Identification and Characterization of Phenotypically Occult Multidrug Resistant *Mycobacterium Tuberculosis* Strains in Pakistan

By

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A thesis submitted in the partial fulfillment of the requirements for the degree of

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In

Microbiology

By

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Dedicated

To my loving father and mother for providing will to aspire.

To the best wife for giving me courage and support

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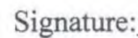
Student Name: **Syed Mehmood Qadir** Signature: 

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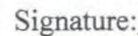
a) External Examiner 1: Signature: 

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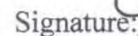
b) External Examiner 2: Signature: 

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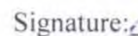
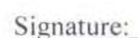
Supervisor Name: **Prof. Dr. Rani Faryal** Signature: 

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature: 

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List of Abbreviations

Abbreviations	Description
AIDS	Acquired immunodeficiency syndrome
AFB	Acid fast bacilli
AG	Arabinogalactan
AM	Amikacin
AMTD	Amplified mycobacteria direct test
BCG	Bacillus-Calmette Guérin
BSL-I	Biosafety Level-I
CRISPR	Clustered regularly interspaced short palindromic repeat
CP	Capreomycin
CRI	Colorimetric redox indicator
CTAB	Cetyltrimethylammonium bromide
DOTS	Directly Observed Treatment Short-Course
DST	Drug susceptibility testing
EMB	Ethambutol
ETM	Ethionamide
FRET	Fluorescence resonance energy transfer
FQs	Fluoroquinolones
GT	Gatifloxacin
HIV	Human immunodeficiency virus
INH	Isoniazid
Kg	Kilogram
KM	Kanamycin
L	Liter
LJ	Löwenstein-Jensen
LAM	Lipoarabinomannan
LEV	Levofloxacin
MTB	*Mycobacterium tuberculosis*
MDR	Multi-drug resistant

Abbreviations	Description
M	Molar
MA	Mycolic acids
MGIT	Mycobacteria growth indicator tube system
MIC	Minimum inhibitory concentration
Min	Minutes
mL	Milliliter
mM	Millimolar
Mm	Millimeter
MODS	Microscopic observation of drug susceptibility
MOX	Moxifloxacin
MTBC	*Mycobacterium tuberculosis* complex
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NGS	Next Generation Sequencing
NRA	Nitrate reductase assay
NTM	Non-tuberculosis Mycobacteria
OFX	Ofloxacin
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimy-ocerosate
PG	Peptidoglycan
PKS	Polyketide synthase
PZA	Pyrazinamide
RIF	Rifampicin
R	Resistant
REMA	Resazurin microtiter assay
ROT	Rothionamide
RR-TB	Rifampicin resistant TB
S	Sensitive
SM	Streptomycin
SNP	Single nucleotide polymorphisms

Abbreviations	Description
TB	Tuberculosis
UV	Ultraviolet
UHC	Universal coverage of health
WHO	World Health Organization
w/v	Weight/Volume
ZN	Ziel Neilson
μg	Microgram
μL	Microliter

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Syed Mehmood Qadir

Abstract

Tuberculosis (TB) is one of the known ancient infectious disease and is still part of the world's disease burden despite vaccination and the availability of anti-tuberculosis drugs for treatment. As per World Health Organization 2020 report on TB, there are an estimated 10 million TB cases and 1.4 million per annum deaths due to TB disease. This situation of TB is further aggravated due to the development of multidrug resistance (MDR) and extensively drug resistance (XDR) TB. Rifampicin (RIF) is one of the most effective anti-tuberculosis first-line drugs prescribed along with isoniazid for its treatment. The resistance to RIF is used as a surrogate marker for MDR TB diagnosis in new as well as previously treated patients. Pakistan is among the top five high TB burdened countries, even for high MDR TB cases. For the diagnosis of drug resistance (DR) TB, WHO endorsed phenotypic and genotypic assays are used, however, all these methods have discrepancies in the diagnosis of drug resistance. Thus, discordance in results for drug resistance is encountered frequently, which is the leading cause of misdiagnosis and treatment failure outcomes.

The current study was designed to identify the extent of discordance in diagnosis based on the results of molecular GeneXpert MTB/ RIF assay and phenotypic rifampicin drug susceptibility testing (DST) on Lowenstein Jensen (LJ) and commercial Bactec MGIT 960 (MGIT). A total of 1986 culture-positive samples out of 6,148 samples were selected for the study which were received from different Programmatic Management of Drug-Resistant TB sites, public sector hospitals of Punjab including Islamabad capital territory and Kashmir. The majority of the TB study population was of the age group 15 to 44 years which accounted for 72.2% of the total TB cases. Among all TB patients included in this study, 1876 (94%) were diagnosed with pulmonary TB, whereas 110 (6%) were extrapulmonary TB cases. Out of 1986 TB cases, concordance in the drug susceptibility results of GeneXpert, MGIT, and LJ DST was found in 1760 Mtb isolates. However, discordance was identified in 226(11.4%) isolates in either of the assays when all three assays' results were compared.

In these identified occult and concordant strains on basis of GeneXpert, MGIT, and LJ DST results were Sanger sequenced for the *rpoB* gene to determine the molecular basis for RIF resistance and discordance results. Sequencing of *rpoB* gene was performed on selected 516 isolates comprising of 140 strains with discordant and 376 isolates with concordant results. The sequencing of these strains found 49 different insertions, deletions, and single double and triple nucleotide polymorphisms in Rifampicin Resistance Determining Region (RRDR) as well as outside RRDR. Among these 49 identified mutations, 03 were novel mutations in the *rpoB* gene in these local Mtb strains. The most frequent mutations were detected at codon 450, 445, and 435 of the *rpoB* gene, which constituted 58%, 14% and 11% prevalence respectively. A high number of mutations (362) were detected in the 81-bp core region of the *rpoB* gene, whereas 2 different mutations were detected outside RRDR at position 170 in one isolate and codon 491 in five isolates. GeneXpert MTB/RIF assay for RIF resistance results was compared with sequencing, a 10.9 % discordance was observed in these two molecular methods' results of 516 selected isolates. The 3.2% discordance was present in GeneXpert sensitive isolates which were identified to be resistant after *rpoB* gene sequencing, while 7.7% discordance results were observed in GeneXpert RIF resistant isolates which were wild type upon Sanger sequencing.

The phenotypic assay MGIT DST results upon comparison with sequencing had 24% discordance, where 23.7% of discordant strains were harboring *rpoB* gene mutations but were RIF susceptible on MGIT DST. The solid-based LJ DST results comparison with sequencing found a discordance of 10.9% which is lower than MGIT DST. Among these 10.6% isolates, which were resistant due to *rpoB* gene mutations after sequencing but were initially detected as susceptible on LJ DST. Most prevalent nonsynonymous mutations altered a codon which resulted in the change of single amino acid in various isolates and was associated with discordant results, such identified mutations were Leu433Pro in 19/20 isolates and 14/20isolates, Asp435Tyr in 17/21 and 5/21, Leu452Pro in 17//20 and 5/20, His445Asn in 15/18 and 11/18, His445Leu in 8/9 and 0/9 and His445Cys in 2/6 and 1/6 on resistant on MGIT and LJ DST respectively. Leu430Arg change was detected in two isolates, while Ser431Gly,

Asn438Lys, and Thr444Ala mutations were found in one isolate each. Also, in the current study mutations were characterized as discordant which were not reported as discordant in previous studies.

Whole Genome sequencing was performed on 25 selected isolates including drug-susceptible (DS) TB, MDR TB, Pre-XDR, and XRD TB isolates. A large number of mutations were identified in *katG*, *inh A*, *gyrA, gyrB*, *embB*, *pncA,* and *rss* genes, which are responsible for drug resistance development to other anti TB drugs used for the treatment of drug-susceptible and DR TB. Upon comparison of phenotypic drug susceptibility testing results for other than RIF anti TB drugs with WGS, phenotypic and genotypic resistance results were in line but in some cases, discordance in results was recorded. Even for newly introduced anti TB drugs Bedaquiline and clofazimine resistance-conferring mutations in the Rv0678 gene were detected through WGS, which highlights serious concern as intrinsic resistance to these newly approved drugs is present already in the local Mtb strain. To further dissect the impact of mutations in the *rpoB* gene on this gene encoded DNA dependent RNA polymerase structure, *in silico* bioinformatics protein prediction tool DynaMut was used. The predicated RNA polymerase structure in most of the identified mutations destabilized protein and increased flexibility of polymerase even binding affinity to the target drug was altered. Phylogenetic analysis for the lineage of Mtb isolates revealed that the majority belonged to Delhi⁄CAS lineage followed by Euro-American Super linage. The discordance between results of different assays was not related to patients' age, gender, and treatment category or lineage identified.

Based on current study findings, among phenotypic methods, LJ DST was found to be less prone to miss rifampicin resistance compared to MGIT DST. Routine WHO endorsed genotypic assay (GeneXpert) used for screening of rifampicin resistance also missed resistance if a mutation in strain was outside RRDR region, hence posing a major setback where strains will be misdiagnosed leading to treatment failures and proliferation of DR strains if were treated as RIF sensitive TB. All three methods (LJ, MGIT, and GeneXpert) had limitations in the drug resistance detection due inability to pick both

previously reported as well as newly identified mutations, which in turn will give high discordance results. Phenotypic methods are the existing gold standard for DST of DR TB but were found mostly failed to compete with sequencing technologies for the diagnosis of rifampicin-resistant TB and MDR TB. Based on this study, it can be proposed that sequencing should be endorsed as the gold standard for the diagnosis of DR TB especially suspected cases of MDR TB and XDR TB. It can be recommended after extensive comparison of WHO endorsed various DST assays, globally as well as in Pakistan gold standard method for detection of RIF resistance and MDR should be revisited, and a new evidence-based DST for TB should be adopted for better treatment, management, and reduction of all types of TB.

PUBLICATION

Mehmood Qadir, Sabira Tahseen, Timothy D. McHugh, Alamdar Hussain, Faisal Masood, Niaz Ahmed, Rani Faryal, 2021. Profiling and identification of novel *rpoB* mutations in rifampicin-resistant *Mycobacterium tuberculosis* clinical isolates from Pakistan**. Journal of Infection and Chemotherapy. ISSN 1341-321X**

1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb) is one of the main worldwide infections persistent for many decades and it is a major cause of deaths globally, even when the world is suffering from the SARS-CoV-2 pandemic. World Health Organization (WHO) estimated 1.4 million annual deaths and 10 million cases due to TB in the year 2019 (WHO, 2020). Although, it has been sixty years since the application of chemotherapy for treating TB and ninety years since the application of vaccines; yet this disease remains a prime cause of global death as the number of deaths by TB exceeds the mortality rate by the human immunodeficiency virus (HIV) infection in recent years (Getahun *et al.*, 2015; Organization, 2017). Alarmingly, still many cases (around 3 million) remain masked and are unable to receive proper healthcare or treatment. This disease primarily damages the lungs and uses the respiratory tract for transfer between humans, however, it can also spread to many other tissues of the body. It has been reported that among the people who contract this disease, about 10% go on to develop tuberculosis while the rest can contain its pathogen (Mtb) successfully. However, the presence of Mtb in the latent stage in most of the people affected by it remains a challenge. In these individuals, the disease does not develop for many years, but when the pathogen reactivates, infection develops and can even progress to a complicated case. This risk of disease progression in TB remains high even from the time it is initially contacted, and this risk is even more aggravated by the presence of any underlying illness or infections like acquired immunodeficiency syndrome (AIDS) by HIV. AIDS or any other disease can cause a co-infection in TB patients, increasing the severity of tuberculosis alarmingly.

