H9 medium, growth factors and antimicrobials, MAS (Amphotericin B, Azlocillin, Nalidixic acid and Polymyxin B and Trimethoprim). At the base of each culture vial there is a gas-permeable sensor with colorimetric indicator which changes colour from green to yellow when CO2 is produced actively growing mycobacteria. Every compartment of the instrument where the incubated vials are kept has a reflectometer and a detection unit. Every 10 minutes the values measured are transferred to a computer, which indicates vials with growth of mycobacteria based on an algorithm (69). It detects resistance to Rifampicin, Isoniazid, Streptomycin, Pyrazinamide and Ethambutol. It's performance parameters are good with the sensitivity of 100%, and time to detection of susceptibility is 7 days (70).

The benefits of this system over the BACTEC system are it provides a high level of automation, with decreased risk of vial inversion or transcription errors. It is a closed system with no cross-contamination risk after specimen inoculation. There is no radioactive waste disposal issues and it has data management capabilities. The maintenance cost is low. For the culture of mycobacteria in liquid media the MB/BacT system can be used as an alternative to other radiometric systems. Disadvantages include high rates of contamination and more time for detection (69) (60). This system has also been withdrawn for performing DST (71).

c) The MGIT 960 System:

The "*Mycobacteria* growth indicator tube (MGIT 960, Becton Dickinson, USA)" introduced in 1995 for the growth and detection of mycobacteria from clinical specimens. The MGIT tube contains modified Middle brook 7H9 broth, with OADC (oleic acid, albumin, dextrose and catalase) and a PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) antibiotic concoction and the bottom of the tube contains a silicon rubber which is impregnated with ruthenium pentahydrate (fluorescence-quenching-based oxygen sensor).When actively growing organisms utilize the dissolved oxygen, the reduced oxygen concentration enables the generation of fluorescence, which is then measured and expressed as growth units (GU). It is a fully automated system, monitoring the growth of microorganisms continuously every hour through fluorescence detection. The system uses a nonradiometric medium and does not require the use of needles for addition of growth supplement or inoculation of organisms. The time to detection was around 13.3 days. The drugs are used in slightly lower concentrations than that used in proportion method which brings down the possibility of false susceptibility results (58).

This system was approved by the FDA since the year 2002 for susceptibility testing of MTBC to Streptomycin, Isoniazid, Rifampicin, and Ethambutol (SIRE) and Pyrazinamide. The susceptibility testing to second line drugs in a multicentre study, for Amikacin, Capreomycin, Ethionamide, Prothionamide, Ofloxacin, Linezolid and Rifabutin was shown to be reliable and reproducible (72).

The inconveniences in using this system are, the user has to make the working solutions of the drugs, resistance or susceptibility is not automatically defined by the instrument, as is the case of primary drugs, and based on the GU values retrieved from the instrument results have to be analysed manually. Growth detection failures are also seen with this system (73) (72).

d) Versa TREK:

This was formerly called "ESP II Culture system". It is also a fully automated system, monitoring for growth and detection of mycobacteria continuously, this system is also approved by FDA for susceptibility of MTBC to all primary drugs. The medium consists of a Middle brook 7H9 broth, which has been enriched with growth supplement (casitone and glycerol), and contains cellulose sponges as a growth support matrix that increases the surface area for exposure to oxygen. Oxygen consumption due to microbial growth leads to pressure changes within the headspace above the broth medium in a sealed bottle. This technology is based on the above principle. It has a special detection algorithm for detecting slowly growing mycobacteria (58) (60).

The advantages of Versa TREK include, it is less labour-intensive, the data management is simple for tracking of results and no cross-contamination risks (71) (74).

3.10.3 Non-commercial Rapid phenotypic methods:

The following are the rapid, non-commercial phenotypic culture methods which have been specifically used in resource limited settings.

Microscopic observation of drug susceptibility (MODS),

Colorimetric redox indicator (CRI) method

Thin-layer agar method

The nitrate reductase assay (NRA) and

Mycobacteriophage-based assays (75) (68).

The WHO endorsed methods are MODS, CRI and NRA. For direct testing of sputum specimens NRA and MODS are recommended. It is also recommended for indirect DST of *Mtb* isolates grown in culture along with CRI method. But the above methods cannot detect XDR-TB. Thus the diagnosis of XDR-TB still relies on conventional culture and DST (75).

a) Microscopically observed drug susceptibility (MODS):

This system is a liquid culture-based DST method. It employs the following principles, the growth of *Mtb* is fast in liquid culture medium compared to a solid medium. At an early stage (7 days), the characteristic cord formation can be visualized in a microscope from the liquid medium and by incorporating drugs into the medium it permits rapid and direct DST along with the bacterial growth detection (76).

The MODS method does not require the use of any proprietary culture media. It employs Middle brook 7H9 broth medium, supplemented with OADC and a selective antibiotic/ antifungal cocktail. It uses a 24-well microtiter plate.12 wells are used for each specimen, including 4 control wells with no antituberculous drug and 8 drug-containing wells including low and high concentrations of INH, RIF, EMB, and STR. The 24-well microtiter plate should be sealed in plastic bags and

the microscopic observations are made without opening them. MODS results are read by examining each well with an inverted microscope (77).

Growth of MTBC is indicated by the presence of cording clumps. The method enables rapid detection of growth and provides a quick indication of susceptibility or resistance to the primary drugs. The percentage agreement for susceptibility testing between MODS and the reference standard is 97%, 100%, 95%, 97% for isoniazid, rifampin, ethambutol, streptomycin respectively (92%) (78).

The benefits of MODS include the lack of any proprietary ingredient and its rapid and accurate detection of *M.tuberculosis* along with detection of MDR-TB. This method is cost effective in resource limited settings with high TB burden (77) (78).

The drawbacks are the MODS method entails some requirements that may be difficult to achieve in limited-resource settings like acquiring and storing labile ingredients such as OADC. It also requires the sophisticated equipment and facilities, like a biological safety cabinet, high-speed centrifuge, incubator, inverted microscope etc., and safety practices of a biosafety level 3 laboratory (58).

b) Colorimetric Redox Indicator:

Colorimetric method works on the following principle Reduction of a coloured indicator added to the culture medium after *Mtb* has been exposed in vitro to different antibiotics. Resistance to a particular drug is detected by reduction of the dye from blue to pink colour (Alamar blue dye), due to oxidation-reduction mechanism of viable organisms, which is directly proportional to the viable number of mycobacteria in the medium. The indicators evaluated have given comparable results in comparison with the gold standard proportional method. The various

indicators used are the tetrazolium salts, the redox indicators resazurin and alamar blue. For isoniazid and rifampicin studies have shown sensitivity and specificity ranging between 89% to 100%. (79) (80).

c) Nitrate reductase assay (Griess method):

M.tuberculosis reduces nitrate to nitrite. Incorporation into the LJ medium of Griess' reagent detects this reaction and produces a coloured product, which determines if the organism is susceptible or resistant. Susceptible organisms, in the presence of antibiotics, are unable to carry out the reduction of nitrate to nitrite, whereas resistant organisms can and produce a red-pink colour of the medium. It can be carried out both on clinical samples and on culture isolates. The average time for results is around 18 to 28 days. For the detection of INH resistance the sensitivity and specificity was 94% and 100% respectively and for RIF resistance, the sensitivity and specificity was 99% and 100% respectively. However, the Griess reagent kills the organisms when added to the tubes, so if further testing is needed multiple tubes are needed to be inoculated. In addition, nitrate-negative AFB does not exclude MTBC, because not all members of the MTBC reduce nitrate, thus requiring further testing (68) (81).

Rapid phenotypic methods – Limitations:

Limitations include the lack of reliable conventional breakpoints for interpretation, reduced accuracy in mixed mycobacterial cultures, reduced fitness, growth of other mutant organisms requiring higher inoculum to increase the sensitivity of the test (82).

3.10.4 The need for rapid testing in drug resistant TB:

Improved TB control mainly relies on early diagnosis of drug resistance and early institution of proper treatment regimen. Globally due to the increasing incidence of MDR-TB and XDR-TB there is an urgent need for rapid methods of DST in *Mtb*. The national TB control programmes are aimed at preventing the spread of drug-resistant TB and treating the patients diagnosed as MDR and XDR-TB. Due to the lack of appropriate diagnostic methods and deficiencies in the laboratory capacities there occurs hurdles in achieving the above aims (63) (61).

A rapid and reliable diagnosis is essential for treating drug-resistant TB. The DST tests are therefore crucial. *M.tuberculosis* bacteria grow slowly - the agar proportion method requires six to eight weeks to give results, while the liquid culture methods gives results in four to five weeks. Molecular methods can cut down the turn-around time for resistance testing to a few days. This reduces periods of infectiousness of TB cases by at least a month, reducing the further spread of the disease to both the community and the health care worker, and better treatment results for the patient with far less morbidity and mortality (5) (61).

The benefits of molecular techniques in the detection of drug resistance are that they are robust, rapid, has high throughput, with lesser technical skills and manipulations as compared to the gold standard conventional culture and DST. These molecular methodologies require only simple-to-use equipment which serve as a platform for testing clinical samples for various other diseases also. Additionally, specimen transport conditions does not impact the outcome of the

molecular tests since they does not require viable bacteria for the purpose of diagnosis (68).

3.11 Genotypic methods:

Real-time PCR coupled to fluorescence detection by molecular beacons or other probes has been used for detecting INH resistance in MTBC. Various molecular methods like Sanger sequencing, Pyrosequencing and Next-generation sequencing, is also being designed for detecting resistance to INH, RIF, EMB, PZA, FQ, and SLI.

Xpert MTB/RIF and Molecular LPA (Line Probe Assays) are presently the two molecular methods endorsed by WHO for the detection of drug resistance in MTBC genotypically. These methods have shortened the time to diagnosis of MDR-TB by detecting INH and RIF resistance. This aids in screening of patients at risk of MDR-TB, identifying patients on inappropriate or inadequate first-line anti-TB regimens, thus interrupting the transmission of MDR-TB. Molecular methods like Hain MTBDR*sl* assays, Pyrosequencing assays have also been widely used for detection of Second-line drug resistance and XDR-TB (63) (58).

Molecular methods are divided into two major categories, which are probe-based methods and sequence-based methods.

Probe-based methods:

Cepheid's GeneXpert MTB/RIF assay using molecular beacon probes, and INNO-LiPA Rif.TB, Hains's MTBDR, Hain's MTBDR*plus* and Hain's MTBDR*sl* assays using line probes are probe based methods.

Sequence-based methods:

Sanger sequencing, Pyrosequencing and Next-Generation Sequencing are sequence-based methods.

Probe-based methods detect the presence or absence of mutations, while sequence based methods provide the exact sequences of either wild-type organisms or mutants. Since all mutations are not always associated with drug resistance, when a mutation is detected by probe-based methods without providing the identity of the mutation, it should be confirmed by a sequence-based method. Revealing the identity of a mutation allows discerning the association of the mutation with drug resistance (58).

3.11.1 Probe based methods:

a) Xpert MTB/Rif (Cepheid, Sunnyvale, USA):

This is a self-sufficient, automated system that can be deployed with basic skills. Decontamination and concentration (using sodium hydroxide and isopropranol) is performed in 15 minutes. The cartridge-based system incorporates an extraction method where the DNA is captured on a filter, washed and lysed via sonication. It is a fully automated hemi nested real time PCR assay using 6 molecular beacons (five of which are for the *rpoB* gene and the last is an internal control). Detection and quantitation of the targeted nucleic acid sequence is done in approximately 1 hour 45 minutes. Rifampicin resistance is detected easily because 95% of all Rifampicin-resistant *M.tuberculosis* strains contain mutations localised within the 81 bp core region of the bacterial RNA polymerase β subunit (*rpoB*) gene, which

codes for the active site of the enzyme. These are highly suggestive of Rifampicin resistance. On either side of this region are *M.tuberculosis* complex-specific DNA sequences. Thus, *M tuberculosis* and Rifampicin resistance can be tested concurrently by targeting one amplicon generated with PCR technology. Moreover, Rifampicin resistance is strongly indicative of MDR-TB.

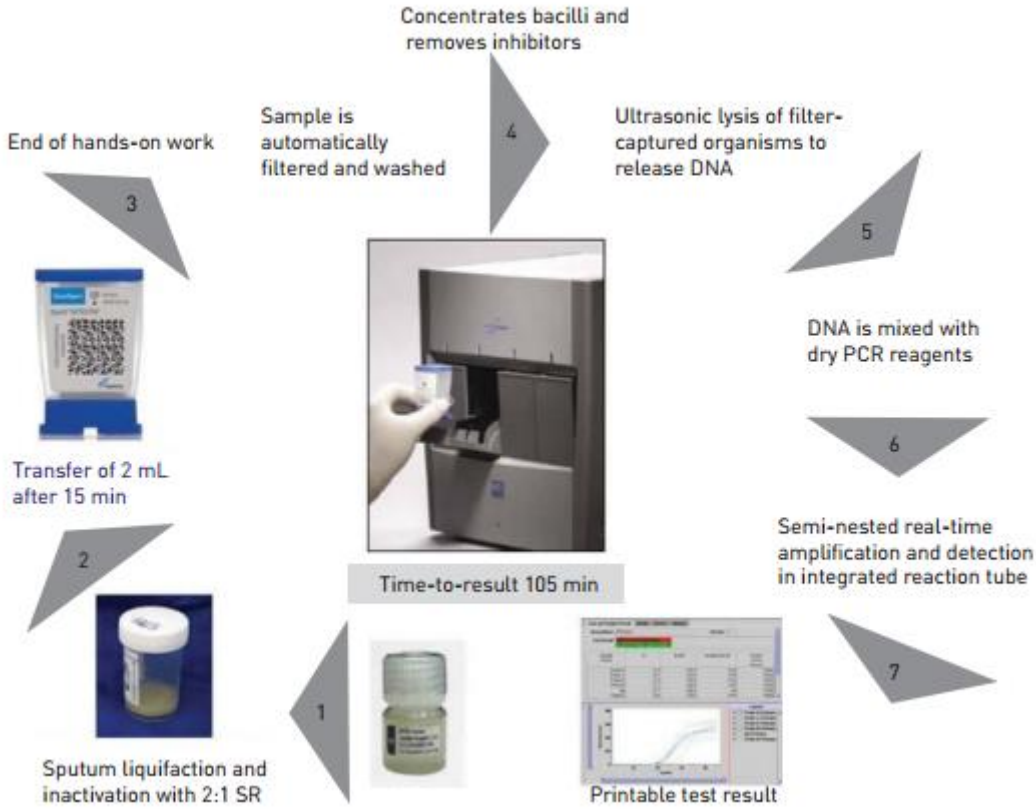


Figure 8: Steps of Xpert MTB/Rif Assay (83).

It has an analytical sensitivity of 5 genome copies of purified DNA with the limit of detection 131 CFU/ml (84) (83), which is much lower than that of smear microscopy (10000 CFU/ml), is similar to solid culture, but is not quite as sensitive

as liquid culture (10 CFU/ml). Studies have shown that the assay poses a minimal biohazard risk and given adequate room ventilation, might be done without the need for a biosafety hood. In sputum positive cases it has high sensitivity ranging from 98%-100% and a high specificity ranging from 97-100%. Rifampicin resistance was detected with 95.1% sensitivity and 98.4% specificity. For detecting extra pulmonary TB the sensitivity and specificity was 86% and 100% respectively (85) (84) (68).

There are few issues restricting the performance of the Xpert MTB/Rif assay at small health care settings. Even though certain centres successfully tested battery operation, this device requires stable electricity supply. Device deployment above 30°C is not recommended currently by the manufacturer and cartridges are stable at temperature 2‑28°C. The Gene Xpert device also needs to be calibrated yearly. It also has a low sensitivity in smear negative tuberculosis ranging from 72-75%, the high cost of cartridge and reagents, and its inability to detect mutations outside the pre-specified target region and inability to detect Isoniazid mono resistance (84) (68).

b) Line probe assays:

The line probe assays are a family of DNA strip tests that uses nucleic acid amplification and reverse hybridization techniques for the rapid detection of drug resistant tuberculosis (70). It is based on the hybridization of labeled amplicons to oligonucleotide probes that have been immobilized on a membrane strip (71). These assays, though not as rapid or as cheap as the Xpert (MTB Rif), have the

advantage of detecting resistance to second line drugs. A meta-analysis for the Genotype MTBDR has shown a high sensitivity and specificity for Rifampicin resistance -98.1% and 98.7% respectively. The sensitivity for Isoniazid resistance was 84.3% while specificity was 99.5% .The meta-analysis also showed an improved sensitivity for the detection of Isoniazid resistance-90%, when it was performed with the newer Genotype MTBDR*plus* assay, which also detects *Inh A* mediated resistance (72). A second version of the Genotype MTBDR*plus* assay has been introduced, which is an improvement over the first version for extrapulmonary samples. A line probe assay for the diagnosis of XDR-TB (Hain's Genotype MTBDR*sl* kit) has also been introduced which picks up resistance to Ethambutol, Aminoglycosides and Fluoroquinolones. The latter test, however has not yet been endorsed by the WHO for testing clinical specimens. Although the line probe assays have several advantages, they cannot be read in isolation and their use does not preclude the need for culture.

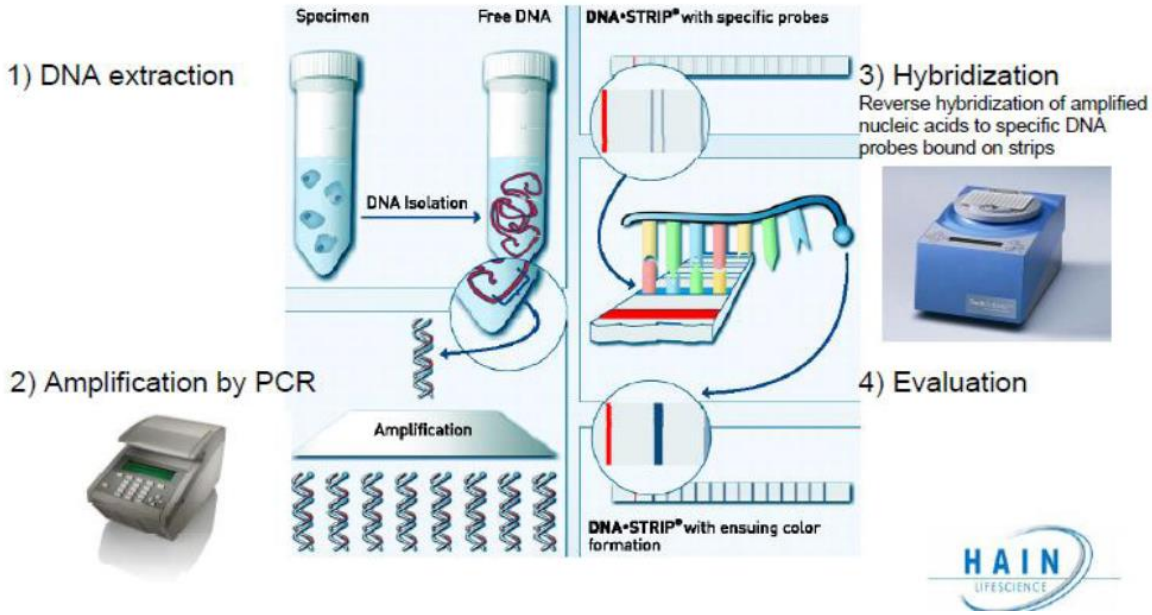


Figure 9: Procedure of Line Probe Assay (86).