This has been an important disease to tackle for humanity since ancient times. In the 1800s, tuberculosis patients were told to "only sleep and consume healthy foods," thus the term "consumption" was introduced for it (Keshavjee and Farmer, 2012). It was the German microbiologist Robert Koch, who proposed that tuberculosis is caused by Mtb in 1882, after that time this difficult and inexplicable rod-shaped bacillus was better understood by scientists (Keshavjee and Farmer, 2012). This groundbreaking discovery, along with the subsequent discoveries including tuberculin in 1890, the development of

the Bacillus-Calmette Guérin (BCG) vaccine in the year 1908, and finally the launch of anti-tuberculosis drugs in 1943 gave hope for the eradication of a TB, which was more deadly than the plague.

This era's discoveries led to a significant reduction in the mortality rates from the early to mid-twentieth century; however, later due to a lack of research funds the drug and vaccine production research work was stalled from 1970 to 1990 (Comas and Gagneux, 2009; Keshavjee and Farmer, 2012). With the advent of the AIDS pandemic along with the emergence of drug resistance among Mtb and the consequent presence of TB resistant strains, tuberculosis infection rates began to rise again, sparking renewed interest in TB research and prevention (Keshavjee and Farmer, 2012). While by this time diagnostic tools and treatment options were available, these techniques had become obsolete. In 1993, the Directly Observed Treatment Short-Course (DOTS) program was developed to monitor and prevent the disease, which was in 1998 upgraded to the DOTS-plus program to combat multi-drug resistant (MDR) TB (Comas and Gagneux, 2009; Keshavjee and Farmer, 2012).

Mycobacterium is classified into three classes for prognosis purposes: *M. leprae*, which causes leprosy; Mtb complex (MTBC), which causes TB in humans and animals; and all other non-pathogenic which includes Mycobacteria and non-tuberculosis Mycobacteria (NTM). There are altogether about 140 species in the genus Mycobacterium (Van Ingen, 2013). Out of these 140, 50 species are responsible for human diseases (Wagner and Young, 2004), even NTM can cause severe infections in immune-compromised individuals (Weiss and Glassroth, 2012; Tang *et al.*, 2018). Mtb complex is a group of Mycobacteria that are closely related. The MTBC includes *M. canettii* and *M. africanum*, known causative agent of African human TB, *M. Bovis* for mammalian TB which infects both cattle and humans, *M. microti* agent for Voles TB, *M. pinnipedii,* and *M. caprae* TB for animals, and finally Mtb agent of human TB (De Aguiar *et al.*, 2015; Miassangoumouka *et al.*, 2021). MTBC members' comparative genomic analysis revealed that despite their high degree of DNA similarity, their members have different phenotypic characteristics, hence cannot be considered as a single species (Grange, 1982; Niemann, Richter and Rüsch-Gerdes, 2000; Brosch *et al.*, 2002; Živanović *et al.*, 2014).

The first strain of Mtb completely sequenced was a laboratory strain Mtb H37Rv discovered cole colleagues in 1998 (Brosch *et al.*, 1998; Živanović *et al.*, 2014). This Mtb H37Rv strain genome comprisess 4.41 million (bp), having almost 4,000 genes, and a high cytosine (C) guanine (G) ratio of about 65% (Camus *et al.*, 2002). Plasmids, as well as other mobile genetic elements, were not found in Mtb but were found in other species (Reva, Korotetskiy, and Ilin, 2015). However, in several Mycobacteria species including *M. tuberculosis*, some additional DNA sequences or repetitive elements and insertion sequences (IS) are present. IS sequences such as IS6110 and CRISPR (clustered regularly interspaced short palindromic repeat) sequences also exist and are now considered essential for molecular epidemiology of these Mycobacteria *(Wiedenheft et al*., 2012).

Mtb is classified as an obligate aerobe but it can survive in a hypoxic environment as well (Bacon *et al.*, 2004). Mtb enter as small-aerosolized droplets called aerosols and after infection enters the alveoli of human lungs. Here, these Mtb rods interact with dendritic cells and macrophages, which are an important component of the immune innate system (Wolf *et al.*, 2007). The bacteria within the alveoli are engulfed by macrophages through phagocytosis; however, this bacterium has developed some escape and survival strategies to escape the macrophages (Hmama *et al.*, 2015). Even these phagocytes which have infected and have engulfed Mycobacteria, assist this Mtb to reach the other parts of the lungs as well as the peripheral lymph nodes. The host generates not only inborn but also adaptive responses after Mtb infection. The adaptive immune responses imparted by the host are both humoral and cell-mediated types of immune responses (Zuiga *et al.*, 2012). Due to its ability to highjack the immune system, TB has a high disease burden and even mortality is high without treatment. Anti-tuberculosis drugs are effective mostly in treating TB cases worldwide.

This disease is not an infection of the modern era but has existed in old times too. Exploration and analysis of ancient sites along with human skeletons have shown that tuberculosis has existed in early times (Hershkovitz *et al.*, 2015). The history of TB in the old-time is proven collectively by DNA analysis and pathological studies of the old mummies. In the pre-historical population, the tubercular defects in the ancient Egyptian

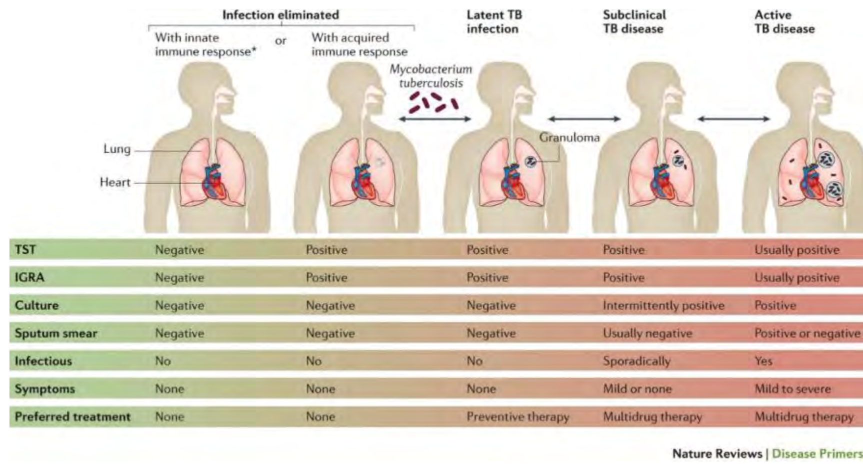
mummies show that TB was common. In old Chinese literature from 4000 BC and Indian religious books from 2000 BC, TB and TB like symptoms have been discussed (Daniel, 2006). Symptoms similar to TB have been found in even older literature, which shows the existence of TB dating back 15000 to 20000 years in various countries of the Middle East, even up to Italy and Denmark in Europe (Sreevatsan *et al.*, 1997).

In general, TB affects the lungs mainly but other parts can also be affected, where infection from pulmonary tuberculosis develops to extra-pulmonary TB. It is estimated that around 2 billion people are infected by Mtb but a relatively small number (5%-10%) develop TB in their lives. However, the probabilities of developing TB disease are much higher among HIV-positive, In the case of HIV, malnutrition, co-morbid, smokers, poor, and alcoholic drinkers chance of TB disease is increased (Habib *et al.*, 2018; MacPherson *et al.*, 2020).

Up to now, various TB diagnostic testing has been developed but sputum-based smear microscopy and molecular testing are rapid assays (Who, 2011) whereas culture strategies, which require as long as about two months for results yet stay the norm for reference. For detection of drug-resistant Mtb against both recommended regimens (first- and second-line anti-TB drugs), both phenotypic and genotypic assays including culture-based methods, polymerase chain reaction (PCR) based, and sequencing technology is applied even after treatment with various medications. Natural TB history studies document that about 70 percent of people who suffer from sputum smear-positive lung TB die within 10 years as well as 20 percent of individuals diagnosed with culturally positive (though smear-negative) pulmonary TB, in the absence of anti-TB therapy. TB diagnostic testing includes sputum-based smear microscopy, rapid molecular testing (first endorsed by the WHO in 2010), and culture methods, which take up to eight weeks to produce results but remain the standard for reference. Mtb resistance to first and second-line anti-TB drugs have been detected and are detected with a rapid test, culture methods, and sequencing technology after treatment with various medications. TB mortality is high without treatment. Natural TB history studies found about 70 percent of people who suffered from sputum smear-positive lung TB were dead in 10 years as well as 20 percent

of those who were diagnosed with culturally positive (though smear-negative) pulmonary TB, in the absence of anti-TB therapy (Tiemersma *et al.*, 2011).

In the 1940s, effective drug therapies were initially developed. A six-month regimen of four drugs which are termed as the first-line drugs: rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) was recommended, which are still recommended for cases of medicinally susceptible TB disease. A complete 6-month course for about 40 dollars per person is provided by the Global TB Drug Facility (WHO, 2020). For patients with medication-sensitive TB, the rate of success for treatment is 85%, and 194 Member States who are receiving Anti-TB drugs from WHO report to the WHO regularly on the treatment rates. Now the major issue is the treatment of multidrug-resistant TB (MDR TB) and rifampicin-resistant TB (RR TB) cases which need prolonged treatment time (Somasundaram, Ram and Sankaranarayanan, 2014). It becomes much more expensive, that is, ≥US$ 1000 per person from 40 US$, the top these drugs are more toxic (WHO, 2020). Bacille Calmette-Guérin vaccine is the only licensed vaccine for the prophylaxis of TB. BCG vaccine was developed nearly 100 years ago, but it is still administered for preventing serious forms of TB in children. No vaccine is currently available for the prevention of adult TB in adults before or after exposure to TB. It has been observed, however, that the results of a trial of the candidate vaccine M72/AS01E for Phase II are promising (WHO, 2020).



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 1.1: Spectrum of TB from Mtb infection to active pulmonary TB disease (Pai *et al.*, 2016)**.**

Mtb can cause disease throughout the body, although it is mainly a pulmonary pathogen. TB can be an asymptomatic or life-threatening disease, hence there is TB (Barry *et al.*, 2009; Esmail *et al.*, 2014). Pragmatically, patients with the TB infection and its latent state (LTBI) can be classified as asymptomatic as well as non-transmissible state versus active TB cases, when they are classified from public health and clinical perspective. Active pulmonary tuberculosis can be diagnosed through culture or molecular tests (Figure 1.1). General symptoms such as fatigue, fever, poor lingering, weight loss, and persistent cough and hemoptysis (coughing up blood) can be present in patients suffering from active TB illness, especially in the advanced stage of the disease. Some patients with cultural-positive active disease may, however, be asymptomatic and they can be classified as subclinical TB cases (Barry *et al.*, 2009; Esmail *et al.*, 2014).

1.2 Epidemiology of Tuberculosis

In 2019, approximately ten million people developed tuberculosis and 1.4 million deaths were reported. Mtb can affect any individual, but the majority (about 90%) of the people developing TB are adults, with the majority being men. In 2019, 30 countries having a high burden of the disease accounted for a majority (87%) of cases of TB. TB is a condition termed as the disease of poor people as individuals suffering from tuberculosis are often experiencing economic turmoil, vulnerability, marginalization, and social exclusion. Mtb has infected approximately one-quarter of the global population. More than 60 million deaths in the recovered TB cases have occurred since 2000, but still many millions of infected people lack access to proper diagnostic as well as to basic health care facilities, the majority have no access to universal coverage of health (UHC). For people affected with TB, preventive treatment is available but there is a need for improvement of multi-sectoral measures for the prevention of tuberculosis which includes poverty, nutrition, HIV infection, diabetes, and smoking. The improvement of these TB determinants can reduce the number of people who develop infections and even die due to this disease (WHO, 2020).

1.3 Classification of *Mycobacterium tuberculosis*

The causative agent of TB, Mtb belongs to the Actinobacteria phylum and is becoming increasingly linked to drug resistance due to mutations and rearrangements in its circular, single chromosome. This acid-fast, slow-growing and facultative intracellular bacterium's key pathogenesis mechanisms include avoiding host clearance by interrupting the usual dendritic cells and macrophage maturation process during phagocytosis, which normally confers innate immunity to the host (Koch and Mizrahi, 2018).

The genus Mycobacterium belongs to the order Actinomycetales, which also contains the other acid-fast bacteria containing mycolic acids in their cell walls like *Corynebacterium*, *Nocardia,* and *Rhodococcus* (Sakamoto, 2012). Microbes in this grouping have a genome characterized with a guanine-cytosine content as high as 61% to 71% (Palomiño *et al*., 2007), along with characteristic cell walls possessing a high amount of fatty acids (Palomiño *et al*., 2007; Knechel, 2009).

Based on the rate of growth of the genus mycobacteria, it is classified further into two subgroups (Helguera-Repetto *et al.*, 2004). Fast-growing bacteria, take one week for growth on solid media (De Groote and Huitt, 2006); while those that are slow-growing, need ten to twenty-eight days for producing visible colonies on petri plate containing solid media. Some members belonging to the Mtb complex also belong to the slow-growing group (Arnold, 2007), an example is *M. leprae* which is a pathogen of humans as well as animals (Pinheiro *et al.*, 2011).

Mycobacterium tuberculosis complex (MTBC) is a group containing eight mycobacterial species which are *Mycobacterium microti, M. caprae, M. canetti, M. pinnipedii, M. mungi, M. africanum M. bovis,* and *M.* tuberculosis (Bayraktar *et al.*, 2011; Živanović *et al.*, 2014). As these members have a similar genomic sequence, therefore, these eight species are grouped as MTBC (Brosch *et al.*, 2002). Despite their genetic relatedness, a difference in the degree of pathogenicity, phenotype, and host tropism exists between these microorganisms (Brosch *et al.*, 2002). Although most of the members present in the MTBC have been reported to cause human diseases; however, Mtb has been known as

the most important causative agent for TB in humans over centuries (Palomiño *et al.*, 2007; Reddington *et al.*, 2011; Forrellad *et al.*, 2013).

In addition, human TB is also caused by *M. africanum* and *M. canetti*, which is prevalent in various African regions. *Mycobacterium bovis* is the cause of zoonotic TB in bovine animals, it can also infect humans after being transferred from the cattle (Forrellad *et al.*, 2013). Various other species, including *M. caprae, M. microti*, and *M. pinnipedii* also cause disease but predominantly in animals (Živanović *et al.*, 2014).

Members belonging to the genus Mycobacteria can exist in a variety of environments, including dust, soil, water, bio-aerosols as well as in extreme conditions like low pH, low nutrients, high oxygen, and extreme temperatures (De Groote and Huitt, 2006). Overall, there are about 150 species that are acid-fast bacilli, and most of them live as free-living species with some being parasitic species (Freidlin, 2013).

1.4 Physiology and Morphology of *Mycobacterium tuberculosis*

Morphologically, Mtb is a Gram-positive little curved or straight rod which are non-spore-forming bacteria, aerobic, non-motile, and acid-fast bacteria (Helguera-Repetto *et al.*, 2004; Knechel, 2009a). These bacteria range from 0.5 µm to 3 µm and possess complex cell walls, which help these bacteria to survive in various conditions (Knechel, 2009a). The cell wall is a rigid, thick, and waxy structure that is highly impermeable and these features help in the existence of bacteria in a variety of environments (He and De Buck, 2010).

Lungs of human beings, particularly, the upper lobes which are well aerated, and have high oxygen pressure help its growth by providing a conducive environment for Mtb to flourish (Palomiño *et al.*, 2007). The best laboratory conditions for the growth of this bacterium include a temperature of 37 ºC, neutral pH, and 5%-10% CO_2 (Palomiño *et al.*, 2007). The cell wall of this bacterium gives it a further advantage for its survival.

1.4.1 Cell-wall composition of *Mycobacterium tuberculosis*

Mycobacterial cell walls as compared to other prokaryotes, slightly differ in their composition chemically as they possess a unique structure, which is shown in Figure 3 (Hett and Rubin, 2008). The difference in chemical composition imparts the ability to this bacteria to resist the antibiotics and immune system (Beran *et al.*, 2006; Hett and Rubin, 2008). The unique composition of mycobacterial cell walls includes arabinogalactan (AG), lipoarabinomannan (LAM), proteins, extractable lipids, peptidoglycan (PG), and mycolic acids (MA) (Beran *et al.*, 2006). The plasma membrane is surrounded by the inner and outer cell wall layer in mycobacteria. Lipomannan, LA, phthiocerol-containing lipids, dimycolate trehalose, and phthiocerol dimycocerosate are the different components present in the outer cell wall of Mtb, as well as many different proteins and other lipids (Hett and Rubin, 2008). An important MA-AG-PG complex is formed by a covalent linkage between AG, PG, and MA, the inner layer is formed by this complex. Arabinogalactan is a polysaccharide present in the cell wall of mycobacteria (Hett and Rubin, 2008). The rigid structure of the cell wall and its diffusion features are attributed to the presence of PG, which is a polymeric compound composed of sugar and a polysaccharide (Knechel, 2009; Yao *et al.*, 2012). In the cell walls of Mtb and other mycobacteria, mycolic acid is present. These make up around half of the dry weight of this bacterium and are also a reservoir of carbon as well as energy (Palomiño *et al.*, 2007).

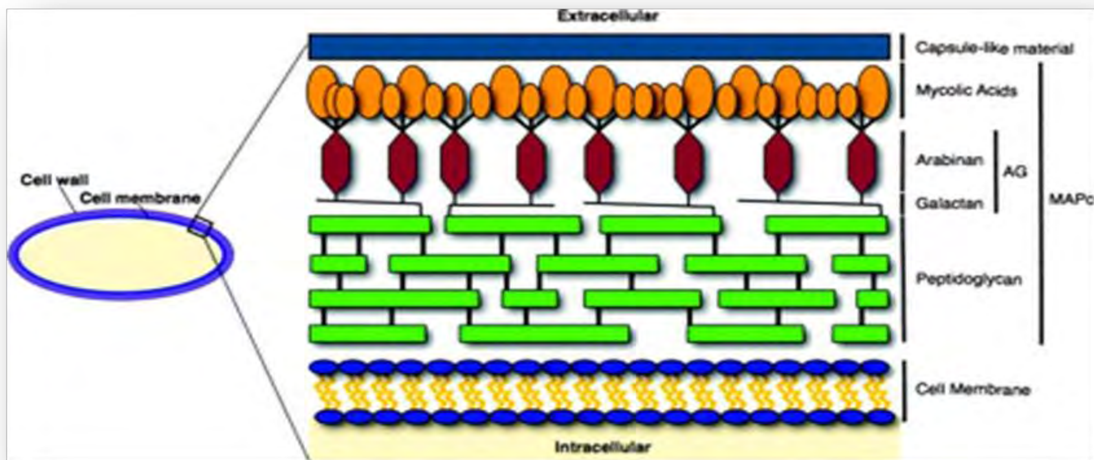


Figure 1.2: Mycobacterial cell wall basic components MAPc, MA-AG-PG complex (Hett and Rubin, 2008).

1.4.2 Genome organization of *Mycobacterium tuberculosis*

Mycobacterial genetics have been extensively explored in recent years and considerable knowledge has been accumulated through the development of molecular methods, especially due to the techniques available to sequence DNA (Smith, 2003). Mtb H37Rv was the first laboratory strain to be fully sequenced (Fleischmann *et al.*, 2002; Ioerger *et al.*, 2010). Its genome contains 4.4×10^6 bp and harbor around 4000 genes (Fleischmann *et al.*, 2002; Smith, 2003) with 65.5% of the sequence consisting of G+C content in which nucleotides are distributed uniformly throughout the whole genome (Palomiño *et al*., 2007). Fatty acid metabolism and an unrelated Pro-Pro-Glu (PPE), as well as Pro-Glu (PE) families of glycine rich and acidic-rich proteins, are metabolized by the enzymes coded by 200 of the genes (Smith, 2003). Survival and growth of mycobacteria are facilitated in extreme environments with the help of these proteins (Palomiño *et al*., 2007). Extensive studies of the genome have revealed a presence of IS elements having 56 copies that belong to IS256, IS110, IS30, IS 21, IS5, IS3, and ISL3 families, their pattern is depicted in Figure 1.3 (Cole, 2002). IS6110 elements are the insertional sequences that are present only in the MTBC members and distinguish them from other species of mycobacteria (Coros, DeConno, and Derbyshire, 2008).

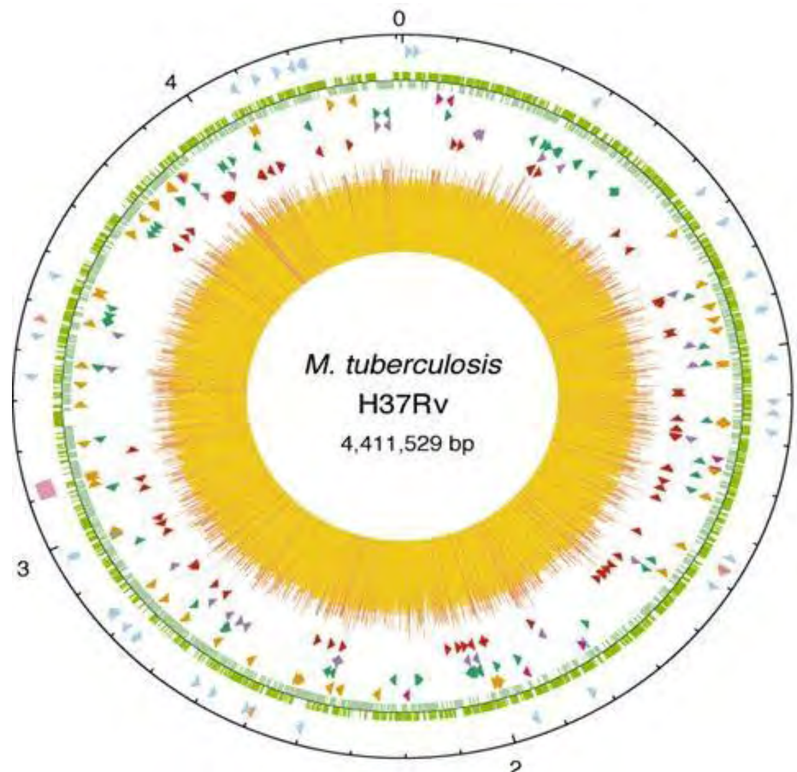


Figure 1.3: Circular chromosomal DNA of Mtb (H37Rv). The outer mot circle shows length in megabytes and 0 is representing replication origin. The exterior first ring represents stable RNA genes position (the blue one is tRNAs, while the other are pink and region with direct repeats are in pink cubes. Coding sequence strand wise is shown in the inward second ring, dark green (clockwise) and light green as anticlockwise. In the third ring orange shows insertion sequences dark pink are 13E12 RFP family and prophage in blue. The position of PPE family members in green is shown in the fourth ring. PE family members are shown in the fifth ring in purple excluding PGRS. Dark red show's location of PGRS in the sixth ring. In center histogram shows G +C content in yellow where G + C content is lower than 65% and in red where more than 65% (Cole *et al*., 1998).