Draw backs of LPA:

Line probe assays are complex and requires biosafety cabinets. LPA is suitable only for regional and national level laboratories. It requires at least three separate rooms, dedicated equipment, reagents and consumables to decrease DNA cross-contamination. WHO recommends LPA to detect resistance to INH and RIF only on specimens which are smear-positive and isolates of *Mtb*. LPA is used in the diagnosis of MDR-TB but conventional phenotypic culture methods (solid or liquid) is required in monitoring treatment response for MDR-TB patients (culture conversion). To detect XDR-TB conventional second-line DST is required because WHO has not currently recommended the use of LPA for diagnosis of resistance to second-line drugs due to sub-optimal performance of the test on clinical specimens (87) (68).

The major problem is the interpretation and readings of different LPA patterns even for a technically skilled laboratory personnel. There can be patterns in LPA which may represent silent mutations (no change in amino acid) and may be susceptible by phenotypic methods. These molecular assays lack the capacity to detect lower levels of resistant organisms in a heteroresistant population (mixed wild-type and mutant) which limits the use of LPA for the early detection of drug resistance. The LPA does not have the ability to differentiate between a high level and low level resistance mutations which may guide the clinicians either to increase the dose of a particular drug or discontinue the drug (68).

3.11.2 Sequence based methods:

a) DNA Sequencing (Sanger's):

The DNA fragments are sequenced by "chain-termination method" in Sangers sequencing. It is considered as the gold standard DNA sequencing method. Here, the primer specifically binds to the single stranded DNA template. DNA polymerase initiates DNA extension which begins at the site of primer binding. DNA extension is then terminated eventually due to a dye-labelled dideoxynucleotide (ddNTP) which interrupts the bond between the two subsequent nucleotides (phosphodiesterase bond). This results in DNA fragments of varied lengths. The DNA fragments are separated by electrophoresis and sequenced later. It remains the gold-standard of DNA sequencing. It's very accurate, and it can read larger amounts of DNA. Due to the lack of time, expertise, and cost Sanger's sequencing is not been used routinely in commercial settings for detecting drug resistance in *Mtb.* It is only used for research purposes (82).

b) Pyrosequencing:

Pyrosequencing (PSQ) is a novel rapid DNA sequencing technology, which was first developed as an alternative to conventional Sanger sequencing for de novo DNA sequencing.

Principle:

PSQ technique is based on "sequencing by-synthesis" principle. The method detects the released pyrophosphate (PPi) formed during DNA synthesis. During DNA synthesis, it uses four enzymes series for the detection of nucleic acid sequences. In

PSQ assay the sequencing primer gets hybridized to a single-stranded biotin-labelled DNA template followed by reaction with the four enzymes – DNA polymerase, Luciferase, ATP sulfurylase and Apyrase. The reaction utilizes the following substrates – Adenosine 5′ Phosphosulfate (APS) and Luciferin. The reaction mixture is added with four deoxynucleotide triphosphates (dNTPs) seperately. Due to nucleotide incorporation by the polymerase enzyme, PPi is released, proportional to the amount of incorporated nucleotide. The PPi is converted quantitatively by ATP sulfurylase to Adenosine Triphosphate in the presence of APS. The Adenosine Triphosphate (ATP) initiates the conversion of luciferin to oxyluciferin in the presence luciferase. Thus visible light is produced which is directly proportional to quantity of ATPs produced. Apyrase (enzyme degrading nucleotides) degrade the ATPs continuously and the non-incorporated dNTPs in the reaction mixture. The sequence of the template DNA can be determined since the added nucleotide is known. The generated light can be seen as a signal peak in the pyrogram which is directly proportional to the quantity of incorporated nucleotides. (88).

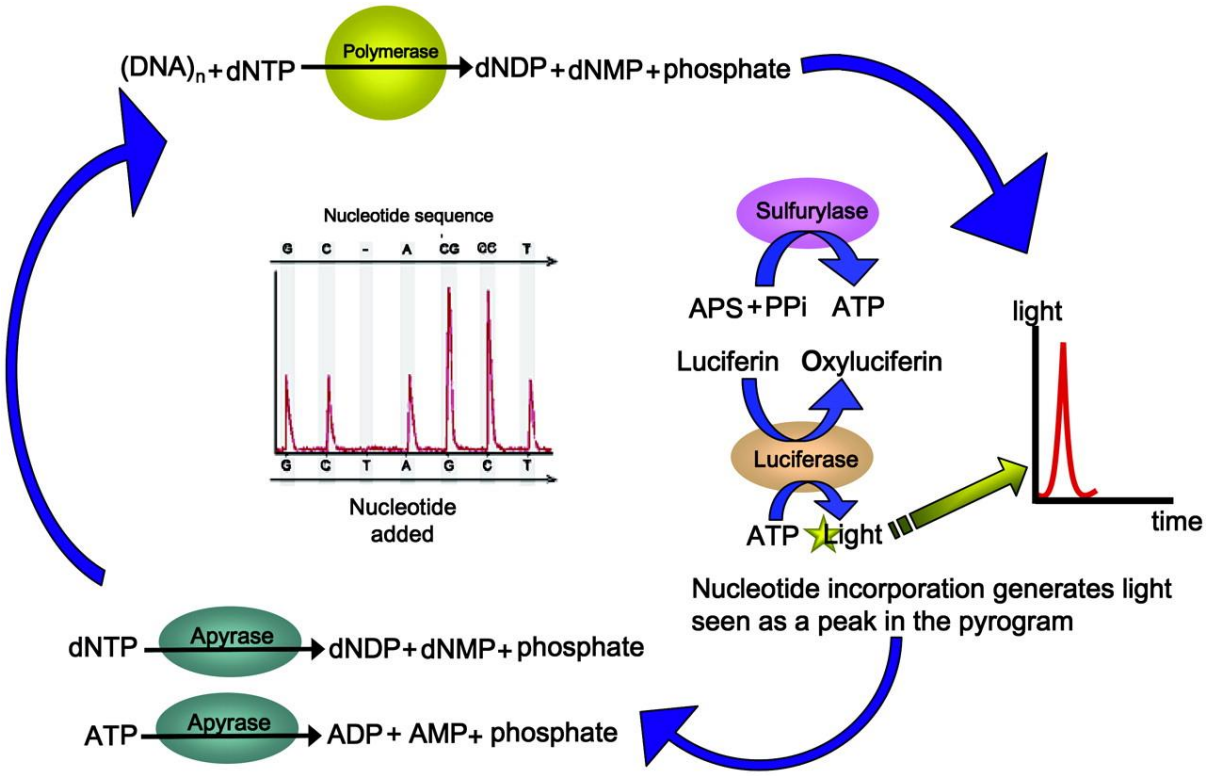


Figure 10: Schematic representation of Pyrosequencing (88).

Similar to Sanger sequencing, PSQ also provides exact DNA sequences, thus detecting both novel and previously known mutations. In case of discrepant results where phenotypic and genotypic results doesn't correlate PSQ can be used to resolve the issue. It can also discriminate between a low and high level resistance mutation thereby guiding the clinician in therapy.

Advantages:

It's is an up-coming latest tool for the rapid identification of *Mtb* in both growth-positive cultures and smear-positive sputum specimens. The sequencing assays can be identified and reported within 1 to 3 days using automated sequencers and well trained staffs. Other advantages include simplicity of processing, speed, reduced cost, ease of interpretability, and relative high throughput (82) (68). For testing

clinical isolates sensitivity of the assay was 94% for INH, 96% for RIF, 93% for FQ, 84% for AMK, 88% for CAP, and 68% for KAN. The specificities of the assay were 100% for RIF, FQ, AMK, and KAN, 96% for Isoniazid and 97% for Capreomycin. With regard to performance in testing clinical specimens, the PSQ assay yielded a sensitivity of 98.4% and specificity of 95.8% for all sub assays (89) (90).

Disadvantages:

A major drawback of Pyrosequencing is that, currently the technique is limited to analysis of short DNA sequence (< 50 nucleotides). The mutations which are located outside the target gene of interest cannot be detected by this assay. The maintenance, installation, and cost limits it's use in poor resource settings (88) (68).

c) Next-Generation sequencing (NGS)

NGS is referred as "Massive-parallel DNA sequencing", "Deep sequencing" or "Second-generation sequencing" because of the high-throughput DNA sequencing technology which is capable of sequencing large numbers of different DNA sequences in a single reaction (in parallel). Basic work flow of NGS includes four phases that is sample collection, template generation, sequencing reactions and detection, and data analysis. In contrast to Sanger sequencing, the sequencing speed and the DNA sequence data generated with NGS are exponentially greater, and are produced at significantly reduced costs (91).

4. Materials and methods

4.1 Study design:

This was a prospective study for a period of 1 year, 10 months done on clinical isolates of *Mycobacterium tuberculosis* obtained from patients with Tuberculosis, for detection of mutations in *rpoB* (RIF), *gyrA* (FQ), *rrs* and *eis* promoter (CAP,KAN) genes by PSQ and the PSQ results were correlated with the conventional gold standard drug susceptibility testing results.

4.2 Ethics approval:

The approval for the study was obtained from the Institutional Review Board, Christian Medical College, Vellore (IRB Min. No. 9131 dated 12.11.2014).

4.3 Study Duration:

The study was conducted over a period of 1year, 10 months from November 2014 to August 2016.

4.4 Study samples:

The study samples comprised of clinical isolates of MDR TB/XDR TB and susceptible MTB isolates. The samples were received in the Microbiology laboratory as part of routine diagnostics for culture and routine mycobacterial drug resistance testing.

4.5 Sample size calculation:

The sample size was determined based on the prevalence of MDR- TB and XDR-TB in the community and sensitivity and specificity of the experimental test.

Consecutive culture isolates which were resistant and sensitive were selected and were evaluated by Pyrosequencing assay.

Number of samples to be evaluated - 50 (40MDR/XDR TB isolates, 10 susceptible isolates)

Based on the formula 4pq/d2 + 4p1q1/d2 (Where p=sensitivity, q =100-sensitivity, p1=specificity, q1=100-specificity and d =10%)

4.6 Inclusion criteria:

1. Clinical isolates which were found to be MDR TB/XDR TB by the gold standard agar proportion method of mycobacterial drug susceptibility testing.

2. Clinical isolates which were found to be susceptible TB by the gold standard agar proportion method of mycobacterial drug susceptibility testing.

4.7 Exclusion criteria:

1. Clinical isolates of those samples which were not requested for mycobacterial drug susceptibility testing.

2. Clinical isolates with no growth on the LJ medium used for mycobacterial drug susceptibility testing.

3. Clinical isolates which fail Pyrosequencing assay while performing the run.

4.8 Data source

Relevant information about the clinical isolates of the patient samples were obtained from the Clinical Work Station and clinical records. Testing of the clinical

isolates were undertaken in the Department of Clinical Microbiology and Department of General Pathology.

4.9 Study algorithm:

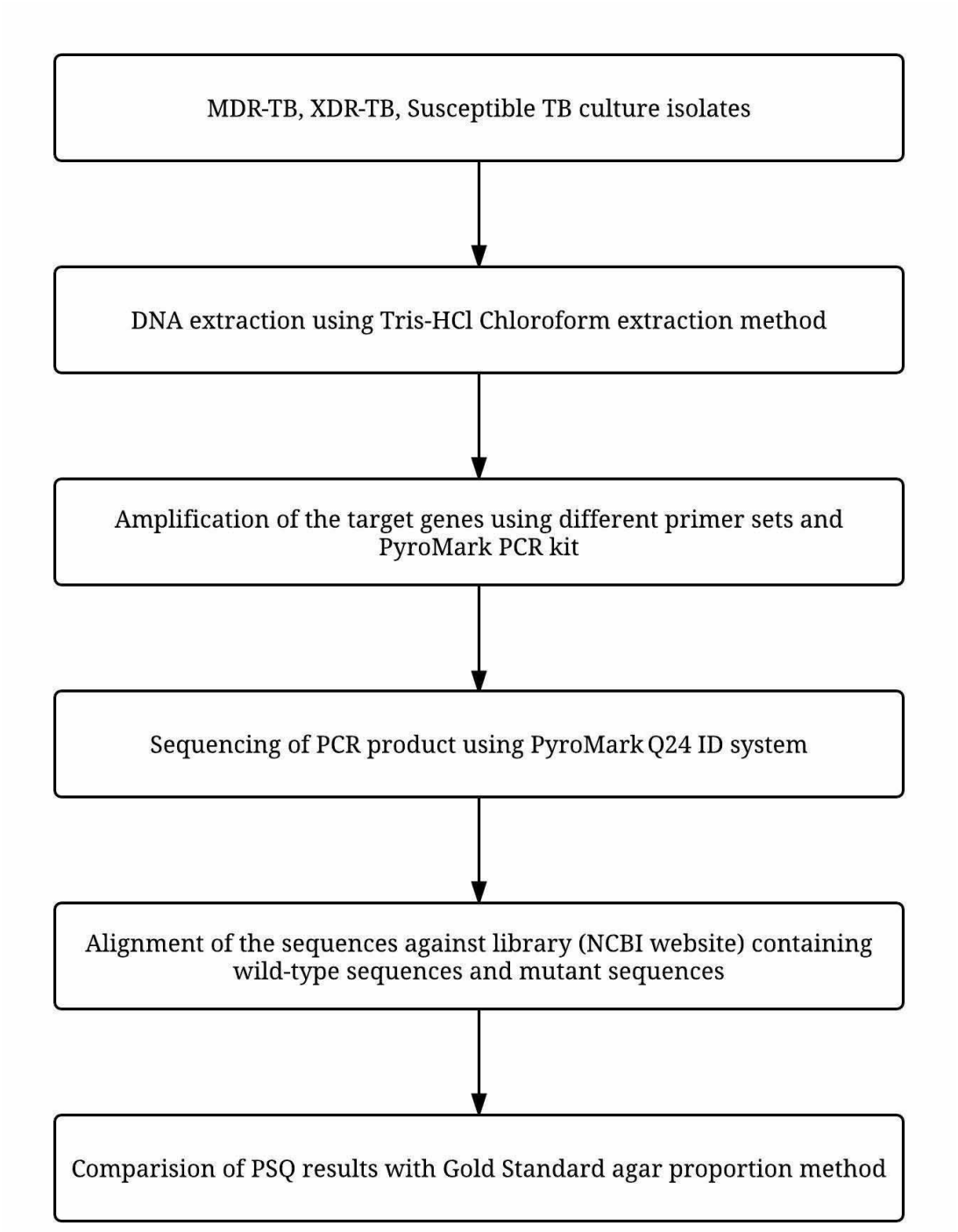


Figure 11: Detailed Study Algorithm

4.10 The Reference Test:

4.10.1. 1% Agar Proportion method:

This method enables precise estimation of the proportion of mutants resistant to a given drug. Ten-fold dilution of inoculums were plated in both control and drug containing media, at least one dilution should yield isolated countable colonies (50-100 colonies). When these numbers were corrected by multiplying by the dilution of inoculums used, the total number of viable colonies observed on the control medium and the number of mutant colonies resistant to drug concentration tested may be determined. Only one concentration per drug is used:

Streptomycin (STM): 4 µg/ml
Isoniazid (INH): 0.2µg/ml
Rifampicin (RIF): 40µg/ml
Ethambutol (ETM): 2 µg/ml
Capreomycin (KAP): 40µg/ml
Ofloxacin (OLOX): 2µg/ml
Kanamycin (KAN): 30µg/ml
Ethionamide (ETH): 40 µg/ml

The suspension (4mg moist weight of representative sample of bacterial mass) was visualized as 2/3 loopful of 3mm internal diameter 24SWG wire-loop into 0.2ml of sterile distilled water in a 7ml Bijou bottle containing 2-3mm glass beads. It was votexed for 30 seconds to make an uniform suspension. 3.8ml of sterile distilled water was added to make the suspension comparable to Mc Farlands opacity tube No. 1. Suspension was allowed to stand on the bench so that coarse particles settle

down. From this suspension a ten-fold dilution was made by adding 0.2 ml to 1.8 ml sterile distilled water (1/10). Two further serial dilutions were prepared in a similar manner. One standard loopful (3mm diameter, 27SWG) was inoculated onto drug free as well as drug containing LJ SLOPES.

Standard Strain "*M.tuberculosis* H37RA" was tested with each new batch of medium.

Incubation: 37degree centigrade for 28 – 42 days

Table 1: Growth recording

+++	Confluent Growth
++	>100 Colonies
1-99 colonies	Actual number of colonies

When number of colonies on a given dilution is <15, the number of colonies with next larger inoculums were counted or estimated if >100.

Interpretation of DST:

Based on the 42 days readings, the number of organisms resistant to each drug concentration was expressed as a percentage of the number of organisms growing on a drug free slope. The slopes were selected for estimating the growth on drug free and drug containing media. The colonies on LJ with 5 to 100 colonies both on drug free and drug containing media were enumerated.

The proportion % is calculated with the following formula:

Average no. of colonies of drug containing media/ Average no. of colonies on drug free media * 100

<1% Susteptible, >1% Resistant.

4.10.2 The index test – Pyrosequencing assay:

The Pyrosequencing assay includes three essential steps –

1) DNA Extraction.

2) Amplification of the target gene segments by Polymerase chain reaction (PCR).

3) Capture of the single-stranded DNA on streptavidin-sepharose beads followed by sequencing with Pyrosequencing technology.

Step 1 – DNA extraction

Requirements:

- Culture isolates of MTB (Resistant/Susceptible)
- Smear loop
- Eppendorf tubes
- 50mM Tris-HCl (pH 8.0)
- pH meter
- Chloroform
- Dry bath (temperature to be set for 85 °C for 30 minutes)

- Nuclease free water
- Pipettes
- Pipette tips (200 µL, 1000 µL)
- Vortex machine and centrifuge
- Nanodrop (Quantification of DNA)

Procedure:

i) A loopful of bacterial colonies were taken from the LJ medium and emulsified in 1 ml of 50 mM Tris-HCl (pH 8.0).

ii) The bacterial cells were inactivated and lysed by placing it in a dry bath at 85°C for 30 minutes.

iii) The suspension of lysed cells were then allowed to cool to room temperature and centrifuged at a relative centrifugal force of 12,000 × *g* for five minutes. Subsequently the supernatant was decanted and the pellet was retained.

iv) The pellet was then suspended in 100µl of 1:1 mixture of chloroform and water and the suspension was vortexed for five minutes. This was followed by centrifugation at a relative centrifugal force of 12,000 × *g* for five minutes.

v) The supernatant containing the extracted DNA was subsequently removed and subjected to quantification by measurement of optical density at 260 nm using Nanodrop.

vi) The extracted DNA of the isolates were diluted to around 20 ng/µl-40 ng/µl and stored at −20°C for further molecular work up (92).

Step 2 – Amplication of target genes by polymerase chain reaction

Requirements:

- DNA Extract
- Pyro PCR master mix (Qiagen)
- Primer set (Forward and Reverse Primers) Lin *et. al.* (90).
- Nuclease free water
- Coral load (10X)
- Microtubes
- Pipettes
- Pipette tips (10µL, 20µL, 200µL)
- Vortex machine and centrifuge
- Thermal cycler
- Electrophoresis gel and tank
- Gel documentation system

Primers:

The insertion sequence "IS6110" was used as the molecular target for identification of *Mtb* Complex. The molecular targets used for determination of drug resistance are as follows –

1. For Rifampicin (RIF) – RIF resistance-determining region (RRDR) of *rpoB,*
2. For Fluroquinolones (FQs) – Quinolone resistance-determining region (QRDR) of *gyrA*

3. For the injectable drugs – Capreomycin (CAP) and Kanamycin (KAN) – *rrs* and *eis* promoter region

Either the forward or the reverse primers were biotinylated. The PCR primers used are listed in table 2.

Table 2: PCR Primer Sequence Lin et. al. (90)

Drug	Locus and primers	Sequence
MTB Complex	*IS6110* Forward Reverse	 Biotin-CCGCCAACTACGGTGTTTA Reverse CAGGCCGAGTTTGGTCAT
RIF	*rpoB* Forward Reverse	 TTTCGATCACACCGCAGACGTT Biotin-AAGGCACGCTCACGTGACAGAC
FQ	*gyrA* Forward Reverse	 AATGTTCGATTCCGGCTTCC Biotin-CGGGCTTCGGTGTACCTCAT
AMK CAP KAN	*rrs* Forward Reverse *eis* Forward Reverse	 TAAAGCCGGTCTCAGTTCGGAAC Biotin-CAGCTCCCTCCCGAGGGTTA Biotin-GGCTACACAGGGTCACAGTC GCCAGACACTGTCGTCGTAATATTC

Master mix preparation:

All the reagents required for performing PSQ assay was obtained from Qiagen. The concentrations of the different components of the PCR mix for one reaction are given below in Table 3.

Table 3: Master mix preparation

Reagent	Volume per reaction
PyroMark PCR Master Mix, 2X	18.75 µL
CoralLoad Concentrate, 10X	3.75 µL
Primer	3.75 µL
Nuclease free water	6.25 µL
DNA Extract	5 µL
Total Reaction Volume	37.5 µL

Procedure for amplification:

1) The master mix was prepared for the appropriate number of reactions with the above template. Separate master mixes were prepared for the each reactions.

2) Appropriate number of 0.5ml PCR tubes were labelled appropriately, and the master mix was distributed in them.

3) DNA extracts were removed from the storage area, brought to room temperature and spun briefly in a micro centrifuge.

4) 5μL of the DNA extract was added to each tube appropriately.

5) Amplification was carried out in Veriti TM Thermal Cycler (Applied Biosystem, California, USA).

Cycling conditions: (Lin *et. al.*) (90).

Table 4: Cycling conditions

Steps	Time	Temperature
Initial activation	15 min	95 °C
Cycling:		
i) Denaturation	15 sec	94 °C
ii) Annealing	30 sec	60 °C
iii) Extension	20 sec	72°C
No. of cycles	50	
Final extension	5 min	72°C

Post amplification analysis:

1) 2% agarose gel containing 0.5 µg/ml ethidium bromide was freshly prepared. Five microliters of each amplicon was mixed well and used for agarose gel electrophoresis to detect the amplified product.

2) The test amplicons were loaded in appropriate wells. 100bp molecular ladder was used. The amplified products were then subjected to electrophoresis. The electrophoresis was performed at 140 volts for 30 minutes.

4) The gel was visualized by ultraviolet radiation using Quantity One® (version 4.6.2) software in the gel documentation system (Bio-Rad, Hercules, California, USA).

5) Isolate amplicons showing discrete desired bands (Fig.12, 13, 14) were taken up for Pyrosequencing assay.

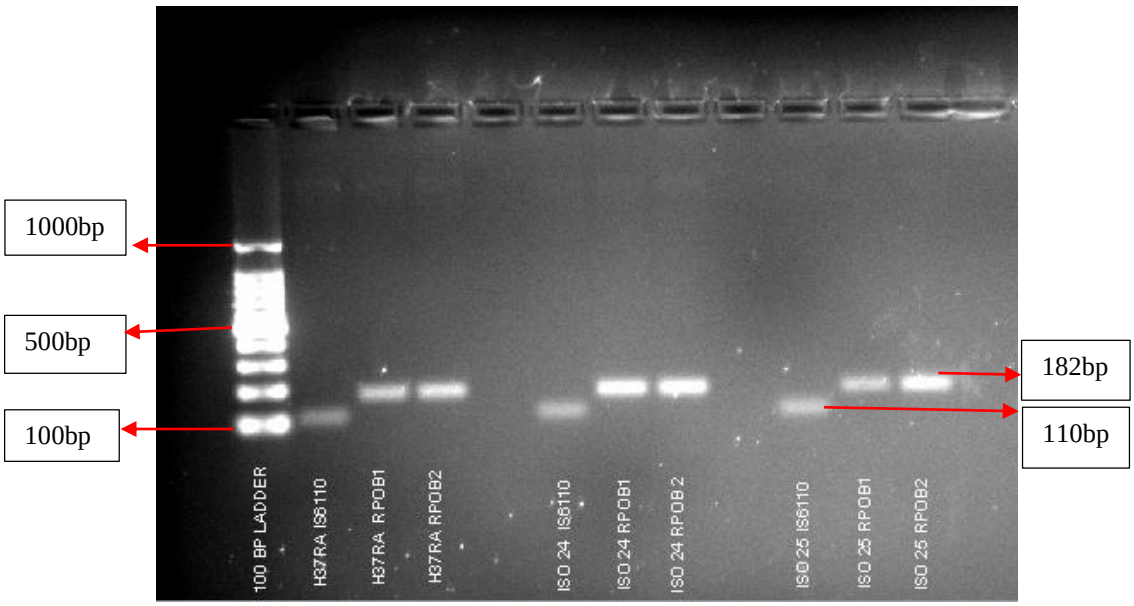


Figure 12: Gel picture showing *rpoB* bands of the control isolate (H37RA) and test isolates.

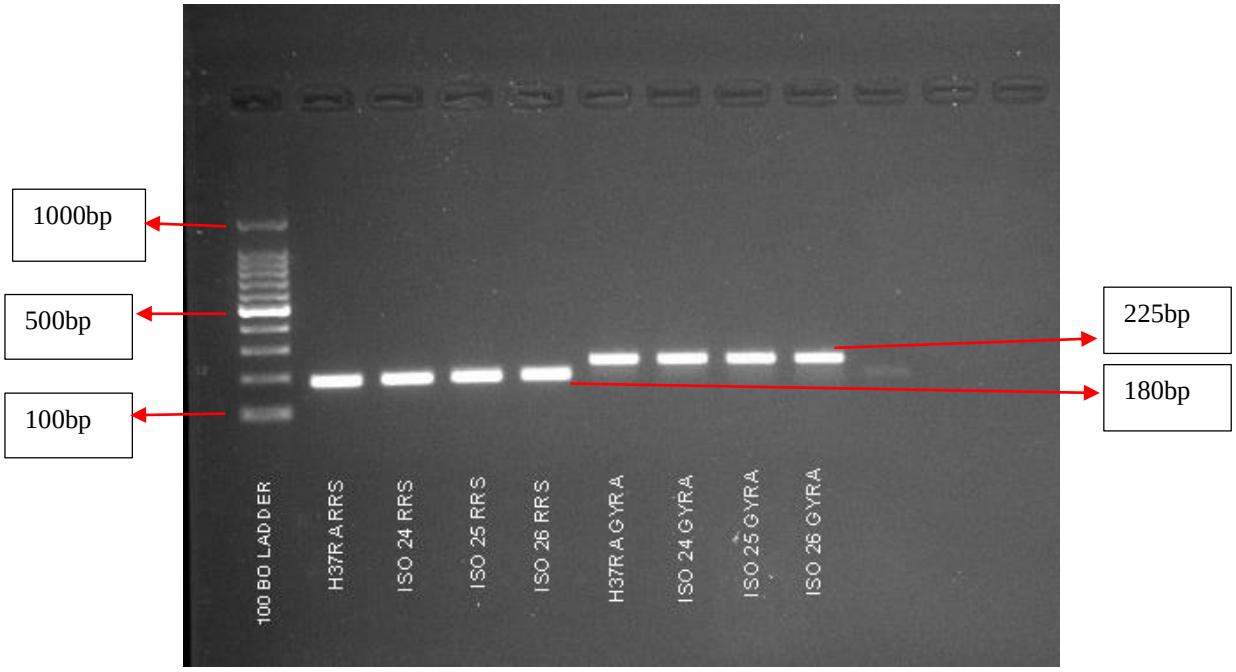


Figure 13: Gel picture showing *rrs* band and *gyrA* band of the control isolate (H37RA) and test isolates.

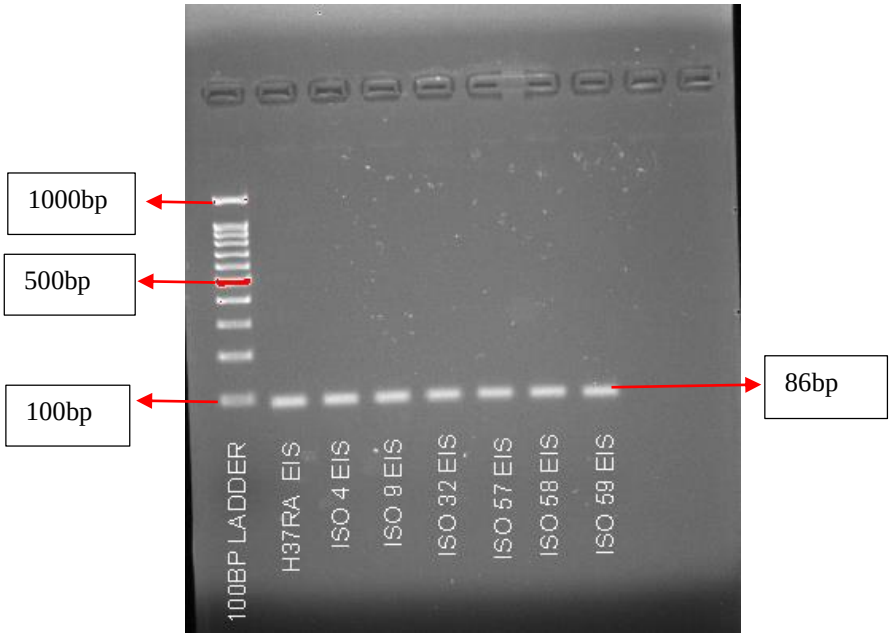


Figure 14: Gel picture showing *eis promoter* region of the control isolate (H37RA) and test isolates.

Step 3: Pyrosequencing assay

The following equipment and reagents were required for sample preparation for Pyro Mark Q24 Instrument. All reagents were allowed to come to room temperature (15–25°C) before starting.

Requirements:

- PyroMark Q24 Vacuum Workstation
- Plate mixer for immobilization of beads
- Heating block – 80°C
- PyroMark Q24 Plate
- 24-well PCR plate or strips
- Strip caps
- Streptavidin Sepharose High Performance (34μm, 5 ml, GE Healthcare)
- Sequencing primer
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Ethanol (70%)
- PyroMark Binding Buffer
- PyroMark Denaturation Solution
- PyroMark Wash Buffer concentrate
- PyroMark Annealing Buffer
- Pyro Mark Q24 Instrument (QIAGEN)

Immobilizing the PCR products to Streptavidin Sapharose High Performance beads:

This procedure was done for immobilization of template DNA to Streptavidin Sapharose High Performance beads (GE Healthcare) prior to analysis on the PyroMark Q24 system.

Procedure:

1) The bottle containing the Streptavidin Sapharose High Performance beads was gently shaken until the solution became homogenous.

2) Master mix for DNA immobilization was prepared according to table 5. A volume 10% greater than that required for total number of reactions to be performed was prepared.

Table 5 : Master mix for DNA immobilization

Component	Volume in µl
Streptavidin Sapharose High Performance	2
PyroMark Binding buffer	40
Water	13
Total volume	55

3) 55µl of the master mix was added to wells of a 24-well PCR plate or strips.

4) 20µl to 30µl biotinylated PCR (based on the brightness if band in gel picture) product was added to appropriate wells containing the master mix to give a total volume of 80µl. PCR plate or strips were sealed using strip caps. The PCR plate was agitated using an orbital shaker at 1400 rpm for 5–10 min at room temperature (15–25°C).

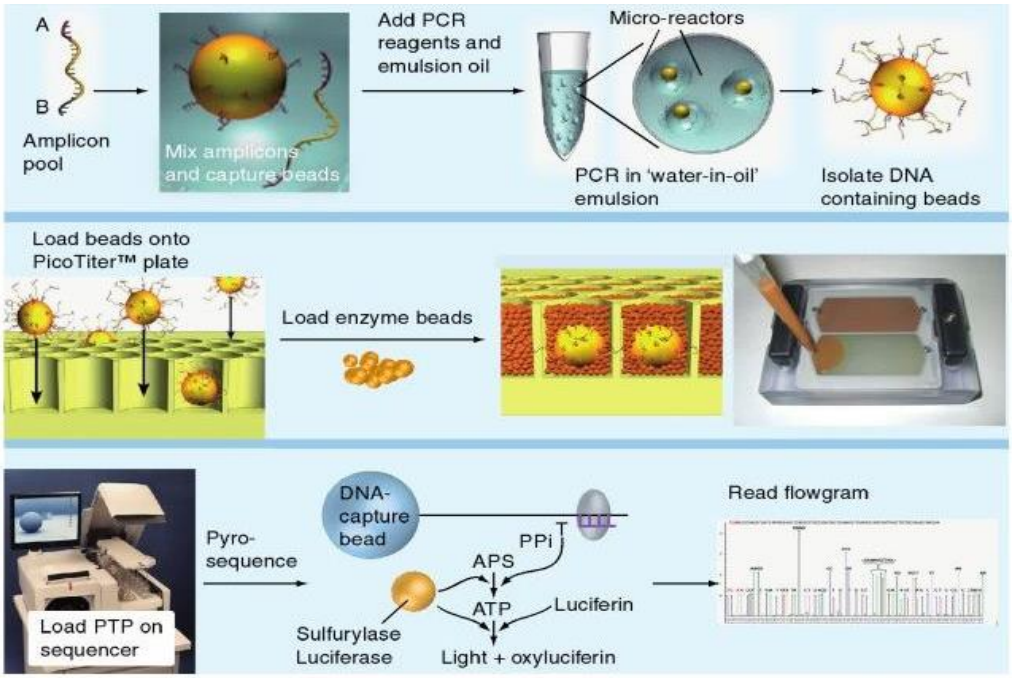


Figure 15: Pyrosequencing Workflow (93).

Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24:

Procedure:

1. A sufficient amount of each sequencing primer was diluted in PyroMark annealing buffer (22.5µl of annealing buffer and 3µl of sequencing primer (10X) were pipetted into each well).

The details of the sequencing primers are in the table 6. (Lin *et.al.*) (90).

Table 6 : Sequencing primers for the target genes

Drug	Locus	Sequencing primers
RIF	*rpoB* Sequencing, for codons 507–521 Sequencing, for codons 522–533	 GCGATCAAGGAGTTCTTC CAGAACAACCCGCTG
FQ	*gyrA*	CAACTACCACCCGCA
AMK, CAP, KAN	*rrs* *eis*	CTTGTACACACCGCC CAGACACTGTCGTCG

2. After diluting the sequencing primers to 0.3µM with PyroMark Annealing Buffer, 25µl was dispensed into appropriate well of the PyroMark Q24 Plate. Then the plate was positioned on the workstation.

3. The workstation troughs were filled according to the Figure 16.

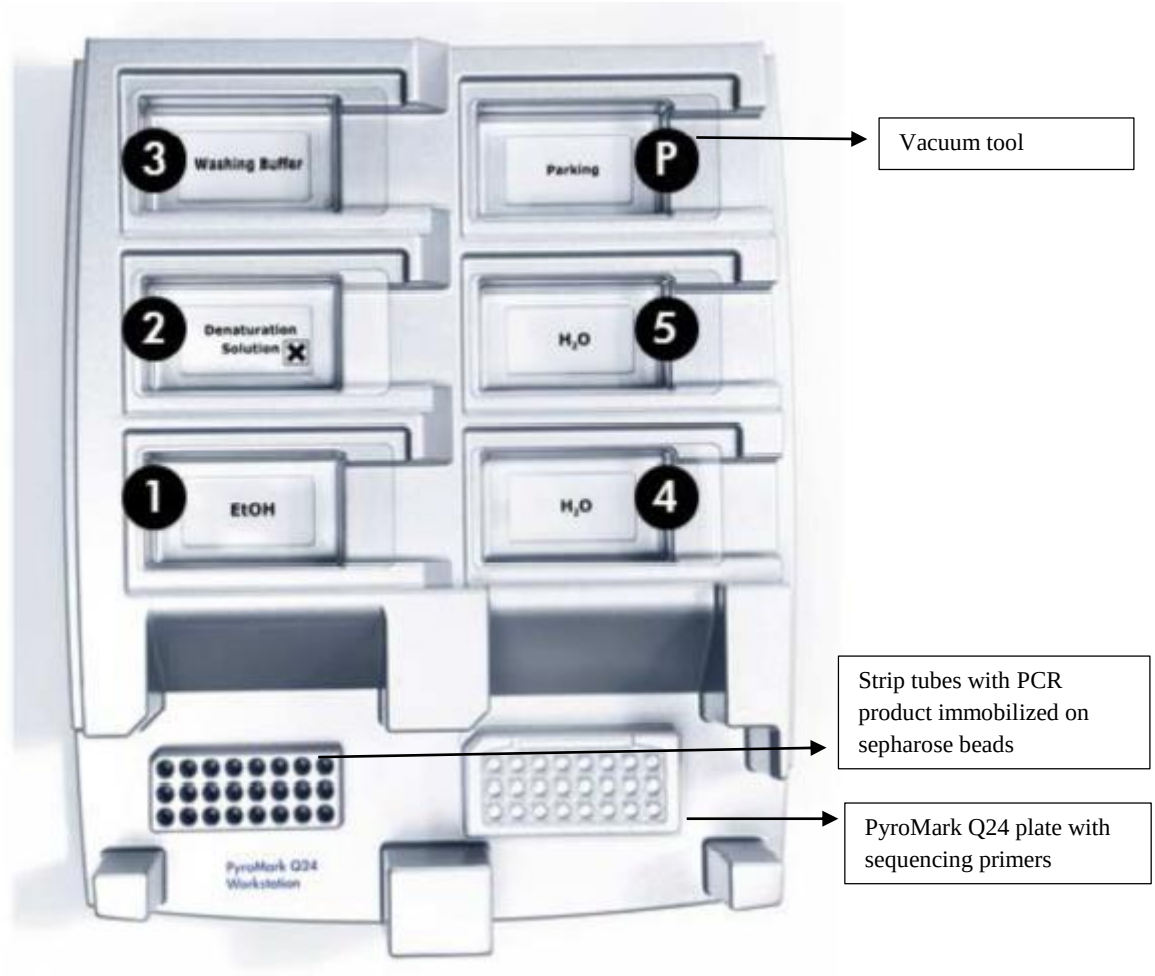


Figure 16: PyroMark Q24 Vacuum station. Placement of PCR strips and PyroMark Q24 plate on the vacuum work station.