1.5 Drug-resistant Tuberculosis

Drug-sensitive TB is a treatable disease through an anti-TB drug regimen, but it has led to the development of the drug-resistant Mtb strain over time. In many countries, a major public health concern is the emergence of resistant TB. Development of resistance against the first line of drugs and second line of anti-TB drugs, even RIF-Resistant TB (RR-TB) is now becoming common. In addition, the resistance from two efficient anti-TB drugs (RIF and INH) has led to multi-drug-resistant TB (MDR TB). In some cases, there is also a need for a modified treatment regimen when resistance to INH is present but not RIF. Various estimates of different forms of drug-resistant TB INH, RIF, and fluoroquinolones are available in the worldwide information revealed annually by WHO up to recent time (WHO, 2020). It has been observed that various individuals showing drug-resistant TB are infected with the drug-resistant pathogenic Mtb strains (Shah *et al.*, 2017). Various factors are associated with the development of MDR TB especially are the nonadherence to the prescribed antibiotics, but it may not be the exclusive cause of acquired resistance and may have a smaller part in the progress of drug resistance. Drug concentrations in insufficient amounts, gradients of the drug in pulmonary tissue, and the role of drug efflux pumps in bacterial membrane along with many genetic alterations in its genome can also be important contributing agents towards the development of drug resistance (Dheda *et al.*, 2018).

New analyses based on the historical information depict that the progression of infection towards the development of full-blown drug-resistant disease is dependent on many risk factors. Scientists have estimated that people infected with Mtb are at the greatest risk of developing the disease during the initial years of infection indicating time as an important determinant for TB infection. The data also shows that Mtb incubation is probably less than previously estimated. If preventive therapy is offered to such infected individuals during the early onset of disease, it can become an effective therapy for the treatment in such cases. Diagnosis is important in such cases and now biomarkers have been recently developed that can help in the early diagnosis in asymptomatic patients of TB, which can prevent the development or progression of disease in such individuals.

1.5.1 Estimates of the drug resistant TB disease burden

In 2019, global cases of MDR/RR TB were reported as 3.3% among the newly infected individuals, and 18% of the cases were reported among the patients who were previously treated for TB. The highest proportion of these cases was observed among the countries that previously belonged to the Soviet Union. About 20% of the newly infected cases, while 50% of the already treated cases were reported among people belonging to former Soviet Union countries. Geographically, 50% of cases of the global MDR/RR TB infections belong to three countries with the highest rate (27%) in India, followed by 14% in China and 8% Russian Federation. There were 182,000 deaths reported in the MDR/RR TB patients alone in 2019. Among them, INH resistant cases were reported to be 17.4% among the treated individuals and 13.1% among the newly infected individuals, it accounts for 1.4 million INH resistant cases. However, 1.1 million out of 1.4 million INH-resistant cases were sensitive to RIF. Therefore, it was concluded that co-resistance to INH and RIF is not present in DR TB cases as 11% of RIF susceptible patients were present. In those settings, where the diagnosis is centered on resistance of RIF resistance, there is a possibility of missing INH resistance, which in turn hinders the proper application of treatment for infected individuals (WHO, 2020). The rates of active TB disease development in exposed infants are very high, but much lower in children of age group 2 to 10 years old; however, the risk increases in these children in their adolescence and plateaus around 25 years of age. This risk remains high throughout adult life later on as well (Marais *et al.*, 2006). In men, the risk of developing TB is higher as compared to women as has been shown by the incidence rate of various regions (Glaziou *et al.*, 2018), while 10% of active TB cases at the global level occur only in children (Swaminathan and Rekha, 2010).

HIV is a major and most important risk factor among several other risk factors that are associated with TB development and progression (Havlir *et al.*, 2008). In HIV-positive individuals, 12% of all new dynamic TB cases and 25% of all deaths related to TB occur. The majority (75%) of active cases of TB and death in Africa are linked to HIV (Getahun *et al.*, 2015). A study showed that HIV-infected adults (18%) and children are the main cause of hospitalization due to active TB disease, which accounts for 10% (Ford *et al.*,

2015). In these hospitalized patients, mortality percentage as high as 25% is among adults and 30% among kids who are HIV infected (Ford *et al.*, 2015).

As HIV-positive people represent just 0.5% of the worldwide populace, the general population is at risk of TB infection due to many other factors too. It is estimated that 27% of tuberculosis cases worldwide are caused by undernutrition and 22% by air pollution especially indoor polluted air (Lönnroth *et al.*, 2010). Type 2 diabetes is another important risk factor for tuberculosis (Jeon and Murray, 2008), excessive use of alcohol triples the risk of acquiring TB along with type 2 diabetes (Rehm *et al.*, 2009), and smoking doubles that risk (Bates *et al.*, 2007; Lönnroth *et al.*, 2010).

Pakistan is among the top five high burden countries along with its bordering countries India and China. Pakistan has an estimated population size of 220 million where estimates of TB burden in 2019 is 570,000, Table 1.1a-e shows data on TB, MDR TB, and MDR RR TB cases regarding treatment and diagnosis from Pakistan. Figure 1.4 shows that it is prevalent in females and the age group of 15-24 years in Pakistan.

Table 1.1a: Estimate of total TB, MDR TB, and MDR RR TB cases in Pakistan (WHO, 2020)

	Number	**(Rate per 100 000 population)**
Total TB incidence	570 000 (404 000-764 000)	263 (187-353)
TB incidence in HIV positive	5 100 (3 400-7 200)	2.4 (1.6-3.3)
MDR/RR TB incidence	25 000 (16 000-36 000)	12 (7.3-17)
HIV-negative TB mortality	42 000 (34 000-51 000)	19 (16-24)
HIV-positive TB mortality	1 900 (1 300-2 800)	0.9 (0.58-1.3)

* WHO Estimates for RR and MDR TB

Table 1.1b: Estimated proportion of TB cases with MDR/RR TB in 2019 (WHO, 2020).

New cases	4.2% (3.2-5.3)
Previously treated cases	7.3% (6.8-7.8)

Table 1.1c: Universal health coverage and social protection in 2019 (WHO, 2020).

Percentage of people treated with TB	58% (43-81)
TB case fatality ratio	8% (5-11)

Table 1.1d: TB case notifications in 2019 (WHO, 2020).

New and relapse cases in Pakistan	328 312
Rapid test diagnosis	42%
HIV history	40%
Pulmonary	81%
Confirmed bacteriologically	50%
0-14 years (children)	14%
Women	41%
Men	45%
Notified cases	334 754

^ *Only pulmonary TB*

Table 1.1e: Drug-resistant TB cases confirmation from Pakistan with various diagnostic assay in 2019 (WHO, 2020).

Number of bacteriologically confirmed New TB cases tested for RIF resistance -	59%
Number of Previously treated TB patients which were bacteriologically confirmed tested for RIF resistance	89%
Number of Laboratory-confirmed MDR/RR TB cases	3 820
Number of MDR/RR TB treatment started for	3 004
Number of Laboratory-confirmed XDR TB cases	60
Number of Patients started XDR Tb treatment	34

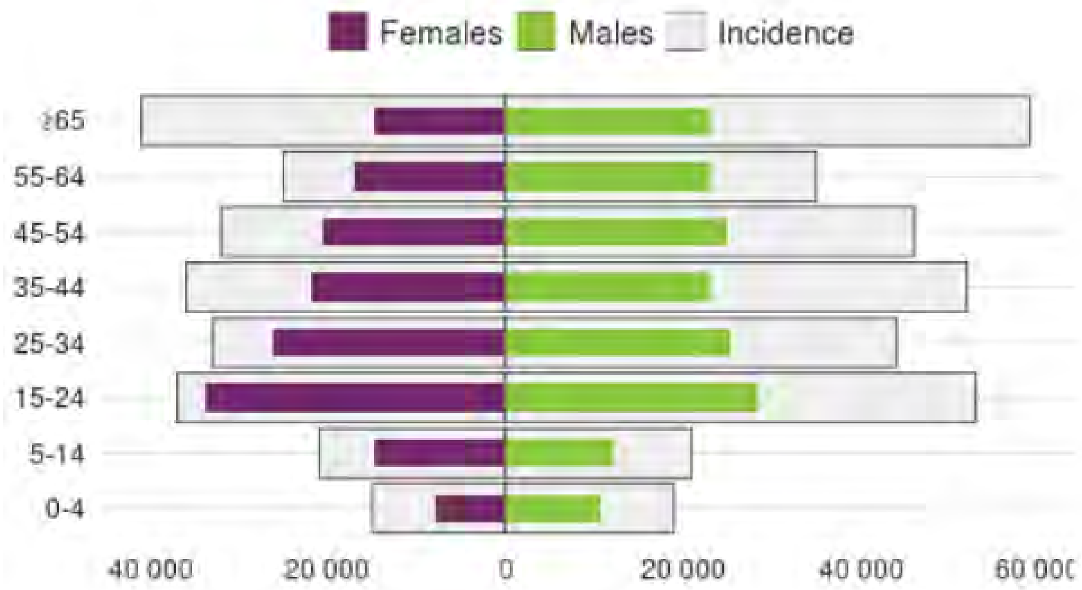


Figure 1.4: Incidence rate in notified cases based on age group and gender in Pakistan (2019)

1.6 Anti-Tuberculosis Drugs Used in First Line and Second Line Regimen

1.6.1 Rifampicin

Rifampicin (RIF) is from the class of rifamycin that was first time used in 1972 as an anti-TB agent. It is among the most important anti-TB antibiotics along with INH, it forms the basis of the multidrug treatment system for tuberculosis. RIF works against replicating as well as non-replicating bacilli, the non-growing bacilli are slow metabolizing bacteria (Mitchison, 1979). RIF is used to inhibit the Mtb messenger RNA extension by binding to the β-subunit of RNA polymerase (Blanchard, 1996). The majority of Mtb RIF-resistant isolates harbor mutations in the β-subunit of the *rpoB* gene which codes for the RNA polymerase. Conformational changes because of these mutations can reduce drug affinity, hence leading to resistance development (Telenti *et al.*, 1993). In the *rpoB* gene, there are hot-spot regions, mutations in these can result in codon alteration in position 507–533 (426-452 Mtb Numbering), 96% of RIF resistant Mtb isolates have a mutation in this hot-spot region of the *rpoB* gene. This region is also called the RIF resistance determining region or RRDR (Ramaswamy and Musser, 1998). In codon 435, 445, and 450, mutations are most commonly reported in many studies and are found to be associated with RIF resistance (Somoskovi, Parsons and Salfinger, 2001; Caws *et al.*, 2006). Mutations also occur outside the hotspot region of the *rpoB* gene but are less common (Heep *et al.*, 2000; Siu *et al.*, 2011). Other RIF resistance may be due to cross-resistance. Certain mutations have been associated with low-level resistance to RIF which were seen in the other codons like 518 or 529, despite these mutations in strains, such strains are still susceptible to certain RIF like rifalazil or rifabutin (Yang *et al.*, 1998; Cavusoglu, Karaca-Derici and Bilgic, 2004). Mono-resistance to RIF is rarely reported, on the other hand, mostly strains resistant to it are generally resistant to other drugs as well.

RIF mono resistance is less common as most cases detected as RIF resistance also has to INH. Hence, RIF resistance is also considered as a proxy marker for MDR-TB (Traore *et al.*, 2000). Genomic studies disclosed that compensatory mutations in *rpoA* and *rpoC* are acquired in rifampin-resistant strains along with *rpoB* mutations. These compensatory

mutations are usually associated with the fitness of strains and can result in increased transmissibility (Brandis and Hughes, 2013; De Vos *et al.*, 2013).

1.6.2 Isoniazid

The INH is the basic component of TB treatment along RIF, which was first introduced in 1952 for treatment of TB, However, it works only against metabolically active bacilli which are also replicating. INH is a prodrug known as an isonicotinic acid hydrazide, which needs activation by the *katG* enzyme for its activity coded by the *katG* gene (Timmins *et al.*, 2004). INH works through *inhA*-encoded NADH-dependent enoyl-acyl carrier protein, (ACP)-reductase and inhibits mycolic acid synthesis, an essential cell wall component of tubercle bacilli (Rawat *et al*., 2003).

Although INH structure is simple, mutations in several genes which include *katG*, *inhA, ksa*, *ahpC,* and *NDH* are linked with INH resistance development. Studies at the molecular level have revealed that mutations in *inhA* and *katG* promoter regions are associated most commonly, which confer resistance to INH in Mtb strains (Ramaswamy *et al.*, 2003; Hazbón *et al.*, 2006).

1.6.3 Ethambutol

Ethambutol (EMB) was initially introduced in 1966 for TB treatment and is now part of the first-line treatment regimen. EMB is a bacteriostatic agent that prevents the biosynthesis of arabinogalactan in the cell wall of actively multiplying bacilli (Takayama and Kilburn, 1989). The three genes *embA, B, and C* code for arabinosyl transferase, which exists in *embCAB* operons in the genome of *M. tuberculosis*, are involved in the synthesis of arabinogalactan, which in turn accumulates Darabinofuranosyl-P-decaprenol intermediate (Mikusova *et al.*, 1995). Mutations in these genes particularly in *emb B* codon 306 and upstream region of *embA* are the prevalent cause of EMB resistance in Mtb cases (Zhang *et al.*, 2013; Brossier *et al.*, 2015).

1.6.4 Pyrazinamide

At the beginning of the 1950s, pyrazinamide (PZA) was introduced for the treatment of TB, and it is still an important part of the standard first-line drug regimen against this disease. Chemically, it is a nicotinamide analogue and after the introduction of this drug the duration of treatment against TB was reduced to six months. Semi dormant tubercle bacilli, which prefer living in the acidic environment are inhibited by this drug. Such an environment is dominant in lesions formed in TB (Mitchison, 1985). Pyrazinamidase/nicotinamidase is the enzyme encoded by the *pncA* gene, which is required for the conversion of pro-drug PZA into pyrazinoic acid, which is an active form of this drug. Majorly mutations in the *pncA* gene are involved in PZA resistance (Konno *et al*., 1967; Scorpio and Zhang, 1996; Sengstake *et al.*, 2017).

1.6.5 Streptomycin

The first antibiotic used successfully against TB was originally isolated from *Streptomyces griseus* a soil microorganism. However, soon resistance was seen against it as it was prescribed for the treatment as monotherapy (Crofton and Mitchison, 1948). It is an aminocyclitol glycoside, which inhibits protein synthesis in actively growing bacilli by blocking the initiation of translation in these bacteria (Moazed and Noller, 1987). It acts on the 30S subunit of the ribosome at the S12 ribosomal protein coded by the *rpsL* gene and the 16rRNA coded by *rrs* (Finken *et al.*, 1993). *rpsL*, *rrs*, and *gidB* genes which encode methyltransferase are believed to cause resistance to streptomycin in clinical Mtb isolates (Wang *et al.*, 2019).

1.6.6 Fluoroquinolones

For MDR TB treatment fluoroquinolones (FQs) are among the most important second-line drugs. Nalidixic acid was discovered as a byproduct of chloroquine, which is an important anti-malarial drug. It has been used as a parent compound for the formation of ofloxacin and ciprofloxacin (Goss, Deitz, and Cook, 1965). In clinical trials, new generation quinolones such as gatifloxacin and moxifloxacin are investigated for use as first-line antibiotics in order to shorten the treatment time in TB (Grace *et al.*, 2019). The two most important enzymes required for the viability of bacteria, that is, topoisomerase

IV and topoisomerase II also called DNA gyrase are inhibited by the FQs. Topoisomerase II is encoded by the genes *gyrA* and *gyrB*, while topoisomerase IV is encoded by *parC* and *parE* (Fàbrega *et al.*, 2009) and mutations have been reported in these genes which are involved in their resistance in Mtb strains.

1.6.7 Kanamycin, Capreomycin, Amikacin and Viomycin

Amikacin and kanamycin are aminoglycosides, while viomycin and capreomycin are cyclic peptides, but all of these inhibit protein synthesis, therefore, are used to treat MDR TB as a second-line drug regimen. Among these, kanamycin and amikacin alter 16S rRNA leading to inhibition of protein synthesis. In the *rrs*, mutations occur at positions 1400 and 1401 besides 1483 positions, which are the most common type of mutations found in the drug-resistant strains. Those harboring these mutations are resistant to amikacin and kanamycin (Alangaden *et al.*, 1998; Suzuki *et al.*, 1998). Previously, it was presumed that a complete cross-resistance occurs between amikacin and kanamycin; however, later it was reported that a variable pattern of resistance occurs having different mechanisms responsible for resistance (Krüüner *et al.*, 2003). An enzyme, aminoglycoside acetyltransferase encoded by *eis* gene has a mutation in its promoter region, which is responsible for low-level resistance against kanamycin (Zaunbrecher *et al.*, 2009). Experimentally, it was proven that the mutations present at position -10 and -35 upstream promoter region of the *eis* gene cause low levels of kanamycin resistance but not resistance against amikacin. In 80% of the clinically isolated strains, such mutations conferring a low level of resistance against kanamycin are present (Zaunbrecher *et al.*, 2009; Campbell *et al.*, 2011).

1.7 Current Treatment Strategies Used Treatment of drug-sensitive resistant TB

Currently, RIF, INH, EMB, and PZA is recommended during the first two months as anti TB cure for TB that is drug-sensitive in 6-month therapy followed by INH and RIF for 4 months (Table 1.2) (Organization and Organization), 2010; Nahid *et al.*, 2016). Although the success rate with standard six months regimen is high (approximately 86%), it also has some limitations among which long treatment duration is the most important (Saukkonen *et al.*, 2006).

The most common adverse effects include a rise in the level of liver enzymes, neurological disorders, skin rash, gastrointestinal intolerance, arthralgia. Serious adverse conditions are immune thrombocytopenia, severe hepatitis, agranulocytosis, renal failure, and ototoxicity. Due to prolonged treatment patient adherence to treatment is also challenging, which needs measures that help treatment adherence continuously. The most common adherence is DOTS (directly observed therapy) in which a health professional observes every dose of treatment directly (Volmink and Garner, 2007). However, therapies in active drug-resistant TB cases have limitation length, uncertain efficacy, and more toxicity (Table 1.3). Due to poor treatment compliance outcomes in endemic countries are poor and have a success rate of around 50%.

Table 1.2: Drug sensitive TB treatment regimen (Pai *et al.*, 2016)

Intensive Phase		**Continuation Phase**			
Drugs*	Interval and Dose (daily)	Drugs	Interval and Dose (18 weeks)	Total Doses	Important Practice Points
INH, RIF, PZA, EMB	8 weeks	INH RIF	Daily for 18 weeks	182 or 130	Newly diagnosed for pulmonary TB preferably
INH, RIF, PZA, EMB	8 weeks or 5 days per week	INH RIF	3 days per week	110 or 94	DOT Compliance is difficult to prefer alternative regimes
INH, RIF, PZA, EMB	3 days per week for 8 weeks	INH RIF	3 days per week	78	Noncompliance in cavitary disease and HIV patients can lead to drug resistance (caution needed)
INH, RIF, PZA, EMB	2 weeks, then 2 days per week for 6 weeks	INH RIF	2 days per	62	In HIV positive and cavitary disease two doses are not recommended

Isoniazid = INH, Rifampicin = RIF, Ethambutol = EMB, pyrazinamide = PZA

Table 1.3: Drug Resistant TB treatment regimen (Pai *et al.*, 2016)

Class	Mechanism	*Drugs	Key Adverse Events	Important practice points of Action
Group A: fluoroquinolones (FQs)				
FQs	Inhibition of gyrase	LEV, MOX, GT	QTc prolongation	QTc Monitoring is required
Group B: injectable				
Aminoglycosides	Inhibition	KM AM CP SM	Nephrotoxicity (all), Electrolyte derangement (all) Ototoxicity (all)	Aminoglycosides and nephrotoxic agents' combination should be avoided Caution needed in patients with diabetes myelitis and kidney disease
Group C: core second-line agents				
Thioamides	Inhibition Synthesis	ETM ROT	Psychosis, depression, confusion (prolongation)(clofazimine) Skin (clofazimine). Peripheral neuropathy Myelosuppression (linezolid)	Thyroid-stimulating hormones should be monitored in patients on ethionamide
Oxazolidinones	Inhibition	Cycloserine Terizidone Linezolid Clofazimine	Confusion, psychosis, and depression (terizidone and cycloserine). Peripheral neuropathy	If myelosuppression occurs, stop linezolid use. Avoid concomitant use of linezolid combination with protein stavudine or

			(linezolid) Myelosuppression (linezolid) toxicity (linezolid) prolongation (clofazimine) Skin and conjunctival pigmentation (clofazimine).	didanosine Monitor QTc while taking clofazimine
Category D: add-on agents				
D1, various classes: isonicotinic acid hydrazide	Inhibition of Acid Synthesis	High-dose INH	Peripheral neuropathy CNS toxicity Hepatotoxicity	Use with pyridoxine for peripheral mycolic neuropathy prevention

*Levofloxacin (LEV), Moxifloxacin (MOX), Gatifloxacin (GT), Kanamycin (KM), Amikacin (AM), Capreomycin (CP), Streptomycin (SM), Ethionamide (ETM), Rothionamide (ROT)

1.8 Assays for detection of *M. tuberculosis*

1.8.1 Microscopy based Assays for detection of *M. tuberculosis*

Proper detection of the causative agent is essential for controlling the infection. The etiological diagnosis of TB is important for both the patient and their surroundings, as well as to reduce the regional burden of the disease. Diagnosis of infection by various conventional and modern techniques is crucial, which is performed in the diagnosis laboratories, which detect mycobacteria. Molecular techniques are proven to be rapid, hence are needed for the detection of acid-fast bacilli (AFB) in samples. The burden of this disease is alarmingly increasing globally due to slow detection. Despite many technological advancements in molecular detection, many diagnostic laboratories still use the traditional method of microscopy and cultivation for its usefulness.

Among many techniques used in the laboratory for diagnosing TB, microscopic examination after staining the sputum specimen using a specialized staining technique called Ziel Neilson (ZN) is the oldest method, which is the easiest and least expensive method for diagnosing TB. During this examination, staining differentiates acid-fast and non-AFB, when viewed under the microscope. This is an important test for early detection of positive cases as every infected person is expected to have 10^8 to 10^{10} bacilli in the sputum sample (Baddeley, A; Dean, A; Monica-Dias, H; Falzon, D; Floyd, K; Garcia, 2013).

Microscopy is still the sole diagnostic method available for the diagnosis of this disease, especially in poor countries of Asia and Africa, where the disease burden is high. Smear microscopy for the detection of AFB can be done in any laboratory setting. As Mtb has unique cell walls composition, therefore, the specific type of stain, that is, Ziehl Neelsen (ZN) stain is red where AFB is seen as bright red that can be easily seen against a blue background. For patients having a high burden of AFB and who have developed lesions in the lungs, TB is detected easily; however, in patients having low levels of Mtb a false negative detection can occur. Therefore, this test has a low sensitivity, and its detection limit ranges from 5000-10,000/mL.