4. Vacuum was applied to the tool by turning ON the vacuum switch.

5. The filter probes of the vacuum tool were carefully lowered into the PCR strips to capture the beads containing immobilized template. The probe was held in place for about 15 seconds.

6. The vacuum tool was then transferred to the trough containing 40ml 70% ethanol (Fig. 16). The filter probe was then flushed for about 5 seconds.

7. The vacuum tool was then transferred to the trough containing 40 ml denaturation solution (Fig.16). The filter probe was then flushed for about 5 seconds.

8. The vacuum tool was then transferred to the trough containing 50 ml wash buffer (Fig. 16). The filter probe was then flushed for about 10 seconds.

9. The vacuum tool was raised up and back beyond 90˚ vertical, for about 5 seconds to drain liquid from the filter probes.

10. The vacuum switch on the tool was closed while the vacuum tool was held over the PyroMark Q24 Plate.

11. The beads were released in the PyromarkQ24 Plate by lowering the filter probes into the diluted sequencing primer and then moving the tool gently from side to side.

12. The vacuum tool was then transferred to the trough containing high-purity water (Fig. 16) and agitated for about 10 seconds.

13. The filter probes were washed by lowering the probes into high purity water (Fig. 16) and applying vacuum. The probes were then flushed with 70ml high purity water.

14. The vacuum tool was raised to beyond 90˚ vertical for about 5 seconds to drain liquid from the filter probes. Then the vacuum was switched OFF and the tool was stored in the “Parking” position (Fig. 16).

Annealing sequencing primers to DNA strands:

The PyroMark Q24 Plate with immobilized DNA was placed in a pre-warmed PyroMark Q24 Plate Holder. The Pyrosequencing samples were heated on a heating block at 80°C for 2 minutes. Then the plate was removed from the holder and the samples were allowed to cool to room temperature (15–25°C) for at least 5 minutes. The cooled plate was then processed in the PyroMark Q24 system.

Setting up the run:

1. In the shortcut browser, the folder in which the run file is to be placed was right-clicked and "New Run" was selected from the context menu. The file name was typed and "Enter" was pressed.

2. "Instrument Method" was selected as per the instructions supplied with the reagents and cartridge used.

3. An assay was added to each well used, e.g., by dragging an assay from the shortcut browser to a well or a selection of wells. A well gets coloured according to the assay loaded into the well.

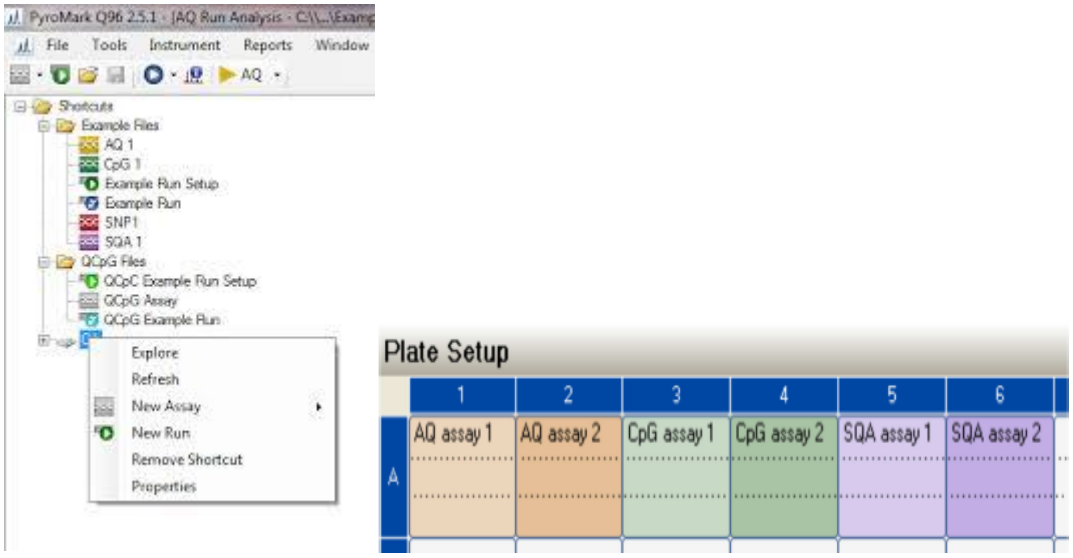


Figure 17: Setting up a run

4. To enter a sample ID or note, the cell was selected and the text was entered. The selected cell gets highlighted with a blue background colour.

6. Click in the toolbar.

7. A list of required volumes of reagents and the plate setup was printed; "Pre Run Information" was selected from the "Tools" menu and then, when the report appeared, "ok" was clicked.

8. The run file was closed and copied in to one of the pen drives supplied. The run file was then processed by inserting the pendrive into the USB port at the front of the PyroMark Q24 Instrument.

<u>Preparation of Pyro Mark Gold Q24 Reagents and Running the PyroMark Q24:</u>

<u>Procedure:</u>

1. The freeze-dried enzyme and substrate mixtures were dissolved each in 620 µl of water and mixed by swirling the vial gently.

2. The reagents and the PyroMark Q24 Cartridge were allowed to reach the ambient temperature.

3. The PyroMark Q24 cartridge was loaded with the appropriate volumes of nucleotides (dATP, dCTP, dGTP, dTTP) enzyme and substrate mixes according to figure 18, as given by the pre-run information.

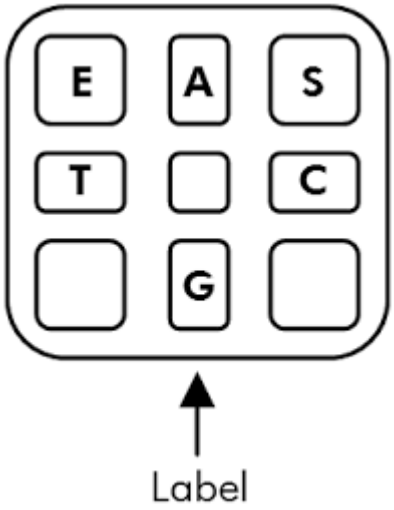


Figure 18: Illustration of PyroMark Q24 Cartridge as seen from above.

4. After loading the cartridge the pyro sequencing plate was loaded on to the PyroMark24 machine. The assay file saved on to the pen drive was selected and the Run was started.

Analysis of run:

1. The processed run file was moved from the USB stick to a computer running PyroMark Q24 Software.

2. The run file was opened by double-clicking the run file in the shortcut browser. If several assay types were included, the analysis mode was selected in the dialog box that opened.

Analysis modes:

1. PyroMark Q24 Software has three analysis modes: AQ, CpG, and SQA.

2. The “SQA” mode was selected in the toolbar.

Result:

Quality colors

Blue: Passed 

Yellow: Check 

Red: Failed 

White: Not analyzed* 

* Either analysis is not supported by the software (e.g., analysis of SNP when in the CpG mode) or the variable position has been deselected by the user (AQ and CpG assays only).

Representative Pyrogram results are shown in Figures 19-26.

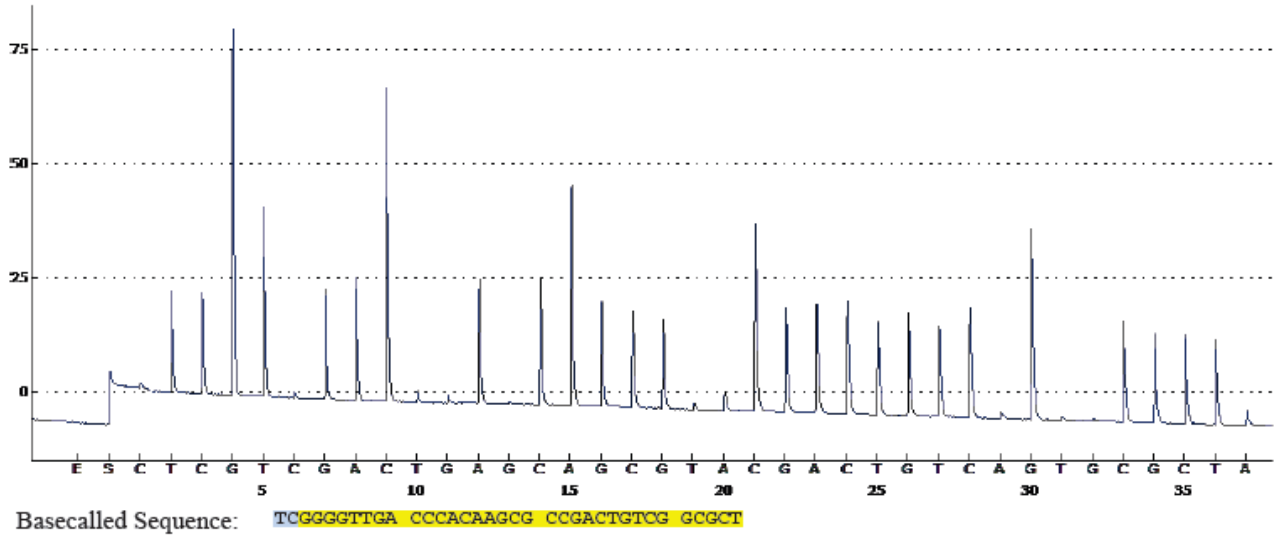


Figure 19: *rpoB* Pyrogram H37RA (Wild type sequence).

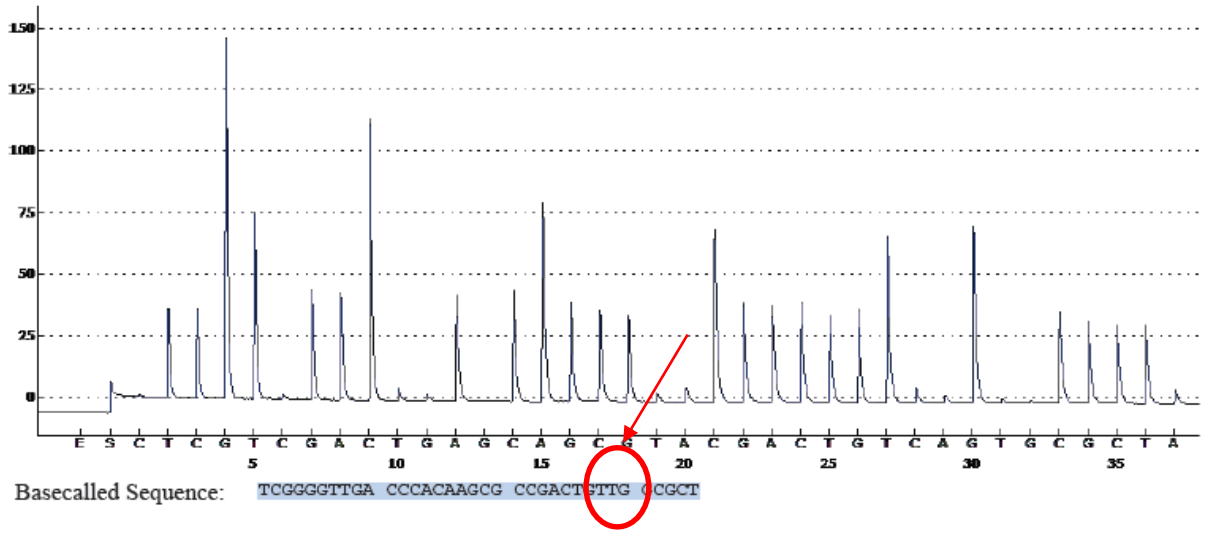


Figure 20: rpoB Mutant sequence TCG531TTG

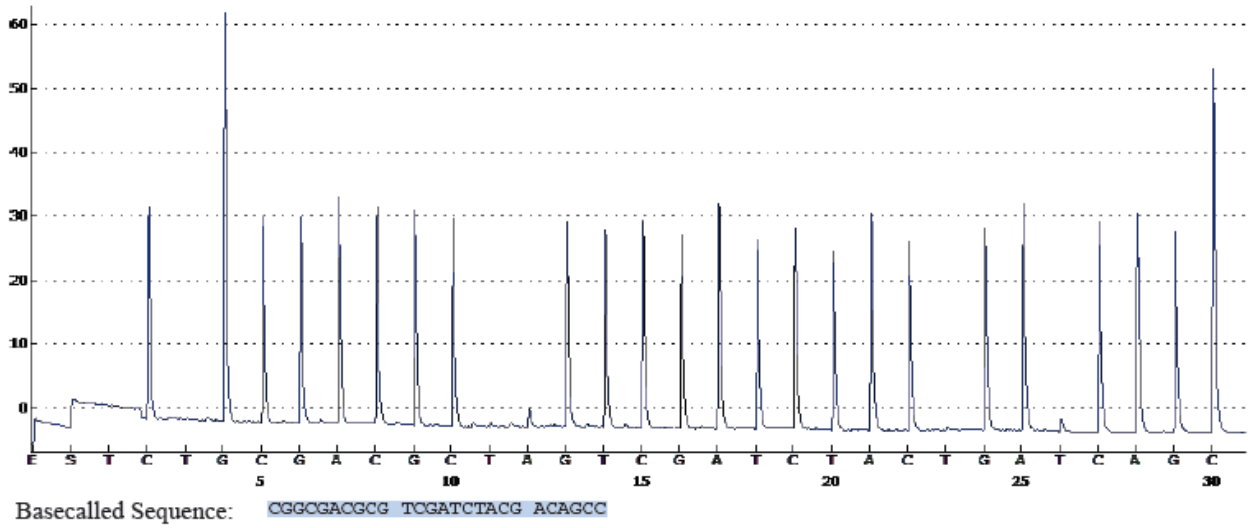


Figure 21: gyrA Pyrogram H37RA (Wild type sequence)

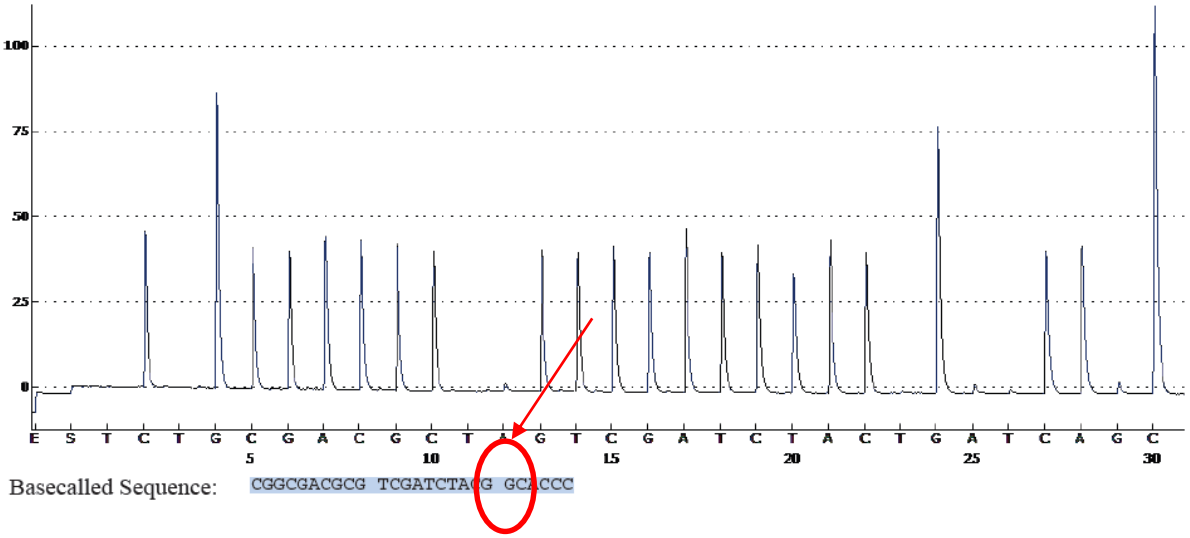


Figure 22: gyrA Mutant sequence (GAC94GGC)

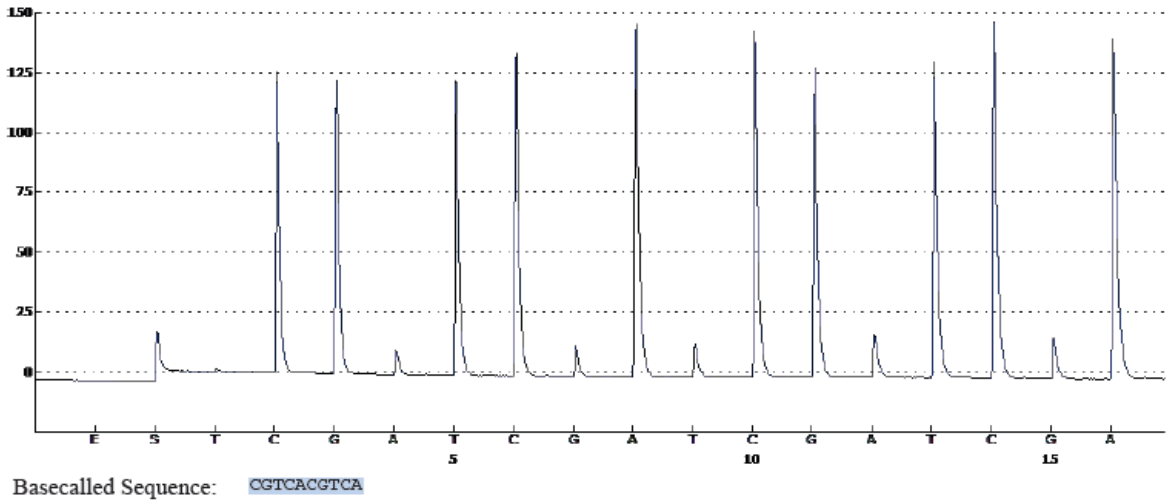


Figure 23: *rrs* H37RA (Wild type sequence)

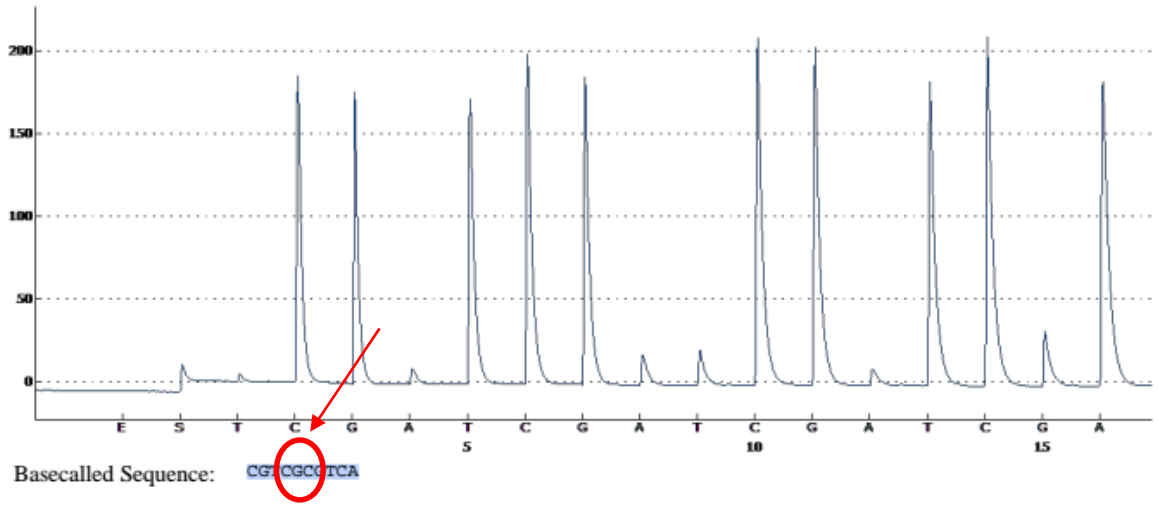


Figure 24: rrs Mutant sequence (A1401G)

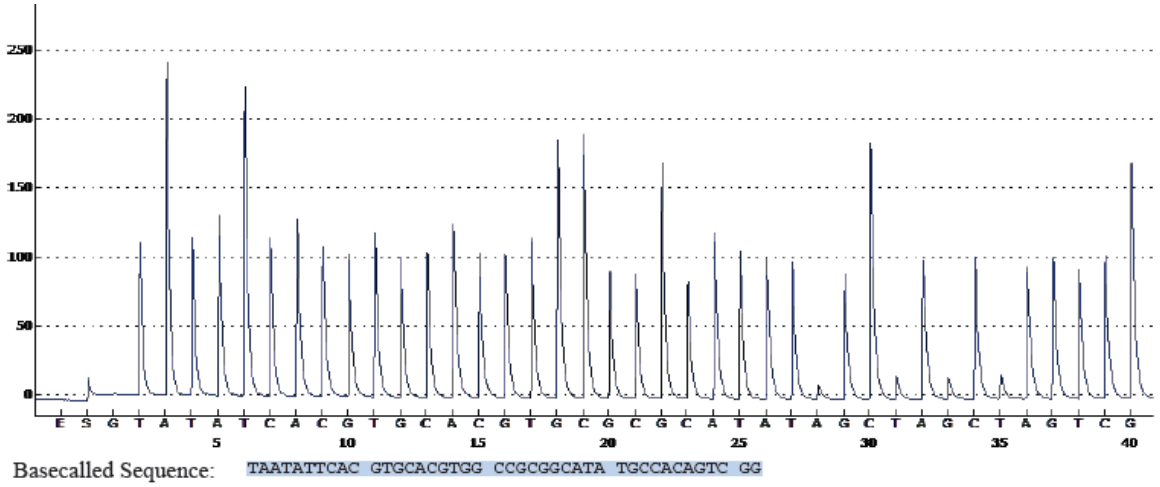


Figure 25: eis promoter H37RA (Wild type sequence)

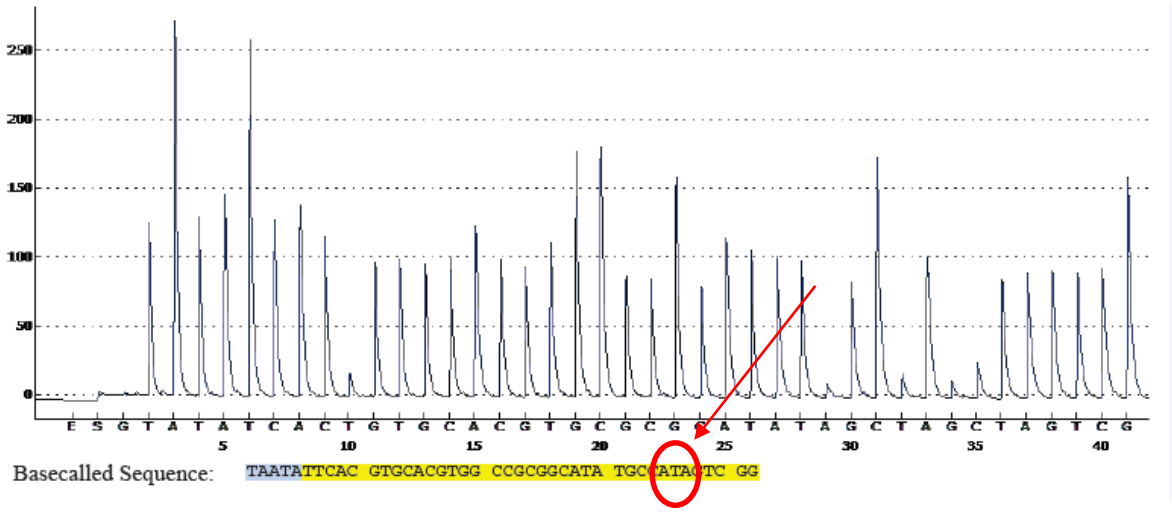


Figure 26: eis promoter mutant sequence (C12T)

5. Results

5.1 Demographic data:

During the study period of 1 year 10 months, from November 2014 to August 2016, 64 MTB clinical isolates were taken for the study. The samples were received in the Microbiology laboratory as part of routine diagnostics for mycobacterial drug resistance testing (agar proportion method).

Based on the inclusion and the exclusion criteria, 64 MTB isolates from clinical samples on which first line (STM, INH, RIF, EMB) and second line (OFLOX, CAP, KAN, ETH) mycobacterial drug susceptibility testing were done, were included in the study.

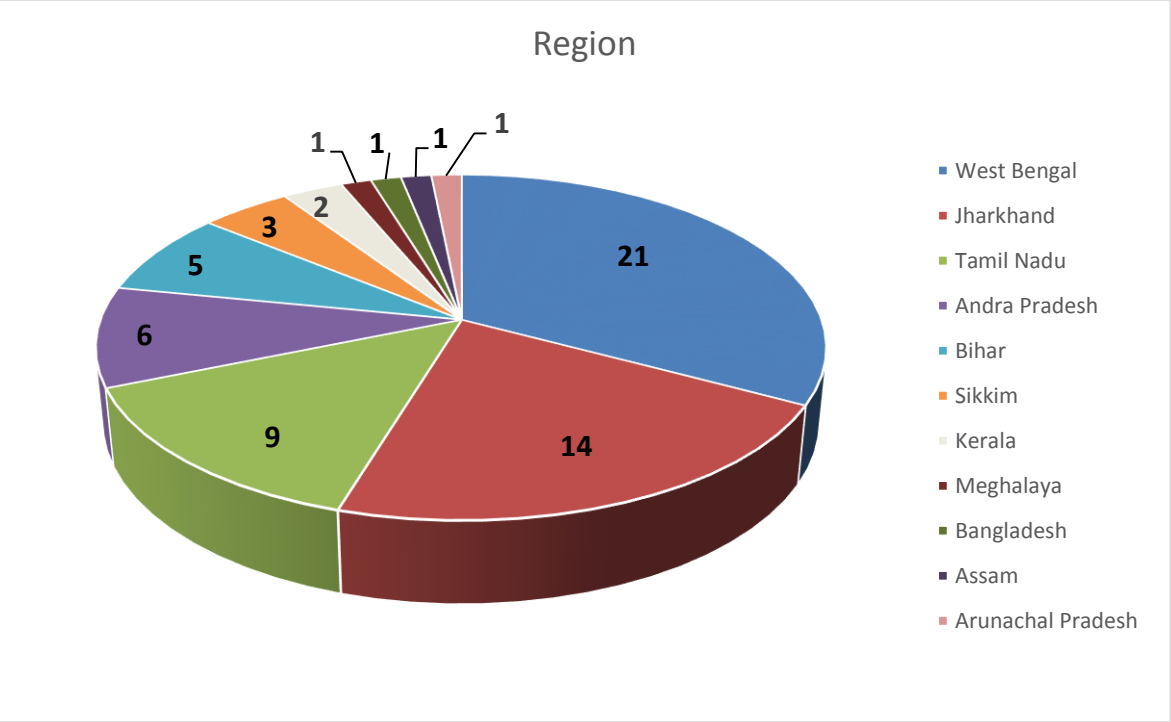


Figure 27: The geographical distribution of MTB clinical isolates included in the study from India.

Thus the following the number of isolates were used for evaluation by PSQ assay:

1. Rifamipicin = 57
2. Fluoroquinolone
    a. Ofloxacin =50
3. Injectable agents
    a. Capremycin = 51
    b. Kanamycin = 51

The MTB clinical isolates were found to be resistant or susceptible based on the agar proportion method. The susceptibility profile of the MTB clinical isolates included in the study is depicted in Figure 28.

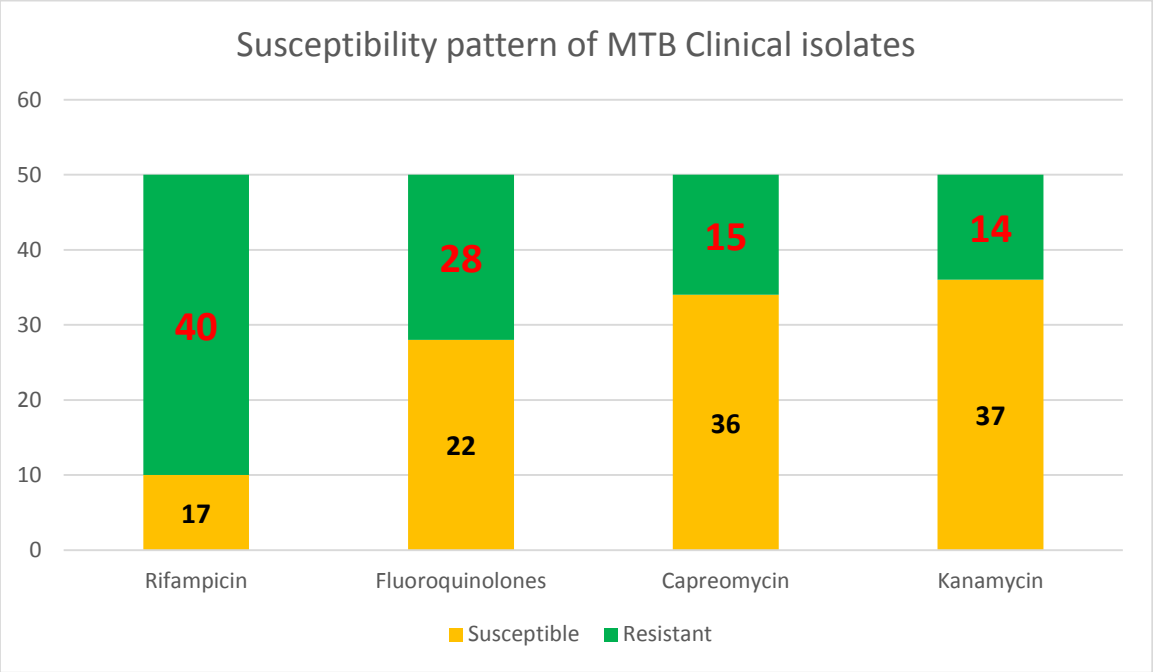


Figure 28: Susceptibility pattern of MTB clinical isolates included in the study.

5.2 Mutations detected by PSQ and their comparison with phenotypic DST:

5.2.1 RIF Resistance:

Drug susceptibility results were available for 57 isolates, for which PSQ was performed. Among the 57 isolates, 40 were phenotypically resistant to RIF. PSQ assay detected a total of 6 unique mutations and combinations of mutation across the RRDR region in the RIF resistant isolates. The most predominant mutation observed in *rpoB* gene was the "TCG531TTG" mutation, which was detected in 33 (82%) of the 40 RIF-resistant isolates.

Three isolates were phenotypically RIF-sensitive, but were found to have mutation in the *rpoB* gene when PSQ was performed. The mutations observed in these isolates were at codon 511 (Leu511Pro). The performance of the PSQ assay for the detection of RIF resistance in comparison to phenotypic DST is summarized in Table 7.

Table 7: Mutations in rpoB gene and their association with phenotypic DST results

Locus	Mutation	Amino acid change	No of isolates	DST Results	
				Resistant	Susceptible
rpoB	TCG531TTG	S531L	33	33	0
	GAC516GTC	D516V	3	3	0
	GAC516TAC + CTG511CGG	D516Y+L511R	1	1	0
	GAC516TAC	D516Y	1	1	0
	CAC526CTC	H526L	1	1	0
	TCG531TGG	S531W	1	1	0
	CTG511CCG	L511P	3	0	3

The diagram below shows the frequency of occurrence of mutations in *rpoB* gene among the RIF resistant isolates included in the study, which were detected by the pyrosequencing assay.

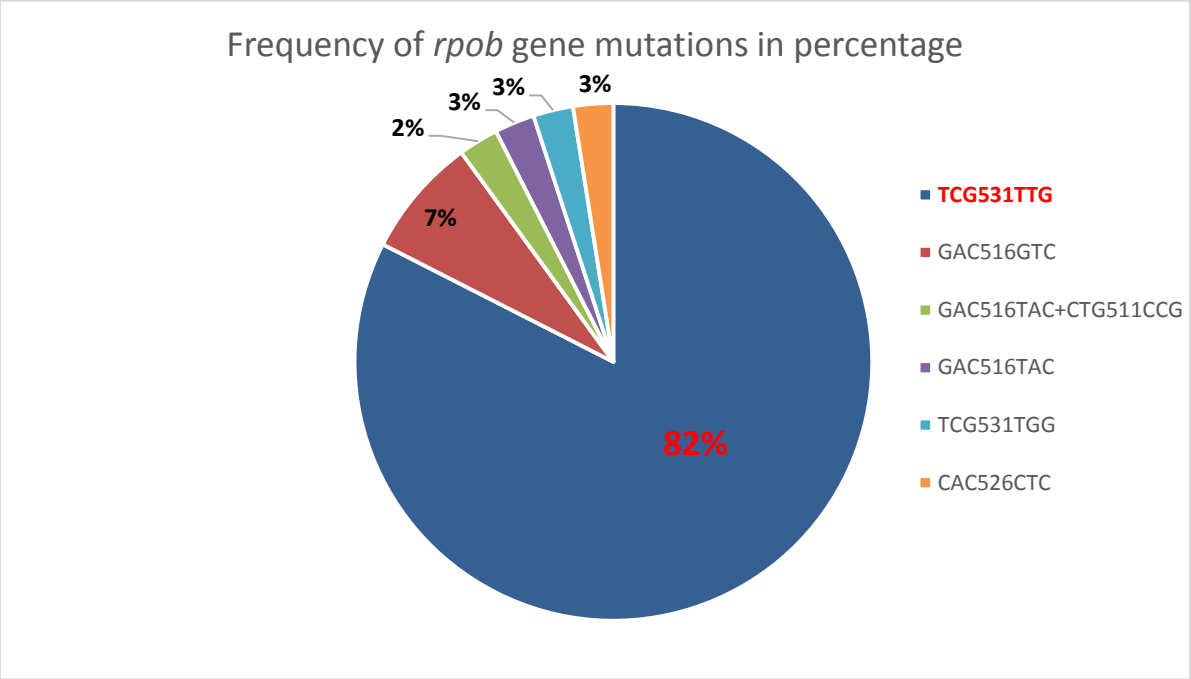


Figure 29: Frequency of occurrence of mutations in *rpoB* gene among the RIF resistant isolates

Table 8: Diagnostic accuracy of PSQ as compared to conventional DST for the detection of Rifampicin resistance in MTB clinical isolates.

	DST (PROPORTION METHOD)		TOTAL
	RESISTANT	SUSCEPTIBLE	
PSQ – RIF RESISTANT	40	0	40
PSQ – RIF SUSCEPTIBLE	0	17	17
TOTAL	40	17	57

The sensitivity and specificity was 100% (87% - 100%) and 100 % (73% - 100%) respectively. The positive predictive value and the negative predictive value was found to be 100%. The agreement between the PSQ results and conventional DST results for RIF was 100% (kappa=1, p value <0.001).

5.3 PSQ in comparison with Xpert MTB/Rif assay:

Xpert MTB/Rif assay results was available for 56 MTB clinical isolates. The agreement between Xpert MTB/Rif assay and PSQ assay was found to be 98.2% (Kappa=0.953, p value < 0.001).

Table 9: Diagnostic accuracy of PSQ in comparison with Xpert MTB/Rif assay

	Xpert MTB/ Rif assay		TOTAL
	RESISTANT	SUSCEPTIBLE	
PSQ – RIF RESISTANT	41	1	39
PSQ – RIF SUSCEPTIBLE	0	14	17
TOTAL	41	15	56

The sensitivity and specificity was 100% (91.4%-100%) and 93.33% respectively. The positive predictive value and the negative predictive value was 97.62 % (87.43-99.94%) and 100% (76.84% - 100%) respectively. One isolate was susceptible by Xpert MTB/Rif assay and resistant by the PSQ. This isolate had a mutation in codon 531 (TCG531TTG), however this isolate was resistant by the gold standard conventional DST testing.

5.2.2 Fluoroquinolone Resistance:

DST results were available for the 50 isolates among which 28 were resistant to fluoroquinolone (FQ). PSQ detected a total of 6 unique mutations, across the *gyrA* gene (QRDR). Twenty resistant isolates showed mutations in the 94th codon of *gyrA*: 15 GAC- GGC, 2 GAC- GCC, 1 GAC- TAC, 1 GAC- CAC, 1 GAC-AAC. All the resistant isolates with these single nucleotide polymorphisms (SNPs) were phenotypically resistant to Ofloxacin (agar proportion method). Eight isolates showed a 90 GCG- GTG mutation. The performance of the PSQ assay for the detection of FQ resistance in comparison to phenotypic DST is summarized in Table 10.

Table 10: Mutations in gyrA gene and their association with phenotypic DST results

Locus	Mutation	Amino acid change	No of isolates	DST Results	
				Resistant	Susceptible
gyrA	GAC94GGC	D94G	15	15	0
	GAC94GCC	D94A	2	2	0
	GAC94CAC	D94H	1	1	0
	GAC94AAC	D94N	1	1	0
	GAC94TAC	D94Y	1	1	0
	GCG90GTG	A90V	8	8	0

The diagram below shows the frequency of occurrence of mutations in *gyrA* gene among the FQ resistant isolates included in the study, which were detected by the pyrosequencing assay.

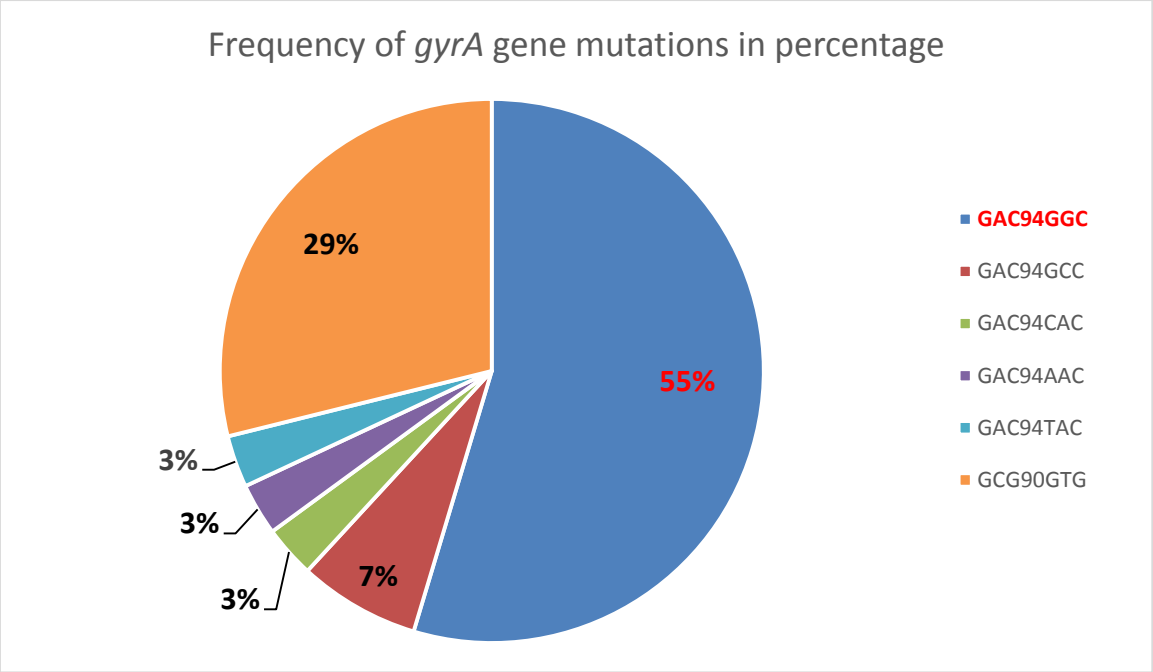


Figure 30: Frequency of mutations detected in *gyrA* gene among the FQ resistant isolates by PSQ assay

Table 11: Diagnostic accuracy of PSQ as compared to conventional DST for the detection of FQ resistance in MTB clinical isolates.