As compared to microscopy, culturing techniques are more sensitive (Hepple, Ford and McNerney, 2012; Mustafa, Devi and Pai, 2017). Fluorescence microscopy is another procedure for the detection of AFB and is a more sensitive technique as compared to simple microscopy (Getahun *et al.*, 2007). Smear microscopy can be used as a tool to detect the response of patients to anti-TB drugs also but cannot differentiate between DS and DR Mtb strains (Getahun *et al.*, 2007).

In the AFB diagnostics, cultivation of bacteria through culture is considered as the gold standard and 50% more sensitive technique as compared to smear microscopy as it is also able to detect in those cases which are rendered negative in the smear microscopy (Salfinger and Kafader, 1987). One of the egg-based media used facilitates the culturing of Mtb; however, the process of growth is quite slow. The colonies of Mtb appear on this media only after 4-8 weeks of culturing. This technique is expensive as compared to smear microscopy because it requires a specific laboratory facility (at least Biosafety Level-II) having trained personnel. Laboratories working as BSL-I labs are equipped with routine equipment, laminar flow, and biosafety cabinets, which are not suitable for the culturing of highly contagious *M. tuberculosis*. If cultured in such facilities, this bacterium cannot be contained, and it may spread through the aerosols. In poor countries, regional and reference laboratories equipped with essential culturing requirements and safe laboratory settings are present at the national and regional levels including national reference laboratories. Therefore, samples for culturing are sent to these laboratories for culturing. This process is very important in those areas that have a high level of drug resistance incidents (Chihota *et al.*, 2010).

Middlebrook (agar-based) is another medium along with LJ medium that can be used for culturing Mtb. After inoculation, this bacterium takes at least 4-8 weeks for growth (Naveen and Peerapur, 2012). It has been reported that bacteria grow faster in liquid media as compared to solid media, hence liquid media provides a faster detection method for bacteria (Chihota *et al.*, 2010).

Noncommercial WHO endorsed assays include microscopic observation of drug susceptibility testing (MODS), colorimetric redox indicator assay (CRI), and nitrate

reductase assay (NRA) as an interim placement where the capacity for automated phenotypic and genotypic DST was developed mostly includes reference laboratories performing under strict control. As these assays were not very helpful in the diagnosis of XDR TB, therefore were unsuccessful to compete with conventional culture and DST methods (Organization, 2011).

1.8.2 Phenotypic Drug Susceptibility Testing (DST)

Drug sensitivity testing and minimum inhibitory concentration (MIC) are the two important techniques that are used for the detection of drug resistance phenotypically. The visible phenotypic nature of resistance gives bacterial cells the ability to exist at certain drug concentrations which normally do not permit bacteria to grow. Many changes occur in the genotypes of bacteria, which can then be transferred to next generations, and which can interfere with detection assays (Wiuff *et al.*, 2005). Most of the antibiotics are only effective against active bacteria and not against dormant bacterial cells due to the altered physiology and metabolism. Bacteria are also physically resistant to several antibiotics in the stationary phase of their growth cycle (Mitchison and Selkon, 1956; Ayrapetyan *et al.*, 2015). There are some bacteria, which are able to survive the exposure of antibiotics, as well as these, can show resistance against them, these bacteria are called Persister (Wakamoto *et al.*, 2013). In all these conditions, most available assays are unable to detect drug-sensitive (DS) and Drug-resistant (DR) TB cases. The term minimum inhibitory concentration refers to the amount of drug which can inhibit bacterial growth but cannot kill all the bacteria present in the sample, and this term was coined because of the persisters cells (Bigger, 1944; Cushnie, Cushnie and Lamb, 2014), In tuberculosis, the presence of persisters and dormant cells results in a prolonged treatment for complete eradication of the pathogen and currently there are no effective drugs against such type of bacteria (Zhao *et al.*, 2012).

In the situation, where there is widespread drug-resistant TB throughout the globe, there is still the importance of DST for the diagnosis of MDR TB for the elucidation of drug susceptibility patterns. There are many areas where drug-resistant TB is prevalent and the current treatment of TB recommended by WHO is ineffective, in such areas the

information of diagnosis as well as drug susceptibility information are equally important (Skrahina *et al.*, 2013; Organization, 2014). An effective drug regimen can be designed based on early diagnosis of drugs resistance will be available, which will affect treatment outcomes along with a reduction in transmission of resistant TB in the surrounding community (Hashmi, Javed and Jamil, 2017). A drug-resistant Mtb strain can be defined as bacteria, which significantly differs from a wild type of bacterium in its drug susceptibility towards any specific drug for which wild type bacteria has not been exposed previously (Canetti *et al.*, 1969). DST determines if a particular bacterium under testing is resistant or susceptible to the tested drug. Traditional petri plate culturing is used for this purpose. For this purpose, one concentration of drug called the critical concentration is typically tested and it distinguishes susceptible from resistant strains. Normally critical concentration should be between the lowest MIC of the resistant strains and highest MIC of the sensitive strains. In phenotypic DST, bacteria are cultured on solid or in liquid media under certain standardized conditions, drugs are added after which inhibition of growth is monitored. As for Mtb a slow growing bacterium, DST is time taking process but still desirable as it shows the presence of resistance regardless of the mechanism that may be responsible for it. It has two main advantages. Firstly, resistance can be detected in the presence of a mutation in any particular resistant gene, and secondly, information about the molecular mechanism of resistance is also not required (Canetti *et al.*, 1969; Yakrus, Metchock and Starks, 2015).

1.8.3 Molecular Diagnostic Assay for Detection of Mtb and MDR TB

For diagnosis of Mtb, several DNA-based techniques are available. These techniques include line probe assay (LPA), amplified mycobacteria direct test (AMTD and gene probe). Although these techniques have been developed these cannot (Meyer *et al.*, 2017) replace the conventional culture or smear microscopy in respect of sensitivity of detection. In 2008, the use of LPA for smear-positive Mtb samples was recommended by WHO. MTBDRplus (Ling, Zwerling and Pai, 2008) and Lipa are the two commercially available assays (based on reverse hybridization technique) present in the market currently. But the diagnosis of TB from sputum samples has been revolutionized by a molecular-based method called the GeneXpert system. This system uses three types of

probes for the detection of mutation in the *rpoB* gene hotspot. These include fluorescence resonance energy transfer (FRET) probes, molecular beacon probes, and Taq Man probes. The detection of mutation by GeneXpert implies that there is the resistance of Mtb to RIF, many studies have reported that it has efficient performance for detection of mutations (Evans, 2011; Meyer *et al.*, 2017; Ejeh *et al.*, 2018).

Molecular-based methods have been developed because conventional DST or culture techniques are not only time consuming but are also quite labor intensive. Molecular techniques detect drug-resistant Mtb rapidly as it is based on the detection of specific genotypes that influence the drug-resistant phenotype (Denkinger *et al.*, 2015).

Molecular methods for detecting drug-resistant chromosomal mutations in Mtb offer several benefits in comparison with non-molecular methods. However, the specificity of these methods can become doubtful, if the samples are heavily colonized or chronic disease is present in the individual due to NTM occurring in the culture-based assay. Nevertheless, these methods have a quick turnaround time and results can be obtained within a few hours or on the day of the test which is a much shorter time as compared to culture-based techniques (Wlodarska *et al.*, 2015). It has been reported that the reproducibility of these methods is also better when it comes to the detection of low or moderate levels of resistance (Plinke *et al.*, 2009).

Molecular tests are also helpful as there is no need for culturing and detection can be done for strains having poor growth. In parallel, molecular methods provide a specific way to identify species too. As molecular testing has many advantages but cannot substitute microscopy and culture completely as they guide regarding the degree of infectiveness that allows practitioners to make decisions on community health care, such as the requirement to isolate a patient where resources are available.

DNA is an extremely stable molecule that is readily detectable from patients, who have received chemotherapy for TB in clinical specimens. Therefore, molecular tests are not recommended for monitoring treatment outcomes (Cole *et al.*, 2005). Culturing is necessary for drugs susceptibility testing especially. Molecular methods are necessary to

trace hetero-resistance which is usually a small population that is resistant to the anti-TB regimen when compared to phenotypically DST methods which are often less sensitive for this detection (Rinder, Mieskes and Löscher, 2001; Folkvardsen *et al.*, 2013).

Over the past few decades, the use of PCR and Sanger sequencing has revolutionized the field of molecular biology (Sanger *et al.*, 1977). This method can be used for the detection of MDR-TB in terms of changes present on the DNA of the strain under consideration. In the current scenario, an ideal diagnostic test for the detection should be highly sensitive and specific, capable of detecting drug resistance, capable of using a variety of samples for detection and inexpensive, in addition, it should also require simple laboratory settings for wide application (Mokaddas *et al.*, 2015).

Furthermore, comparative genomic analysis has shown that the genome of Mtb is quite stable, therefore, genetic polymorphism inside the genome of this bacterium can differentiate different clinical strains from each other besides drawing a phylogenetic relationship between the isolated clinical strains, hence can prove efficient for detection purposes (Ramaswamy and Musser, 1998b; Maus *et al*., 2005). Such analysis is majorly based on studying single nucleotide polymorphisms (SNPs) in strains. There are two types of SNPs found in this bacterium, nonsynonymous and synonymous single nucleotide polymorphism. Nonsynonymous single nucleotide polymorphism (nsSNP) involves a change in the amino acid sequence which results in changes in drug target proteins and as a consequence, phenotypic resistance against the dug occurs; however, in synonymous SNP (sSNP), no change in the sequence of amino acids occurs. This difference provides valuable evidence for the differentiation of strains of *M. tuberculosis* (Sreevatsan *et al.*, 1997; Wade and Zhang, 2004). Therefore, it can be concluded that sSNPs are important in understanding the phylogenetic relationship between different Mtb strains while nsSNPs are important in understanding the molecular basis of drug resistance. These can be used as a genetic marker for the epidemiology of Mtb (Mathema *et al.*, 2006). If this genetic information is targeted, an accurate and rapid detection method can be developed based on these markers.

The PCR amplification of various candidate genes is now available to detect the presence or absence of a certain mutation, as an indication of the susceptibility or resistance of isolate to a particular medication. Also, different methods are available for the detection of drug-resistant TB, such as enzyme-restriction analytics for digestion (Castiblanco *et al.*, 2008).

1.8.4 Polymerase chain reaction-based techniques

For the detection of MDR TB, rapid detection is available by using techniques based on real-time PCR (Espasa *et al.*, 2005; Javed *et al.*, 2017). It allows amplification as well as detection of mutation in specimen simultaneously (Higuchi *et al.*, 1992). This technique has the advantage of low contamination as well as rapid detection when compared with conventional PCR. Many such molecular-based assays are available which are listed in Table 1.4.

1.8.5 Loop-mediated isothermal amplification (TB-LAMP)

Loopamp MTBC kit was manufactured by Eiken chemical company Ltd. (Tokyo, Japan) for MTBC detection. The assay is performed manually, and results are obtained in almost one hour using loop-mediated isothermal amplification. Results reading is taken using ultraviolet (UV) light with the naked eye. The method comprises of three steps which include; preparation of sample in 10-20 min, the second step is amplification for 40 min and the third step is reading under UV light in 1-2 min (Boehme *et al.*, 2007).

1.8.6 GeneXpert MTB/RIF and GeneXpert Ultra

An automated real-time PCR instrument, commercially available and widely used through the cartridge-based amplification of DNA is called GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA). It has been designed for the detection of RIF's resistance and MTBC. The identification of species and mutation is done through hybridization analysis of the molecular markers used for the PCR product. These target regions in the genome of Mtb is the RRDR region of the *rpoB* (Helb *et al.*, 2010). Although this assay cannot detect the mutation directly, it can detect the loss of genotype present in the wild type strain.