	DST (PROPORTION METHOD)		TOTAL
	RESISTANT	SUSCEPTIBLE	
PSQ – FQ RESISTANT	28	0	28
PSQ – FQ SUSCEPTIBLE	0	22	22
TOTAL	28	22	50

The sensitivity was 100% (82.8% - 100%) and specificity was 100% (78.9% - 100%). The positive predictive value and negative predictive value was 100% and 100% respectively. The agreement between the two test was 100% (kappa = 1.00 and p value <0.001).

5.2.3 Second Line Injectable (SLI) Drug Resistance:

For the second line drugs Capreomycin (CAP) and Kanamycin (KAN), DST results were available for 51 isolates among which 15 were phenotypically resistant to CAP and 14 were phenotypically resistant to KAN. Among these 10 isolates were resistant to both the SLI (CAP, KAN). In these 10 isolates PSQ detected a 1401 A-G mutation in 5 isolates and 1402 C- T mutation in 1 isolate in the *rrs* gene region. The remaining 5 isolates did not show any mutation in the *rrs* gene segments evaluated suggesting that mutation might exist in other sites of *rrs* gene. Thus *eis* promoter gene region was also evaluated by the PSQ assay for the isolates resistant to SLI, since *eis* promoter mutations confer lower levels of CAP resistance and low to moderate levels of KAN resistance The *eis* promoter mutation C-12T was found in one of the isolates which was susceptible to CAP and resistant to KAN.

The performance of the PSQ assay for the detection of SLI resistance in comparison to phenotypic DST is summarized in Table 12.

Table 12: Mutations in *rrs* and *eis* promoter regions and their association with phenotypic DST results

Locus	Mutation	No of isolates	DST Results (CAP)		DST Results (KAN)	
			Resistant	Susceptible	Resistant	Susceptible
rrs	A1401G	5	5	0	5	0
	C1402T	1	1	0	1	0
eis	C12T	1	0	0	1	0

The diagram below shows the frequency of occurrence of mutations in *rrs* and *eis* promoter region among the SLI resistant isolates included in the study.

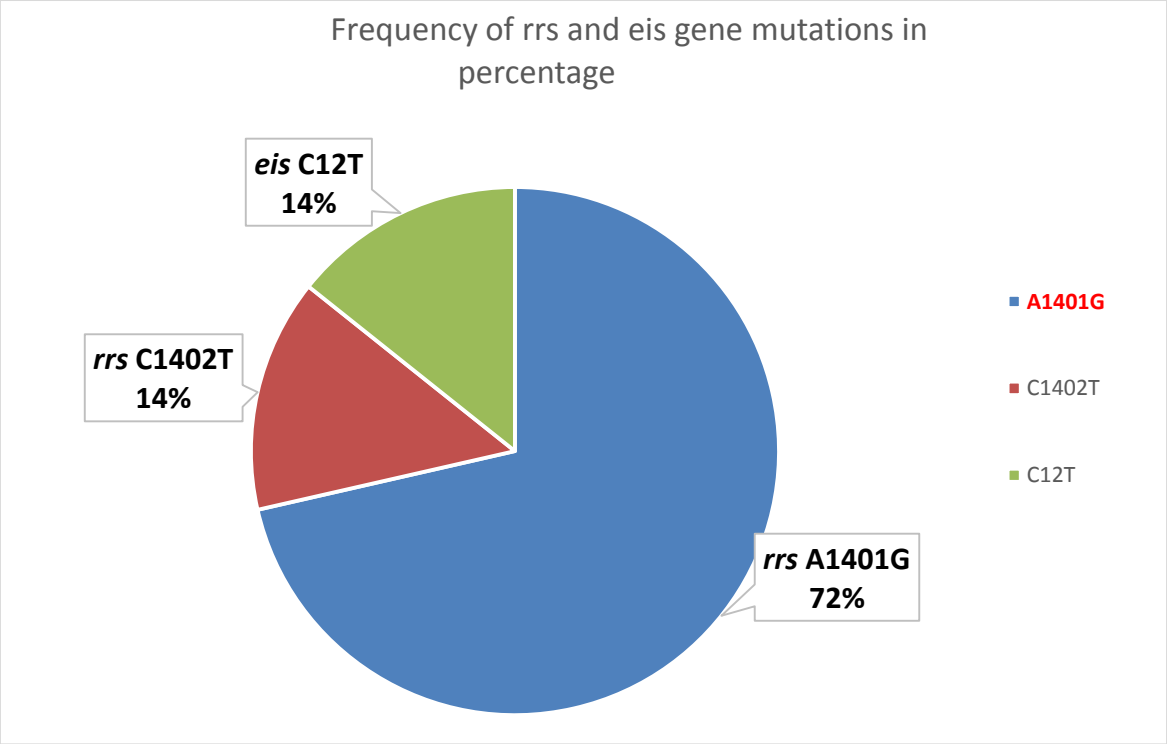


Figure 31: Frequency of mutations detected in *rrs* and *eis* promoter regions among the SLI resistant isolates by PSQ assay

Table 13: Diagnostic accuracy of PSQ as compared to conventional DST for the detection of CAP resistance in MTB clinical isolates.

	DST (PROPORTION METHOD)		TOTAL
	RESISTANT	SUSCEPTIBLE	
PSQ – CAP RESISTANT	6	0	6
PSQ – CAP SUSCEPTIBLE	9	36	45
TOTAL	15	36	51

The sensitivity was 40% (16.3% - 67.7%) and specificity was 100% (85.8% - 100%). The positive predictive value and negative predictive value was 100% (42.1% - 100%) and 80% (65.4% - 90.4%) respectively. The agreement between the two test were 82.35% (kappa = 0.48 and p value <0.001).

Table 14: Diagnostic accuracy of PSQ as compared to conventional DST for the detection of KAN resistance in MTB clinical isolates.

	DST (PROPORTION METHOD)		TOTAL
	RESISTANT	SUSCEPTIBLE	
PSQ – KAN RESISTANT	7	0	7
PSQ – KAN SUSCEPTIBLE	7	37	44
TOTAL	14	37	51

With the addition of *eis* promoter region in the PSQ assay, the sensitivity for detecting SLI (KAN) resistance increased to 50% (23.04% - 76.96%) and

specificity was 100% (90.51% - 100%). The positive predictive value and negative predictive value was 100% (59.04% - 100%) and 84.09% (69.93% - 93.36%) respectively. The agreement between the two test were 86.27% (kappa = 0.59 and p value <0.001).

5.4 Comparison of PSQ assay and Line Probe assay in detecting drug resistant mutations:

A prospective study was conducted in our setting, Christian Medical College, a tertiary care centre in South India, where the performance of line probe assay (GenoTypeMTBDR*plus*) was compared with culture and the Xpert MTB/Rif assay on sputum specimens of suspected MDR pulmonary tuberculosis patients (January 2013 to June 2013). In this study, among the patients who were resistant to Rifampicin, we observed predominantly absence of a wild-type 8 band (covering codons 530-533) without a corresponding mutation band in 16 of the sputum samples, along with occurrence of MT3 band (S531L) in 10 of the sputum samples (n=35). In comparison to our study, PSQ also showed predominantly TCG531TTG (S531L) mutation in 82% of the *Mtb* clinical isolates

Another prospective study (unpublished data) being done in our setting also evaluating the performance of GenoType MTBDR*sl* in detecting resistance of M*tb* clinical isolates to 2nd line anti-TB drugs. In this study among the FQ resistant isolates we observed predominantly MUT3 band (D94G) with the absence of the corresponding wild-type 3 band (covering codons 92-97) in 12 (41.37%) of the clinical MTB isolates (n=29) and among the aminoglycoside resistant isolates we predominantly observed MUT1 band (A1401G, C1402T) with the absence of

corresponding wild-type 1 band (covering codons 1401, 1402) in 3 of the clinical MTB clinical isolates (n=4).

The results were similar to our study, where the most common mutation was GAC94GGC (D94G) in 15(55%) of the FQ resistant clinical isolates (n=28) and among the aminoglycosides A1401G being the most common mutation in 5 of the clinical isolates (n=10 i.e. resistant to both CAP & KAN).

5.5 Turnaround time of tests:

Test	**Turnaround time**
1st line & 2nd line DST	6 weeks
Xpert MTB/Rif assay	2 hours
Line probe assay	6-8 hours
Pyrosequencing	6 hours

6. Discussion

Bacteriological methods are labour-intensive and time-consuming for the detection of resistance to most of the anti-TB drugs, but they are highly sensitive and specific and considered the gold standard DST methods. WHO approved methods such as Xpert MTB/Rif assay and Line Probe assays are rapid but have fixed targets for detection and cannot detect silent mutations or mutations outside the target region. Sequencing techniques overcome these limitations. Therefore we evaluated Pyrosequencing, one of the sequencing techniques for detection of mutations associated with resistance to Rifampicin and Second-line anti-TB drugs in clinical isolates of *M. tuberculosis*.

Pyrosequencing is a rapid, robust and high-throughput diagnostic sequencing technique that is easy to perform. In this method, multiple strains can be simultaneously sequenced in a 24-well plate. The assay is capable of simultaneously detecting *Mtb* and the most common mutations conferring phenotypic resistance to Rifampicin, Fluoroquinolones and Second line injectable agents. It can also be used to detect novel mutations within the targeted gene regions. PSQ also provides a detailed sequence information, thereby the sequence can be interpreted based on the current and evolving knowledge on phenotypic expression of the drug-resistant *Mtb* strains. The gold standard for the detection of drug resistance in tuberculosis is the bacteriological methods. Thus, we compared the results of Pyrosequencing with the 1% agar-proportion method of drug susceptibility testing in MTB clinical isolates.

6.1 PSQ for the detection of Rifampicin resistance:

Rifampicin is a tuberculocidal drug used for treating TB. RIF resistance is used as a surrogate marker for MDR-TB. The accuracy of molecular diagnostics for the detection of RIF resistance is critical, since it can influence in taking early clinical decisions. The frequency of mutations detected in *rpoB* gene in RIF-resistant isolates by the PSQ assay are consistent with the other reports published in the literature, thus demonstrating the importance of the RRDR hotspot region in the resistance of MTB clinical isolates (94) (95) (96) (97). Several studies have indicated that RRDR region contributes to around 90–95% of RIF-resistance (97,98). Notably, majority of MTB clinical isolates (82%) had mutations in codon 531, which is similar to the previously published reports (92) (99) (100). The other mutations were observed in codons 516, 526, and 511.

The PSQ assay detected mutation in all the Rifampicin resistant isolates (n=40) included in the study. Additionally it also detected mutation in 3 isolates that were determined to be phenotypically susceptible. The mutation observed in these 3 isolates were at codon 511 (CTG511CCG). This mutation can occur in other RIF resistant strains along with other mutation, but the occurrence of CTG511CCG mutation alone confers low predictive value for rifampicin resistance (101). This mutation is known to be documented as “disputed mutations” conferring “low-level” Rifampicin resistance (Van duen *et. al.*) (102). Since these mutations are already known to have inconsistent RIF-DST results, they were excluded for specificity analysis of our study (92) (102) (103).

The sensitivity and specificity of the PSQ assay for detecting RIF resistance was 100% (91.19% - 100%) and 100% (69.15 – 100%) respectively, which was in concordance with previously published studies (90) (89) (101) (104).

The sensitivity and specificity of the PSQ assay in comparison with the Xpert MTB/Rif assay was 100% and 93.33% respectively. PSQ detected mutation in one of the isolates at codon 531 (TCG531TTG), which was susceptible by Xpert MTB/Rif assay. However it was resistant by the gold standard conventional DST testing. This may be a false-negative result of Xpert MTB/RIF assay due to mixed *M.tuberculosis* complex infections. Studies have shown that the Xpert assay failed to detect RIF resistance in vitro when <90% of the organisms in the sample were RIF resistant (105).

6.2 PSQ for the detecting of fluoroquinolone resistance

The main cellular target for fluoroquinolone in *Mtb* is "the DNA gyrase enzyme" (type II topoisomerase). It has two A and two B subunits which are encoded by *gyrA* and *gyrB* genes, respectively. The genetic mechanism of resistance to FQs is due to QRDR region mutations of *gyrA* and *gyrB* resulting in changes in the DNA gyrase enzyme. Studies have shown that around approximately 60% to 90% of *Mtb* clinical isolates with FQ resistance have mutations in the QRDR of *gyrA*, commonly in "codons 88, 90, 91, and 94" (106,107) which is similar to our study, roughly 55% of the mutations occurred in the 94th codon region. The *gyrB* locus was not included in our study, as mutations in this gene occur at a much less frequency with lower sensitivity and specificity. They commonly co-occur with *gyrA* mutations. Among the susceptible and resistant clinical isolates there was

uniform distributions of "*gyrA* S95T mutation" (92%), which is similar to the previous studies published. This mutation is regarded as polymorphism which does not confer FQ resistance (104,108).

The sensitivity and specificity of the PSQ assay for FQ was 100% and 100% respectively. This may be due to the smaller sample size. However there are studies in regard with reduced sensitivity of the PSQ assay in detecting FQ resistance. This is due to the varied frequency of occurrence of mutations of the *M.tuberculosis* in a particular geographic region or may be due other resistance mechanisms like efflux pumps (101,104,109).

6.3 PSQ for the detection of Second line injectables:

With regard to the SLI, the mutations most commonly associated with resistance are the "A1401G mutation", followed by "C1402T and G1484T mutations" in the *rrs* gene. Our assay can only detect the "A1401G and C1402T mutations", and another primer set is needed to detect the "G1484T mutation". Due to the low prevalence of the above mutation we did not include it in our study. With less number of resistant strains tested, our assay showed 100% specificity for detecting CAP and KAN resistance. But, the sensitivity was low for detecting CAP resistance (40%). For KAN, there was an increase in the sensitivity to 50% due to the addition of *eis* promoter gene in the PSQ assay. Other PSQ studies have also shown similar increase in sensitivity with the inclusion of *eis* promoter gene in the PSQ assay (109,110)

The review article published by Georghiou *et.al.* has shown that the rrs 1401 mutation did not detect greater than 80% of *Mtb* strains globally that were resistant to CAP and AMK. It also did not detect 60% of KAN resistant *Mtb* strains. For improving the sensitivity and specificity in the detection of SLI, other SNPs in the "*tlyA* and *gidB* genes" can be considered as promising markers in improving the clinical and diagnostic utility of future molecular methodologies to the second line injectable drugs (111).

6.4 PSQ versus LPA:

The WHO had approved Line probe assay (LPA) for the detection of mutations conferring resistance to INH and RIF, commercially available as GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany). LPAs targeting resistance to second-line anti-TB drugs, GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany) detect mutations in the *gyrA* gene, *rrs* gene and *embB* gene and they complement the Hain GenoType MTBDR*plus* LPA for the detection of XDR-TB. The sensitivity of these tests depends on the inclusion of various mutations conferring resistance to *Mtb* and the frequency at which these mutations are found in MTB strains in different geographical settings (112).

A study conducted in our setting, evaluated the performance of GenoType MTBDR*plus* (113) and GenoType MTBDR*sl* (unpublished data) showed a similar result in regard to frequency of occurrence of mutation in Rifampicin and Second line drugs. Among the samples (n=35) evaluated by MTBDR*plus*, 46% had absence of a wild-type 8 band without a corresponding mutation band and 29% had presence of MUT3 band.

With regard to GenoType MTBDR*sl* evaluated on M*tb* clinical isolates, the fluoroquinolone resistant isolates (n=29) predominantly had a MUT3 band (D94G) with the absence of the corresponding wild-type 3 band (41.37%) and the aminoglycoside resistant isolates predominantly had MUT1 band (A1401G, C1402T) with the absence of corresponding wild-type 1 band in 3 out of 4 the clinical *Mtb* isolates (n=4).

The most common mutation picked up by LPA for rifampicin resistance covered the codon regions 530 to 533. GAC94GGC (D94G) for fluoroquinolone resistance and A1401G for second line injectable resistance. This was similar to the mutations picked up PSQ assay in our study.

Though LPA detected the most common mutations, PSQ has an added advantage that it can pick up additional mutations. In a study published by Nikam *et.al* shows that, in the case of discrepant results, PSQ can be used as a valuable tool for rapidly evaluating LPA-indeterminate results instead of repeating phenotypic DST, as the PSQ technology appears to resolve discrepancies in line with phenotypic DST results (114) .

7. Limitations of the study

a) Sample size:

The sample size was limited in our study due to a lack of funds. A larger sample size would have helped us to evaluate PSQ better.

b) Sensitivity of PSQ assay in detection of SLI:

The lower sensitivity rates of PSQ for detection of second line injectables (CAP, KAN) reflect that the incomplete understanding of resistance mechanisms associated with SLI investigated in the study. Therefore sequencing of other genes encoding for other resistance mechanisms in SLIs would improve sensitivity of the PSQ assay.

c) PSQ on *Mtb* clinical isolates:

The real usefulness of Pyrosequencing would have been effective if it was performed on clinical samples, but due to the lack of fund and time, the PSQ assay could not be standardised on clinical samples.

d)Technical aspects:

Sequencing failures was also a limitation, due to which certain isolates were excluded from the study, even though they had DST results. Georghiou *et.al*. in his study had stated that sequencing failures may be associated with the sequencing length of the given gene target (longer sequencing targets had more sequencing failures) (115).

8. Summary & Conclusion

In summary, Of the 57 isolates tested for RIF resistance, 40 were phenotypically resistant to Rifampicin. By PSQ the most predominant mutation observed in *rpoB* gene was TCG531TTG (82%). Twenty eight of the 50 isolates were phenotypically resistant to FQ. Of these, 20 isolates showed mutations in the 94th codon of *gyrA* gene. The predominant mutation observed was GAC94GGC (55%). Of the 51 isolates tested for resistance to aminoglycosides 10 were phenotypically resistant to CAP and KAN. Five of the 10 isolates had A1401G mutation. However, 4 of the resistant isolates had no mutation in the *rrs* gene segments evaluated suggesting that mutations exist in other sites of *rrs* gene.