The GeneXpert MTB / RIF due to the detection of loss of genotype within a short time has led to its rapid rolled out in many high-burden TB countries in Africa and Asia, since its endorsement by WHO in December 2010 (WHO, 2013). The GeneXpert MTB / RIF roll-out is not without challenges despite its advantages. The limitation to its use is costly and does not eliminate the requirement of phenotypic DST. The cost-effectiveness of GeneXpert MTB / RIF was estimated (Vassall *et al.*, 2011) in a meta-analysis. Where it was found that pooled sensitivity was 88% while specificity was 95% for detection of TB in the initial screening. In TB patients with HIV, co-infection pooled sensitivity was 80%. In the case of RIF, resistance detection pooled sensitivity was 94% while specificity was 98%. Overall, the study concluded that assay is sensitive as well as specific for initial diagnosis of TB and RR TB (Steingart *et al.*, 2013).

In 2017 on world TB day which is the 24th of March, Cepheid launched a new version of GeneXpert and named it GeneXpert Ultra (Cepheid, 2017). The sensitivity of the assay was recorded higher especially for the diagnosis of smear-negative and HIV positive TB patients, however, with lower specificity compared to GeneXpert MTB/RIF assay. A multicenter study was conducted in 2016 in low and middle-income countries at 10 sites to evaluate the performance of ultra. WHO technical group of experts concluded that performance of new assay is better than old assay among TB detection in children, HIV positive patients as well as in case of diagnosis of extra-pulmonary TB and suggested more research should be done to improve the specificity of the assay (Organization, 2017).

1.8.7 Line Probe Assays for TB and Drug Resistant TB Diagnosis

The occurrence of resistance to the primary anti-TB drugs stimulates the need for fast methods to identify Mtb strains which have successfully developed resistance to the disease and stop the spread of resistant strains (Organization, 2008). The control and prevention programs of TB have been truly threatened by the emergence of MDR Mtb. In the *rpoB* gene, the line probe test detects core mutations, which have RIF resistance in the sharp 81bp core region (Borisov *et al.*, 2017; Rigouts *et al.*, 2013). It is estimated that

almost 95% of rifampicin resistance is due to changes in this region, which allow for rapid disclosure of resistance to this drug (Telenti *et al.*, 1993; Ramaswamy *et al.*, 2003).

In contrast, the molecular origin of INH resistance is much difficult to detect because it is due to multiple gene mutations, including the genes *inhA*, *katG*, *kasA,* oxyR, and ahpC (Ramaswamy and Musser, 1998; Somoskovi, Parsons and Salfinger, 2001). Innovative molecular approaches are now available based on PCR for quick recognition and identification of genetic changes and SNPs that cause INH resistance (Meaza *et al.*, 2017). However, in everyday clinical exercise, it is very hard as this assay is not fully standardized and inadequate to detect all SNPs.

Now different kits are available that are easy to use for commercial purposes. Another DR TB detection assay is a multiplex PCR technique that is combined with the technique of reverse hybridization. MTBDRplus identifies RIF and INH mutations which are identified by amplification of *rpoB*, *inhA,* and *katG* gene and then nonhybridization of probes with wild type sequence or by binding of probes with altered sequence (Meaza *et al.*, 2017). The MTBDR test is a quick molecular test that detects second-line anti-TB mutations in one day. It detects FQs mutations and second-line injection medications which involve different genes as *gyrA*, *gyrB*, *rrs,* and *eis* (Jagielski *et al.*, 2016). The assay consists of basically three steps including isolation of DNA and the reverse hybridization process involving multiplex PCR. These tests not only use the cultivated strains from the samples but also can directly use samples that are decontaminated and do not affect the quality of results (Lacoma *et al.*, 2008).

The resistance level due to less common mutations varies; therefore, proficiency testing is performed in the supranational TB reference laboratory. It has been observed that in several countries, and even in a single country, various assays are applied for identification and detection of TB and MDR TB cases depending upon resources available and cost. Now, it is well recognized that there are discrepancies in the detection of MDR TB strains by different methods. Strain despite being resistant to the first line drug regimen is detected as drug sensitive, such TB strains are termed occult strains. As strains carrying the *rpoB* gene mutation give discordant results when those are tested with

the critical concentration on liquid medium based Mycobacteria growth indicator tube system (MGIT). These strains are RIF resistant genetically but when MGIT is used these are classified as susceptible (Van Deun *et al.*, 2013). This is especially true for codons 430Pro, 435Tyr, 452Pro, 491Phe, and 445Asn mutations (Rigouts *et al*., 2013; Kang *et al.*, 2019). Mtb strains that are INH resistant and susceptible to RIF using DST methods phenotypically and resistant to RIF genotypically are referred to as phenotypically occult MDR strains. Inadequate information about the significance and prevalence of such phenotypic genotypic discordance has a role in clinical failures which needs to be addressed (Van Deun *et al.*, 2013). Over time, occult strains have been also named discordant or disputed or van Deun strain in literature.

The presence of such mutations and limitation in the detection of occult strains probably explains that there is a discrepancy between phenotypes and genotypes of these strains (Al-Mutairi *et al.*, 2019). The prevalence of such discordant results based on phenotypic detection method could be an underestimate of the real picture of MDR TB as molecular tests plus MIC testing is not routinely performed simultaneously (Van Deun *et al.*, 2013). Due to this disparity in detection assay, the real extent of the spread of such MDR TB strains is underestimated in different countries.

Besides public health concerns, there are major challenges for clinicians in the rise and transmission of Mtb with discordant results because it creates misinterpretation of DST results. In addition, a gap in the knowledge about the prevalence of these strains and the degree of RIF resistance prevalence is leading to adverse clinical outcomes due to inappropriate management of patients even such patients are hard to treat. Another problem is that the diagnostic accuracy of phenotypic and genotypic assays varies as the frequency of mutations in different geographic regions is varied.

DST on MGIT and solid LJ Media is routinely performed in our country on WHO defined critical concentrations of 1 µg/mL and 40 µg/mL in defined conditions. However, a critical concentration of 1 µg/mL is shown in some studies in strains harboring discordant mutations are detected as RIF susceptible, as this critical concentration is above the epidemiological cut-off values. In addition, phenotypic MGIT DST and

MTBDRplus when are performed on cases diagnosed as RIF resistant by Xpert MTB/RIF assay show varied susceptible patterns of drugs. In Pakistan, discordant results have been encountered, causing clinical and diagnostic dilemmas but its true prevalence and underlying genetic determinants are unknown.

Up to now studies done point towards the issue that none of the methods endorsed by WHO can completely identify the drug resistance to both first-line and second-line drugs used for the TB treatment. Genotypic assays like GeneXpert and LPA produce fast and reliable results but cannot still detect resistance completely as mutations outside the targeted region for amplification are missed. In addition, drug resistance due to efflux pumps, cell wall permeability, etc. are altogether not detected by Genotypic assays. Also, low-level RIF resistance linked to specific *rpoB* mutations is easily missed by liquid and solid culture-based methods. Due to these facts, there is discordance between phenotypic and genotypic DST, which can lead to uncertainty in the appropriate treatment strategy and accounts for high relapse TB cases, hence treatment of TB cases is hard. In Pakistan, both genotypic and phenotypic methods are recommended for drug susceptibility testing of TB. This concurrent use of different tests has generated discordance results challenges resulting in diagnostic dilemmas for laboratories and attending clinicians.

In our region, phenotypically occult strains results have been encountered but not highlighted. The current study is designed to identify the extent of this problem, along with investigation of missed mutations in the *rpoB* gene through sequencing which is the cause of discordant results within phenotypic and genotypic assays. Furthermore, whole-genome analysis of selected strains for the detection of mutations in other anti TB drugs imparting resistance (*katG, inhA, pncA, rrs, embB, gyrA and gyrB*) for comparison of genotypic and phenotypic methods. I*n silico* structure prediction of selected *rpoB* gene mutations for its impact on RNA polymerase. This will enable devising a strategy for accurate detection of resistance strains for better treatment options in the future**.**

The current study was designed to identify the extent of discordance, understand its molecular basis and develop strategies for antibiotic resistance detection for the recommendation of better diagnostic methods to reduce the load of TB and MDR TB.

1.9 Aim of the study

Comparative analysis of different available assays for diagnosis of MDR Mtb and identify mutations in occult clinical isolates for discordance using *rpoB* gene sequencing and whole genome analysis approach.

1.9.1 Objectives of the study

- Comparison of RIF DST on LJ medium, MGIT and genotypic RT-PCR based assay GeneXpert.
- Identification of mutations responsible for RIF resistance in Pakistani Population.
- Identification of mutations responsible for occult results in different phenotypic and genotypic assays in RIF resistant strains detected in the Pakistani population.
- Whole genome sequencing of selected isolates to determine the mutations imparting resistance to other important anti TB drugs, phylogenetic analysis of strains along with RNA polymerase structure prediction due to detected mutations.

2. Literature review

The prevalence of tuberculosis is on the rise in recent years. Despite all the efforts made to reduce the global cases of tuberculosis, 10 million people are still reported to be infected with tuberculosis globally (WHO, 2020). Although the fatality of this disease i more amHIV-positive patients, however, deaths in HIV-negative people wwere reported to be 1.2 million in 2019 (WHO, 2020). The prevalence ofdrug-resistant tuberculosis is a major barrier towards the Global TB Control Program. Among the drug-resistant TB, half a million cases were reported to be suffering from RIF-resistant TB whereas about 78% of the cases were multidrug-resistant tuberculosis (MDR TB). These cases not only include the newly diagnosed TB patients but also previously treated cases. About 3.3% of cases were newly reported in 2019; however, a considerably large number (17.7%) of these TB cases were reported among the previously treated individuals who were suffering from MDR/ RIF resistant TB (RR TB). In Pakistan, the prevalence of TB has been reported as 5.7%, which place Pakistan on 5th position among eight high burden countries. Currently, India, Indonesia, China, Philippines, Pakistan, Nigeria, Bangladesh, and South Africa are eight high burden countries in term of TB, which account for 66% of total TB globally. In case of DR TB, the highest disease burden exists in India, China, and Russia. The emergence of drug resistant tuberculosis, which include RIF resistant (RR TB) and multi drug resistant TB (resistant to INH and RIF) and extensively drug-resistant TB (XDR TB), which is MDR TB plus fluoroquinolone and at least one second line injectable drug have aggravated the situation. Geographical distribution of the new infections as well as the previously treated tuberculosis infections has been shown in the Figures 2.1 and 2.2.