To conclude the data published by other molecular studies is comparable to our data (89,90,101,104,106,116) demonstrating that PSQ assay can be used as a rapid and effective method for identification of *Mycobacterium tuberculosis* and also to detect mutations associated with drug resistance in *Mtb* clinical strains. The whole technique can be accomplished within 6 hours (from DNA extraction to reporting results) and integrated easily into the diagnostic laboratory work flow. It's quite flexible, as the assay number and the choice of the target included can be adjusted easily. It can be used for investigation of a larger number of specimens. Another benefit of sequencing assays compared to probe based assays is the "discriminatory power at the genotypic level". Sequencing can differentiate between silent and missense mutations can differentiate mutations not associated with resistance (104).

The accuracy of PSQ is comparable to that of other sequencing methods like Sanger sequencing (90,98) and the sequence information provided by PSQ assay

enables the users to know the association with MICs (minimum inhibitory concentration) of the drugs with respect to each mutation, thus guiding treatment decisions.

Pyrosequencing, however, cannot be an alternative to conventional DST. Phenotypic resistant strains lacking mutation in known "resistance-associated loci", where there are multiple genetic bases for resistance or where all the mechanisms of resistance have not been identified, presents a problem for all molecular technique designed to detect mutations associated with resistance. However, issues like costs and complexities of testing additional sites limit the assays numbers that can be performed for each sample in clinical practice. This emphasizes the need for research that aims at identifying novel resistance mechanisms in *M. tuberculosis*.

The rapidity along with a "multilocus sequencing" in parallel of several isolates makes Pyrosequencing an effective technique for screening of drug resistance in *Mtb*. As this technology is based on PCR, it has a greater utility to be further modified for application directly on clinical specimens.

9. Bibliography

1. Daniel TM. The history of tuberculosis. Respir Med. 2006 Nov;100(11):1862–70.

2. History of Tuberculosis (TB) [Internet]. National Jewish Health. [cited 2016 May 25]. Available from: https://nationaljewish.org/healthinfo/conditions/tb/history

3. CDC | TB | Drug-Resistant TB [Internet]. [cited 2016 May 25]. Available from: http://www.cdc.gov/tb/topic/drtb/

4. Zignol M, van Gemert W, Falzon D, Sismanidis C, Glaziou P, Floyd K, et al. Surveillance of anti-tuberculosis drug resistance in the world: an updated analysis, 2007–2010. Bull World Health Organ. 2012 Feb 1;90(2):111–119D.

5. WHO | TB diagnostics and laboratory strengthening - WHO policy [Internet]. WHO. [cited 2016 May 15]. Available from: http://www.who.int/tb/areas-of-work/laboratory/policy_diagnosis_pulmonary_tb/en/

6. WHO | WHO guidelines on tuberculosis [Internet]. WHO. [cited 2016 May 25]. Available from: http://www.who.int/publications/guidelines/tuberculosis/en/

7. Tuberculosis (TB) and other mycobacterial diseases: diagnosis, screening, management and data - GOV.UK [Internet]. [cited 2016 May 25]. Available from: https://www.gov.uk/government/collections/tuberculosis-and-other-mycobacterial-diseases-diagnosis-screening-management-and-data

8. SEARO | Revised National Tuberculosis Control Programme (RNTCP) – Guidelines for TB Control in India [Internet]. SEARO. [cited 2016 May 25]. Available from: http://www.searo.who.int/india/tuberculosis/topic/tb_rntcpguidelines/en/

9. Organization WH. Global tuberculosis report 2015 [Internet]. World Health Organization; 2015 [cited 2016 May 8]. Available from: http://www.who.int/iris/handle/10665/191102

10. Millard J, Ugarte-Gil C, Moore DAJ. Multidrug resistant tuberculosis. BMJ. 2015 Feb 26;350:h882.

11. WHO | What is multidrug-resistant tuberculosis (MDR-TB) and how do we control it? [Internet]. WHO. [cited 2016 May 9]. Available from: http://www.who.int/features/qa/79/en/

12. Notice to Readers: Revised Definition of Extensively Drug-Resistant Tuberculosis [Internet]. [cited 2016 May 9]. Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5543a4.htm

13. Organization WH, others. Extensively drug-resistant tuberculosis (XDR-TB): recommendations for prevention and control. Wkly Epidemiol Rec. 2006;81(45):430–432.

14. Organization and others - 2014 - Drug-resistant TB surveillance and response supp.pdf [Internet]. [cited 2016 May 9]. Available from: http://www.who.int/tb/publications/global_report/gtbr14_supplement_web_v3.pdf

15. Annual Reports :: Central TB Division [Internet]. [cited 2016 May 9]. Available from: http://tbcindia.nic.in/index1.php?sublinkid=4160&level=1&lid=2807&lang=1

16. annual-tb-report-2015.pdf [Internet]. [cited 2016 May 9]. Available from: http://www.searo.who.int/tb/annual-tb-report-2015.pdf

17. Michael JS, John TJ. Extensively drug-resistant tuberculosis in India: A review. Indian J Med Res. 2012 Oct;136(4):599–604.

18. Longo D, Fauci A, Kasper D, Hauser S, Jameson J, Loscalzo J. Harrison's Principles of Internal Medicine, 18th Edition. McGraw Hill Professional; 2011. 4322 p.

19. admin. Manson's Tropical Diseases 23rd Edition | PDF | 2014 | Medical Ebooks Free [Internet]. [cited 2016 May 9]. Available from: http://www.medbookzz.com/2015/01/manson-tropical-diseases-23rd-edition.html

20. Ernst JD, Trevejo-Nuñez G, Banaiee N. Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis. J Clin Invest. 2007 Jul 2;117(7):1738–45.

21. Fu LM, Fu-Liu CS. Is Mycobacterium tuberculosis a closer relative to Gram-positive or Gram-negative bacterial pathogens? Tuberc Edinb Scotl. 2002;82(2–3):85–90.

22. Tille P. Bailey & Scott's Diagnostic Microbiology. Elsevier Science Health Science; 2013. 1038 p.

23. Gill WP, Harik NS, Whiddon MR, Liao RP, Mittler JE, Sherman DR. A replication clock for Mycobacterium tuberculosis. Nat Med. 2009 Feb;15(2):211–4.

24. Bennett JE, Dolin R, Blaser MJ. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. Elsevier Health Sciences; 2014. 5095 p.

25. Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, et al. The Peptidoglycan of Stationary-Phase Mycobacterium tuberculosis Predominantly

Contains Cross-Links Generated by l,d-Transpeptidation. J Bacteriol. 2008 Jun;190(12):4360–6.

26. Kaur D, Guerin ME, Škovierová H, Brennan PJ, Jackson M. Biogenesis of the cell wall and other glycoconjugates of Mycobacterium tuberculosis. Adv Appl Microbiol. 2009;69:23–78.

27. Bansal-Mutalik R, Nikaido H. Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. Proc Natl Acad Sci U S A. 2014 Apr 1;111(13):4958–63.

28. Hett EC, Rubin EJ. Bacterial Growth and Cell Division: a Mycobacterial Perspective. Microbiol Mol Biol Rev MMBR. 2008 Mar;72(1):126–56.

29. Kumar P, Arora K, Lloyd JR, Lee IY, Nair V, Fischer E, et al. MEROPENEM INHIBITS D,D-CARBOXYPEPTIDASE ACTIVITY IN MYCOBACTERIUM TUBERCULOSIS. Mol Microbiol. 2012 Oct;86(2):367–81.

30. Girardin SE, Travassos LH, Hervé M, Blanot D, Boneca IG, Philpott DJ, et al. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem. 2003 Oct 24;278(43):41702–8.

31. Kieser KJ, Rubin EJ. How sisters grow apart: mycobacterial growth and division. Nat Rev Microbiol. 2014;12(8):550–62.

32. Tuberculosis (TB) Transmission and Symptoms [Internet]. [cited 2016 May 12]. Available from: https://www.niaid.nih.gov/topics/tuberculosis/understanding/pages/transmission.aspx

33. CDC | TB | Basic TB Facts [Internet]. [cited 2016 May 12]. Available from: http://www.cdc.gov/tb/topic/basics/default.htm

34. Guidelines for Preventing the Transmission of Mycobacterium tuberculosis in Health-Care Settings, 2005 [Internet]. [cited 2016 May 12]. Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5417a1.htm?s_cid=rr5417a1_e

35. Tuberculosis: From an incurable scourge to a curable disease - journey over a millennium Sharma SK, Mohan A - Indian J Med Res [Internet]. [cited 2016 May 12]. Available from: http://www.ijmr.org.in/article.asp?issn=0971-5916;year=2013;volume=137;issue=3;spage=455;epage=493;aulast=Sharma

36. Narasimhan P, Wood J, MacIntyre CR, Mathai D. Risk Factors for Tuberculosis. Pulm Med. 2013 Feb 12;2013:e828939.

37. Zumla A, Raviglione M, Hafner R, Fordham von Reyn C. Tuberculosis. N Engl J Med. 2013 Feb 21;368(8):745–55.

38. Crevel R van, Ottenhoff THM, Meer JWM van der. Innate Immunity to Mycobacterium tuberculosis. Clin Microbiol Rev. 2002 Apr 1;15(2):294–309.

39. Hunter RL. PATHOLOGY of POST PRIMARY TUBERCULOSIS of the LUNG: AN ILLUSTRATED CRITICAL REVIEW. Tuberc Edinb Scotl. 2011 Nov;91(6):497–509.

40. Knechel NA. Tuberculosis: Pathophysiology, Clinical Features, and Diagnosis. Crit Care Nurse. 2009 Apr 1;29(2):34–43.

41. Sharma SK, Mohan A, Sharma A, Mitra DK. Miliary tuberculosis: new insights into an old disease. Lancet Infect Dis. 2005 Jul;5(7):415–30.

42. Desikan P. Sputum smear microscopy in tuberculosis: Is it still relevant? Indian J Med Res. 2013 Mar;137(3):442–4.

43. Rieder HL, Kam KM, Kim SJ, Chonde TM, others. Priorities for tuberculosis bacteriology services in low-income countries: International Union Against Tuberculosis and Lung Disease. Paris Fr. 2007;

44. WHO | International Standards for Tuberculosis Care (ISTC) [Internet]. WHO. [cited 2016 May 15]. Available from: http://www.who.int/tb/publications/2006/istc/en/

45. Organization WH. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MT [Internet]. World Health Organization; 2013 [cited 2016 May 15]. Available from: http://www.who.int/iris/handle/10665/112472

46. Harries A. 11. What is the additional yield from repeated sputum examinations by smear microscopy and culture? TOMAN'S Tuberc. 2004;46.

47. WHO | Same-day diagnosis of tuberculosis by microscopy [Internet]. WHO. [cited 2016 May 15]. Available from: http://www.who.int/tb/publications/2011/tb_microscopy_9789241501606/en/

48. Singhal R, Myneedu VP. Microscopy as a diagnostic tool in pulmonary tuberculosis. Int J Mycobacteriology. 2015 Mar;4(1):1–6.

49. Tuberculosis Division International Union Against Tuberculosis and Lung Disease. Tuberculosis bacteriology--priorities and indications in high prevalence countries: position of the technical staff of the Tuberculosis Division of the International Union Against Tuberculosis and Lung Disease. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2005 Apr;9(4):355–61.

50. Karstaedt AS, Jones N, Khoosal M, Crewe-Brown HH. The bacteriology of pulmonary tuberculosis in a population with high human immunodeficiency virus seroprevalence. Int J Tuberc Lung Dis. 1998 Apr 1;2(4):312–6.

51. Khan EA, Starke JR. Diagnosis of tuberculosis in children: increased need for better methods. Emerg Infect Dis. 1995 Dec;1(4):115–23.

52. Manual for Sputum Smear Fluorescence Microscopy , RNTCP 2007: http://tbcindia.nic.in/pdfs/Flourescence Microscopy%20Manual.pdf - Google Scholar [Internet]. [cited 2016 May 16]. Available from: https://scholar.google.co.in/scholar?hl=en&q=Manual+for+Sputum+Smear+Fluorescence+Microscopy+%2C+RNTCP+2007%3A+http%3A%2F%2Ftbcindia.nic.in%2Fpdfs%2FFlourescence+Microscopy%2520Manual.pdf&btnG=

53. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006 Sep;6(9):570–81.

54. Cuevas LE, Al-Sonboli N, Lawson L, Yassin MA, Arbide I, Al-Aghbari N, et al. LED Fluorescence Microscopy for the Diagnosis of Pulmonary Tuberculosis: A Multi-Country Cross-Sectional Evaluation. PLOS Med. 2011 Jul 12;8(7):e1001057.

55. Reza LW, Satyanarayna S, Enarson DA, Kumar AMV, Sagili K, Kumar S, et al. LED-Fluorescence Microscopy for Diagnosis of Pulmonary Tuberculosis under Programmatic Conditions in India. PLOS ONE. 2013 Oct 9;8(10):e75566.

56. RICHARDS OW. The Staining of Acid-Fast Tubercle Bacteria. Am Assoc Adv Sci Sci. 1941 Feb 21;93(2408):190.

57. Brodie D, Schluger NW. The Diagnosis of Tuberculosis. Clin Chest Med. 2005 Jun;26(2):247–71.

58. Jorgensen JH, Carroll KC, Pfaller MA. Manual of Clinical Microbiology. ASM Press; 2015. 2892 p.

59. Rodrigues C, Shenai S, Almeida D, Sadani M, Goyal N, Vadher C, et al. Use of bactec 460 TB system in the diagnosis of tuberculosis. Indian J Med Microbiol. 2007;25(1):32.

60. R Ramachandran. What is new in the diagnosis of tuberculosis? Part 1: techniques for diagnosis of tuberculosis. [Internet]. [cited 2016 May 13]. Available from: http://icmr.nic.in/

61. WHO | Policy guidance on TB drug susceptibility testing (DST) of second-line drugs (SLD) [Internet]. WHO. [cited 2016 May 18]. Available from: http://www.who.int/tb/features_archive/xdr_mdr_policy_guidance/en/

62. Kim SJ. Drug-susceptibility testing in tuberculosis: methods and reliability of results. Eur Respir J. 2005 Mar 1;25(3):564–9.

63. WHO | Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis [Internet]. WHO. [cited 2016 May 18]. Available from: http://www.who.int/tb/publications/pmdt_companionhandbook/en/

64. Canetti G, Froman S, Grosset J, Hauduroy P, Langerová M, Mahler HT, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ. 1963;29(5):565–78.

65. What is new in the diagnosis of tuberculosis ? PART II Techniques for Drug Susceptibility Testing Indian Council of Medical Research [Internet]. [cited 2016 May 19]. Available from: http://icmr.nic.in/

66. Klingeren B van, Dessens-Kroon M, Laan T van der, Kremer K, Soolingen D van. Drug Susceptibility Testing of Mycobacterium tuberculosis Complex by Use of a High-Throughput, Reproducible, Absolute Concentration Method. J Clin Microbiol. 2007 Aug 1;45(8):2662–8.

67. Acharya S, Ghimire P, Khadka D, Nepali S. Comparison of Proportion and Resistance Ratio Methods for Drug Susceptibility Testing of <i>Mycobacterium tuberculosis</i> Isolated from Patients Attending National Tuberculosis Centre, Nepal. SAARC J Tuberc Lung Dis HIVAIDS [Internet]. 2010 May 7 [cited 2016 May 19];5(1). Available from: http://www.nepjol.info/index.php/SAARCTB/article/view/3078

68. Parsons LM, Somosköví Á, Gutierrez C, Lee E, Paramasivan CN, Abimiku A 'le, et al. Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities. Clin Microbiol Rev. 2011 Apr;24(2):314–50.

69. Rohner P, Ninet B, Metral C, Emler S, Auckenthaler R. Evaluation of the MB/BacT system and comparison to the BACTEC 460 system and solid media for isolation of mycobacteria from clinical specimens. J Clin Microbiol. 1997 Dec 1;35(12):3127–31.

70. Díaz-Infantes MS, Ruiz-Serrano MJ, Martínez-Sánchez L, Ortega A, Bouza E. Evaluation of the MB/BacT Mycobacterium Detection System for Susceptibility Testing of Mycobacterium tuberculosis. J Clin Microbiol. 2000 May 1;38(5):1988–9.

71. Espasa M, Salvadó M, Vicente E, Tudó G, Alcaide F, Coll P, et al. Evaluation of the VersaTREK System Compared to the Bactec MGIT 960 System for First-Line Drug Susceptibility Testing of Mycobacterium tuberculosis. J Clin Microbiol. 2012 Feb 1;50(2):488–91.

72. Rüsch-Gerdes S, Pfyffer GE, Casal M, Chadwick M, Siddiqi S. Multicenter Laboratory Validation of the BACTEC MGIT 960 Technique for Testing Susceptibilities of Mycobacterium tuberculosis to Classical Second-Line Drugs and Newer Antimicrobials. J Clin Microbiol. 2006 Mar 1;44(3):688–92.

73. Growth Detection Failures by the Nonradiometric Bactec MGIT 960 Mycobacterial Culture System [Internet]. [cited 2016 May 22]. Available from: http://jcm.asm.org/content/50/6/2092.short

74. Piersimoni C, Olivieri A, Benacchio L, Scarparo C. Current Perspectives on Drug Susceptibility Testing of Mycobacterium tuberculosis Complex: the Automated Nonradiometric Systems. J Clin Microbiol. 2006 Jan;44(1):20–8.

75. WHO | Noncommercial culture and drug-susceptibility testing methods for screening patients at risk for multidrug-resistant tuberculosis [Internet]. WHO. [cited 2016 May 22]. Available from: http://www.who.int/tb/publications/2011/mdr_tb_diagnostics_9789241501620/en/

76. Moore DAJ, Evans CAW, Gilman RH, Caviedes L, Coronel J, Vivar A, et al. Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of TB. N Engl J Med. 2006 Oct 12;355(15):1539–50.

77. Michael JS, Daley P, Kalaiselvan S, Latha A, Vijayakumar J, Mathai D, et al. Diagnostic accuracy of the microscopic observation drug susceptibility assay: a pilot study from India. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2010 Apr;14(4):482–8.

78. Moore DAJ, Evans CAW, Gilman RH, Caviedes L, Coronel J, Vivar A, et al. Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of TB. N Engl J Med. 2006 Oct 12;355(15):1539–50.

79. Martin A, Portaels F, Palomino JC. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in Mycobacterium tuberculosis: a systematic review and meta-analysis. J Antimicrob Chemother. 2007 Feb 1;59(2):175–83.

80. Singh S, Kumar P, Sharma S, Mumbowa F, Martin A, Durier N. Rapid Identification and Drug Susceptibility Testing of Mycobacterium tuberculosis: Standard Operating Procedure for Non-Commercial Assays: Part 3: Colorimetric Redox Indicator Assay v1.3.12. J Lab Physicians. 2012 Jul;4(2):120–6.

81. Singh S, Kumar P, Sharma S, Mumbowa F, Martin A, Durier N. Rapid Identification and Drug Susceptibility Testing of Mycobacterium tuberculosis: Standard Operating Procedure for Non-Commercial Assays: Part 2: Nitrate Reductase Assay v1.3.12. J Lab Physicians. 2012 Jul;4(2):112–9.

82. Kalokhe AS, Shafiq M, Lee JC, Ray SM, Wang YF, Metchock B, et al. Multidrug-resistant tuberculosis drug susceptibility and molecular diagnostic testing. Am J Med Sci. 2013 Feb;345(2):143–8.

83. Weyer K, Mirzayev F, Migliori GB, Van Gemert W, D'Ambrosio L, Zignol M, et al. Rapid molecular TB diagnosis: evidence, policy making and global implementation of Xpert MTB/RIF. Eur Respir J. 2013 Jul;42(1):252–71.

84. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, et al. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. The Lancet. 2011 May 6;377(9776):1495–505.

85. Suzana S, Shalini B, Rupali P, Venkatesh K, Christopher D, Michael JS. Rapid diagnosis of extra pulmonary tuberculosis by automated Xpert MTB/RIF assay. BMC Infect Dis. 2012;12(1):1–1.

86. WHO | The Global Plan to Stop TB 2006–2015 [Internet]. [cited 2016 May 9]. Available from: http://www.who.int/tb/features_archive/global_plan_to_stop_tb/en/

87. Organization WH, others. The use of molecular line probe assay for the detection of resistance to second-line anti-tuberculosis drugs: expert group meeting report, Geneva, February 2013. 2013 [cited 2016 May 24]; Available from: http://apps.who.int/iris/handle/10665/78099

88. Fakruddin M, Chowdhury A, Hossain MN, Mannan KS, Mazumda RM. Pyrosequencing-principles and applications. Int J Life Sci Pharma Res. 2012;2:65–76.

89. Ajbani K, Lin S-YG, Rodrigues C, Nguyen D, Arroyo F, Kaping J, et al. Evaluation of Pyrosequencing for Detecting Extensively Drug-Resistant Mycobacterium tuberculosis among Clinical Isolates from Four High-Burden Countries. Antimicrob Agents Chemother. 2015 Jan 1;59(1):414–20.

90. Lin S-YG, Rodwell TC, Victor TC, Rider EC, Pham L, Catanzaro A, et al. Pyrosequencing for rapid detection of extensively drug-resistant Mycobacterium tuberculosis in clinical isolates and clinical specimens. J Clin Microbiol. 2014 Feb;52(2):475–82.

91. Rizzo JM, Buck MJ. Key Principles and Clinical Applications of "Next-Generation" DNA Sequencing. Cancer Prev Res (Phila Pa). 2012 Jul 1;5(7):887–900.

92. Jureen P, Engstrand L, Eriksson S, Alderborn A, Krabbe M, Hoffner SE. Rapid Detection of Rifampin Resistance in Mycobacterium tuberculosis by Pyrosequencing Technology. J Clin Microbiol. 2006 Jun;44(6):1925–9.

93. Gega A, Kozal MJ. New technology to detect low-level drug-resistant HIV variants. Future Virol. 2011 Jan;6(1):17–26.

94. Zhang Y. The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol. 2005;45:529–564.

95. Mani C, Selvakumar N, Narayanan S, Narayanan PR. Mutations in the rpoB Gene of Multidrug-Resistant Mycobacterium tuberculosis Clinical Isolates from India. J Clin Microbiol. 2001 Aug;39(8):2987–90.

96. Yue J, Shi W, Xie J, Li Y, Zeng E, Wang H. Mutations in the rpoB gene of multidrug-resistant Mycobacterium tuberculosis isolates from China. J Clin Microbiol. 2003 May;41(5):2209–12.

97. Palomino JC, Martin A. Drug Resistance Mechanisms in Mycobacterium tuberculosis. Antibiotics. 2014 Jul 2;3(3):317–40.

98. Hamze M, Ismail MB, Rahmo AK, Dabboussi F. Pyrosequencing for rapid detection of tuberculosis resistance to Rifampicin and Isoniazid in Syrian and Lebanese clinical isolates. Int J Mycobacteriology. 2015 Sep;4(3):228–32.

99. Wang S, Zhao B, Song Y, Zhou Y, Pang Y, Ou X, et al. Molecular characterization of the rpoB gene mutations of Mycobacterium tuberculosis isolated from China. J Tuberc Res. 2013;1(1):1–8.

100. Patra SK, Jain A, Sherwal BL, Khanna A. Rapid Detection of Mutation in RRDR of rpo B Gene for Rifampicin Resistance in MDR-Pulmonary Tuberculosis by DNA Sequencing. Indian J Clin Biochem. 2010 Jul;25(3):315–8.

101. Zheng R, Zhu C, Guo Q, Qin L, Wang J, Lu J, et al. Pyrosequencing for rapid detection of Tuberculosis resistance in clinical isolates and Sputum samples from re-treatment Pulmonary Tuberculosis patients. BMC Infect Dis. 2014 Apr 13;14(1):200.

102. Van Deun A, Maug AK, Bola V, Lebeke R, Hossain MA, de Rijk WB, et al. Rifampicin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol. 2013;JCM–00553.

103. Deun AV, Barrera L, Bastian I, Fattorini L, Hoffmann H, Kam KM, et al. Mycobacterium tuberculosis Strains with Highly Discordant Rifampin Susceptibility Test Results. J Clin Microbiol. 2009 Nov 1;47(11):3501–6.

104. Engström A, Morcillo N, Imperiale B, Hoffner SE, Juréen P. Detection of First- and Second-Line Drug Resistance in Mycobacterium tuberculosis Clinical Isolates using Pyrosequencing. J Clin Microbiol. 2012 Mar 29;JCM.06664-11.

105. Zetola NM, Shin SS, Tumedi KA, Moeti K, Ncube R, Nicol M, et al. Mixed Mycobacterium tuberculosis complex infections and false-negative results for

rifampin resistance by GeneXpert MTB/RIF are associated with poor clinical outcomes. J Clin Microbiol. 2014 Jul;52(7):2422–9.

106. Bravo LTC, Tuohy MJ, Ang C, Destura RV, Mendoza M, Procop GW, et al. Pyrosequencing for Rapid Detection of Mycobacterium tuberculosis Resistance to Rifampin, Isoniazid, and Fluoroquinolones. J Clin Microbiol. 2009 Dec 1;47(12):3985–90.

107. Hillemann D, Rüsch-Gerdes S, Richter E. Feasibility of the GenoType MTBDRsl Assay for Fluoroquinolone, Amikacin-Capreomycin, and Ethambutol Resistance Testing of Mycobacterium tuberculosis Strains and Clinical Specimens. J Clin Microbiol. 2009 Jun 1;47(6):1767–72.

108. Avalos E, Catanzaro D, Catanzaro A, Ganiats T, Brodine S, Alcaraz J, et al. Frequency and Geographic Distribution of gyrA and gyrB Mutations Associated with Fluoroquinolone Resistance in Clinical Mycobacterium Tuberculosis Isolates: A Systematic Review. PLOS ONE. 2015 Mar 27;10(3):e0120470.

109. Georghiou SB, Seifert M, Lin S-Y, Catanzaro D, Garfein RS, Jackson RL, et al. Shedding light on the performance of a pyrosequencing assay for drug-resistant tuberculosis diagnosis. BMC Infect Dis. 2016;16:458.

110. Kambli P, Ajbani K, Nikam C, Sadani M, Shetty A, Udwadia Z, et al. Correlating rrs and eis promoter mutations in clinical isolates of Mycobacterium tuberculosis with phenotypic susceptibility levels to the second line injectables. Int J Mycobacteriology. 2016 Mar;5(1):1–6.

111. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of Genetic Mutations Associated with Mycobacterium tuberculosis Resistance to Amikacin, Kanamycin and Capreomycin: A Systematic Review. PLOS ONE. 2012 Mar 29;7(3):e33275.

112. Barnard M, Warren R, Van Pittius NG, van Helden P, Bosman M, Streicher E, et al. GenoType MTBDRsl Line Probe Assay Shortens Time to Diagnosis of Extensively Drug-Resistant Tuberculosis in a High-Throughput Diagnostic Laboratory. Am J Respir Crit Care Med. 2012 Dec 15;186(12):1298–305.

113. Ninan MM, Gowri M, Christopher DJ, Rupali P, Michael JS. The diagnostic utility of line probe assays for multidrug-resistant tuberculosis. Pathog Glob Health. 2016 Aug 8;1–6.

114. Nikam C, Patel R, Sadani M, Ajbani K, Kazi M, Soman R, et al. Redefining MTBDRplus test results: what do indeterminate results actually mean? Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Feb;20(2):154–9.

115. Georghiou SB, Ajbani K, Rodrigues C, Rodwell TC. Performance of a pyrosequencing platform in diagnosing drug-resistant extra-pulmonary

tuberculosis in India. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2016 Feb;20(2):160–5.

116. Huang W-L, Chen H-Y, Kuo Y-M, Jou R. Performance Assessment of the GenoType MTBDRplus Test and DNA Sequencing in Detection of Multidrug-Resistant Mycobacterium tuberculosis. J Clin Microbiol. 2009 Aug 1;47(8):2520–4.

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 05, 2015

Dr. Aishwarya. G
PG Registrar
Department of Clinical Microbiology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research Grant Project:**
To evaluate Pyrosequencing for the rapid detection of Multidrug drug resistant and extensively drug resistant tuberculosis. A pilot study.
Dr. Aishwarya. G. PG Registrar, Dr. Joy Sarojini Michael, Clinical Microbiology, Dr. Rekha Pai, General Pathology, CMC, Vellore.

Ref: IRB Min No. 9131 [DIAGNOSE] dated 12.11.2014

Dear Dr. Aishwarya. G,

I enclose the following documents:

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy Sarojini Michael, Clinical Microbiology, CMC, Vellore.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 05, 2015

Dr. Aishwarya. G
PG Registrar
Department of Clinical Microbiology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research Grant Project:**
To evaluate Pyrosequencing for the rapid detection of Multidrug drug resistant and extensively drug resistant tuberculosis. A pilot study.
Dr. Aishwarya. G, PG Registrar, Dr. Joy Sarojini Michael, Clinical Microbiology, Dr. Rekha Pai, General Pathology, CMC, Vellore.

Ref: IRB Min No: 9131 [DIAGNOSE] dated 12.11.2014

Dear Dr. Aishwarya. G,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To evaluate Pyrosequencing for the rapid detection of Multidrug drug resistant and extensively drug resistant tuberculosis. A pilot study." on November 12th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. Aishwarya. G, Joy Sarojini Michael, Rekha Pai.
3. Informed Consent form & Information Sheet (English, Tamil & Telugu)
4. Clinical Research Form
5. No of documents 1-4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on November 12th 2014 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Name	Qualification	Designation	Other Affiliations
Dr. Chandra Singh	MS, MCH, DMB	Professor, Urology, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS M Ch	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Bobby John	MBBS, MD, DM, Ph D, MAMS	Professor, Cardiology, CMC, Vellore	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Colorectal Surgery, CMC, Vellore	Internal, Clinician
Dr. Rajesh Kannangai	MD, Ph D.	Professor & In-charge Retrovirus Laboratory (NRL under NACO), Department of Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Anup Ramachandran	Ph. D	The Wellcome Trust Research Laboratory Gastrointestinal Sciences, CMC	Internal, Basic Medical Scientist
Dr. Anand Zachariah	MBBS, PhD	Professor, Medicine, CMC, Vellore	Internal, Clinician
Dr. Simon Pavamani	MBBS, MD,	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Dept of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. T. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Denise H. Fleming	B. Sc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMC, Vellore	Internal, Scientist & Pharmacologist
Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Addl. Deputy Nursing Superintendent, Professor of Nursing in Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Legal Expert, Vellore	External, Legal Expert
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist & Epidemiologist
Rev. Joseph Devaraj	B. Sc, BD	Chaplaincy Department, CMC	Internal, Social Scientist

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Nihal Thomas,	MD, MNAMS, DNB(Endo), FRACP(Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2.5 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/ Annual report and subsequent submission of it to the IRB.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD,MNAMS,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy Sarojini Michael, Clinical Microbiology, CMC, Vellore.

IRB Min No: 9131 [DIAGNOSE] dated 12.11.2014

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

ISOLATE NO.	STATE	AGE	SEX	RIF (LJ DST)	OFLOX (LJ DST)	CAP (LJ DST)	KAN (LJ DST)	Gene Xpert	PYRO *rpoB*	PYRO *gyrA*	PYRO *rrs*	PYRO *eis*
ISO 1	West Bengal	27	M	R	R	S	S	RESISTANT	GAC516GTC	GAC94CAC	WT	
ISO 2	Tamil Nadu	21	M	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	WT
ISO 3	West Bengal	15	F	R	R	S	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 4	West Bengal	33	M	R	R	S	R	RESISTANT	TCG531TTG	GAC94AAC	WT	WT
ISO 5	Jharkhand	16	M	R	R	S	S	RESISTANT	TCG531TTG	GCG90GTG	WT	
ISO 6	West Bengal	44	M	R	S	S	S	RESISTANT	FAIL	WT	WT	
ISO 7	Sikkim	23	M	R	R	S	S	RESISTANT	TCG531TTG	GAC94GCC	WT	
ISO 8	Assam	25	M	R	R	S	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 9	Bihar	25	F	R	R	R	R	RESISTANT	GAC516GTC	GAC94GGC	WT	WT
ISO 10	Jharkhand	44	M	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	WT
ISO 11	Jharkhand	21	M	R	R	S	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 12	West Bengal	19	F	R	R	R	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 13	Tamil Nadu	46	M	S	S	S	S	SUSCEPTIBLE	WT	WT	WT	
ISO 14	West Bengal	54	M	R	NOT DONE			RESISTANT	TCG531TTG	NOT DONE	NOT DONE	
ISO 15	Jharkhand	18	M	S	S	S	S	SUSCEPTIBLE	WT	FAIL	WT	
ISO 16	Jharkhand	22	M	S	S	S	S	SUSCEPTIBLE	WT	WT	WT	
ISO 17	West Bengal	34	F	S	S	S	S	SUSCEPTIBLE	WT	WT	WT	
ISO 18	West Bengal	25	M	R	R	S	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 19	Sikkim	28	F	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	
ISO 20	Jharkhand	14	F	S	S	S	S	SUSCEPTIBLE	WT	WT	FAIL	
ISO 21	Tamil Nadu	34	F	R	S	S	S	RESISTANT	GAC516TAC	WT	FAIL	
ISO 22	H37RA			S	S	S	S	SUSCEPTIBLE	WT	WT	WT	WT
ISO 23	West Bengal	57	M	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	
ISO 24	West Bengal	40	M	S	NOT DONE			SUSCEPTIBLE	WT	NOT DONE	NOT DONE	
ISO 25	Jharkhand	23	M	R	R	S	S	RESISTANT	TCG531TTG	GCG90GTG	WT	
ISO 26	Andra Pradesh	54	M	S	S	S	S	SUSCEPTIBLE	WT	WT	WT	
ISO 27	Kerala	54	M	S	NOT DONE			SUSCEPTIBLE	WT	NOT DONE	NOT DONE	
ISO 28	Tamil Nadu	54	M	S	NOT DONE			SUSCEPTIBLE	WT	NOT DONE	NOT DONE	
ISO 29	Bihar	54	M	R	NOT DONE			RESISTANT	TCG531TTG	NOT DONE	NOT DONE	
ISO 30	West Bengal	54	M	R	S	S	R	RESISTANT	CAC526CTC	WT	WT	WT
ISO 31	West Bengal	54	M	R	S	S	S	RESISTANT	GAC516GTC	WT	WT	
ISO 32	Sikkim	54	F	R	R	R	R	INDETERMINATE	TCG531TTG	GAC94GGC	WT	WT
ISO 33	Jharkhand	54	F	R	R	R	R	RESISTANT	TCG531TTG	GAC94GGC	WT	WT
ISO 34	Orrisa	54	M	R	R	R	R	RESISTANT	TCG531TTG	GCG90GTG	A1401G	WT
ISO 35	Arunachal Pradesh	54	M	R	R	R	R	RESISTANT	TCG531TTG	GAC94GGC	A1401G	WT
ISO 36	West Bengal	54	M	R	R	R	S	NOT DONE	FAIL	GAC94GCC	WT	
ISO 37	Jharkhand	54	M	R	R	R	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 38	West Bengal	54	M	R	R	R	R	NOT DONE	CTG511CGG,GAC516TAC	GAC94GGC	WT	WT
ISO 39	West Bengal	54	M	R	R	R	S	RESISTANT	TCG531TTG	GAC94TAC	WT	
ISO 40	Jharkhand	54	F	R	R	R	S	RESISTANT	TCG531TTG	FAIL	WT	
ISO 41	West Bengal	54	M	R	R	R	S	RESISTANT	TCG531TTG	GAC94GGC	FAIL	

ISO 42 (D)	Tamil Nadu	54	F	R		NOT DONE		SUSCEPTIBLE	TCG531TTG	NOT DONE	NOT DONE	
ISO 43 (D)	Bihar	54	M	S	R	S	S	RESISTANT	FAIL	GAC94GGC	FAIL	
ISO 44 (D)	West Bengal	54	F	S	S	S	S	RESISTANT	CTG511CCG	WT	WT	
ISO 45 (D)	Andra Pradesh	54	M	R	R	S	S	SUSCEPTIBLE	FAIL	FAIL	WT	
ISO 46 (D)	Andra Pradesh	54	F	S	S	S	S	RESISTANT	CTG511CCG	WT	WT	
ISO 47 (D)	Andra Pradesh	54	F	S		NOT DONE		RESISTANT	FAIL	NOT DONE	NOT DONE	
ISO 48 (D)	Andra Pradesh	54	F	S	S	S	S	RESISTANT	FAIL	NOT DONE	NOT DONE	
ISO 49	West Bengal	54	M	S	S	S	S	SUSCEPTIBLE	WT	FAIL	WT	
ISO 50	Andra Pradesh	54	F	R	R	S	R	RESISTANT	TCG531TTG	GCG90GTG	WT	WT
ISO 51	Bangladesh	54	M	S	S	S	S	SUSCEPTIBLE	WT	FAIL	WT	
ISO 52	Tamil Nadu	54	M	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	
ISO 53	Bihar	54	F	R	R	S	S	RESISTANT	TCG531TTG	GCG90GTG	WT	
ISO 54	Meghalaya	54	F	R	R	S	S	RESISTANT	TCG531TTG	GAC94GGC	WT	
ISO 55 (D)	Jharkhand	54	M	S	S	S	S	RESISTANT	CTG511CCG	WT	WT	
ISO 56	Jharkhand	54	M	S	S	S	S	SUSCEPTIBLE	FAIL	WT	WT	
ISO 57	West Bengal	54	M	R	R	R	R	RESISTANT	TCG531TTG	GCG90GTG	C1402T	WT
ISO 58	West Bengal	54	M	R	R	R	R	RESISTANT	TCG531TTG	FAIL	A101G	WT
ISO 59	Tamil Nadu	54	F	R	R	S	R	RESISTANT	TCG531TTG	GCG90GTG	WT	C12T
ISO 60	Jharkhand	54	F	R	R	R	R	RESISTANT	TCG531TTG	GCG90GTG	A1401G	WT
ISO 61	Bihar	54	M	R	R	R	R	RESISTANT	TCG531TTG	GAC94GGC	A1401G	WT
ISO 62	West Bengal	54	M	S	S	S	S	SUSCEPTIBLE	WT	WT	WT	
ISO 63	Tamil Nadu	54	M	R	S	S	S	RESISTANT	TCG531TTG	WT	WT	
ISO 64	Jharkhand	54	M	S	S	S	S	SUSCEPTIBLE	WT	WT	FAIL	

M: Male F: Female

LJ DST: Lowenstein-Jensen (Conventional) Drug Susceptibility Testing

R: Resistant S: Susceptible

WT: Wild Type