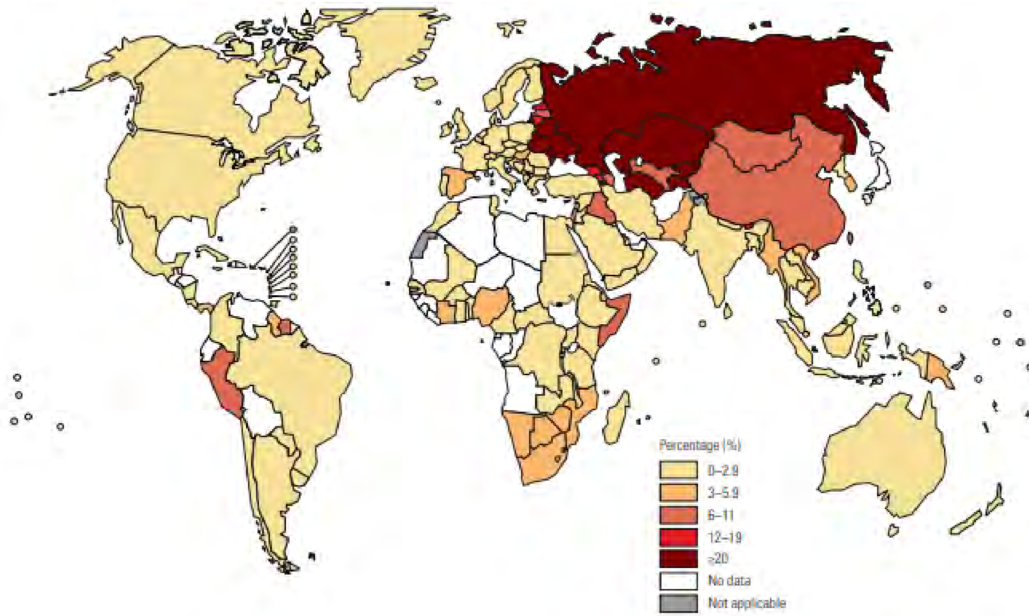


Figure 2.1: New cases of RR/MDR TB at global level (WHO, 2020).

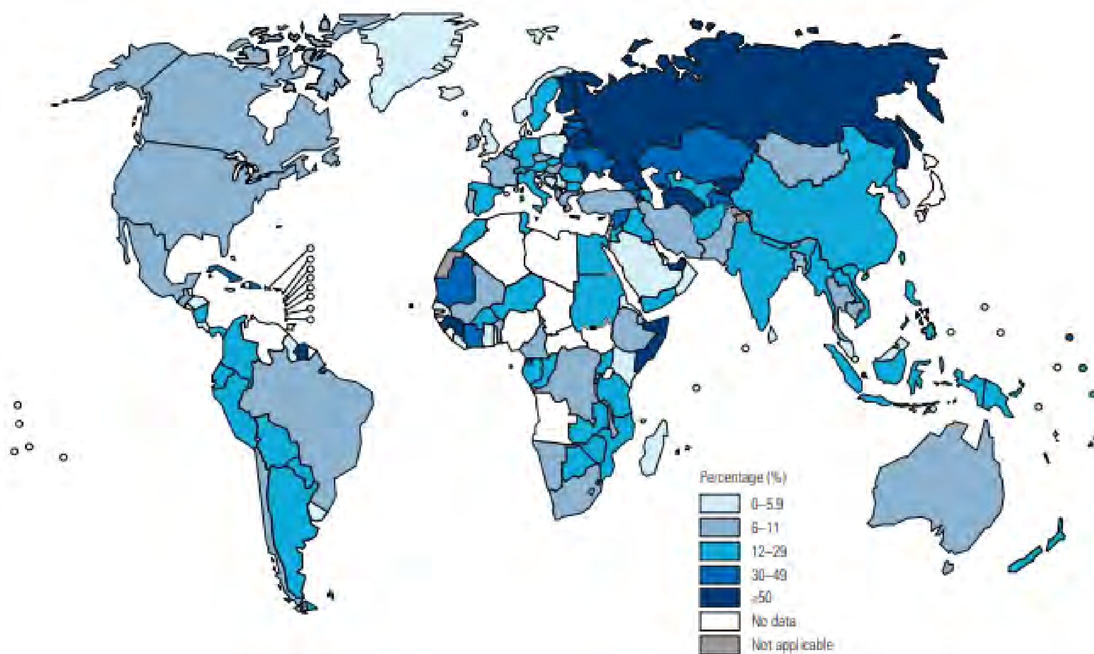


Figure 2.2: Previously treated TB cases with RR/MDR TB (WHO, 2020).

Mtb is the causative agent of TB belonging to the large family of pathogenic bacteria. It is a non-spore forming bacteria that has a curved rod like shape and is classified as an acid-fast bacterium. Their size ranges from 0.5 to 3 μm and possesses cell walls with complex structures. The cell wall of Mtb is a waxy thick structure and is rigid, which helps these bacteria to survive in a variety of environments (Forrellad *et al.*, 2013).

The disease is transmitted due to airborne sputum particles containing Mtb. These particles are termed droplet nuclei and spread into the air after sneezing and coughing of the person suffering from pulmonary TB. Inhaling of these 1-5 μm droplet nuclei results in contracting of infection (Mathema *et al.*, 2006; Ahmad, 2011). It has been reported that these particles remain in the air after being suspended in air, the duration can last from min to hours (Knechel, 2009; Ahmad, 2011). Besides inhalation of such particles as a source of infection, there are many factors, which influence the development of disease. It can vary among different individuals depending on the bacterial load that is inhaled, UV light exposure of bacteria, the nature, duration, and distance of contact as well as the strength of the immune system of the infected person (Knechel, 2009; Ahmad, 2011). It has been estimated that although 30% of people become exposed to Mtb; however, all of them don't develop the disease and in the absence of secondary infection like HIV, only 5 to 10% of people contract the disease (Lin and Flynn, 2010).

Mtb genome constitutes many genes for metabolism, in addition to genes for various virulence factors that help Mtb in the invasion, attachment and pathogenesis. Another important role played by these virulence factors is that after infection with Mtb these factors suppress the immune response of the host (Prozorov *et al.*, 2014). After these bacteria make their entry into the alveoli, macrophages and dendritic cells phagocytize the bacteria (Smith, 2003; Lin and Flynn, 2010). In the lungs, mycobacterial adhesins help bacilli to attach alveolar macrophages (Govender *et al*., 2014; Prozorov *et al.*, 2014), this attachment of bacilli to macrophages activates the immune system for self-defense against infection (Govender *et al*., 2014). The engulfed Mtb bacilli in phagocytes' phagosomes face a harsh environment, Mtb develops some strategies for its survival in these conditions (Forrellad *et al.*, 2013). One of these survival mechanisms is to stop the progression of phagosomes development (Forrellad *et al.*, 2013; Prozorov *et al.*, 2014),

Increased resistance to toxic compounds of the host and prevention from apoptosis are some other survival mechanisms in Mtb (Forrellad *et al.*, 2013). In addition, these bacteria cannot only survive but also manage to escape macrophages with the help of phospholipase (Ahmad, 2011).

Phagocytes produce organic peroxides and H_2O_2 against Mtb but enzymes and proteins of bacteria like *katG* protein A hydrolyze these oxides (Forrellad *et al.*, 2013). Another enzyme, that is, *Ahp*C, which is a hydroperoxide reductase, helps in the detoxification of hydroperoxides within the macrophages (Smith, 2003). The survival of mycobacterial bacilli within macrophages is facilitated by all the mentioned factors. Several other genes like *kat*G, *sod*A/*sec*A2*, Rv3654cl/RV3655C, nuoG,* and *pkn E* are responsible for the prevention of apoptosis (Forrellad *et al.*, 2013). If most of the mycobacterial bacilli can be killed in the granulomas, the progression of the disease is inhibited (Ahmad, 2011); however, some of these bacteria survive and escape from the immune system that later survives and persist in the host in a dormant state (Ahmad, 2011). In macrophages, Mtb is released from lysosomes to the cytosol where these bacilli escape the immune response and remain alive to infect new cells (Prozorov *et al.*, 2014). Along with developing pathogenesis, Mtb has also developed some mechanisms to invade the immune system and resist antimycobacterial drugs. After the development of resistance against the first and second line of drugs, there is now a high mycobacterium population that is exhibiting DR TB, MDR TB, and even XDR TB condition.

Sometimes resistance in bacteria is developed by some transmissible elements like plasmids and transposons but it is still not confirmed if the resistance in the case of Mtb has been emerged because of acquired plasmids or (Nachega and Chaisson, 2003). It has been reported that the result of non-compliance with prescribed treatment and poor disease management, the mutant bacilli which are resistant to antibiotics are selected (Campos *et al.*, 2003). Different studies have reported that if these bacteria are exposed to the sub-lethal concentration of antibiotics, it can lead to drug resistance as this practice stimulates mutagenesis, resulting in mutation in various genes which impart drug resistance (Smith *et al.*, 2012). Malabsorption of antibiotics and sub-optimal dosing are also other major factors contributing to the development of resistance (Shenoi and

Friedland, 2009) which are further compounded by factors like host genetics, health status, drug usage, and genetics mechanisms evolving the Mtb strains like RIF resistance. Some of the mechanisms involved in conferring RIF resistance in Mtb (Figure 2.3) are drug efflux pumps, mutations in the *rpoB* gene (Sharma *et al.*, 2010), compensatory mutations, alteration in target like RNA polymerase (Figure 2.4), and reduction in the permeability of bacterial envelope (Poon and Chao, 2005).

Spontaneous mutation, deletion, and additions of nucleotides in different genes whose products are the target of anti TB drugs result in drug resistance, for example, change in base-pair of *rpoB* gene which causes RIF resistance in Mtb. Bacterial RNA polymerase continues their functions even in the presence of these mutations and also confer RIF resistance (Sharma *et al.*, 2010). The *rpoB* gene in Mtb encodes beta subunit for DNA-dependent RNA polymerase of 1173 amino acids as shown in Figure 2.4. The majority of mutations, almost 95% are in the *rpoB* gene located within the 81 bp region which makes Mtb RIF resistant (Viveiros *et al.*, 2005; Daum *et al.*, 2012; Veluchamy *et al.*, 2013). In clinical Mtb isolates, classical mutations in the *rpoB* gene are not solely responsible for RIF resistance (Louw *et al.*, 2009), rather it is reported in the literature that 5% RIF resistant isolates do not even have any mutation in the hotspot region (81 bp) of *rpo*B gene (Louw *et al.*, 2009). So, there are some other possible but less studied mechanisms that might be responsible for RIF resistance (Louw *et al.*, 2009; Sharma *et al.*, 2010), which might be involved in altering lipid composition in the cell wall and efflux pumps.

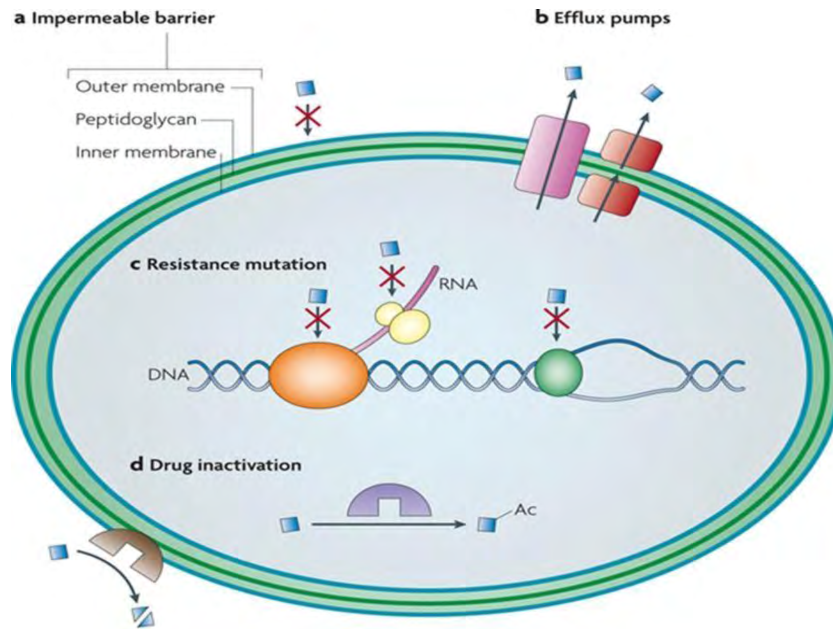


Figure 2.3: Mechanisms of drug resistance in Mtb including mutations in the *rpoB* gene of Mtb (Allen *et al.*, 2010)

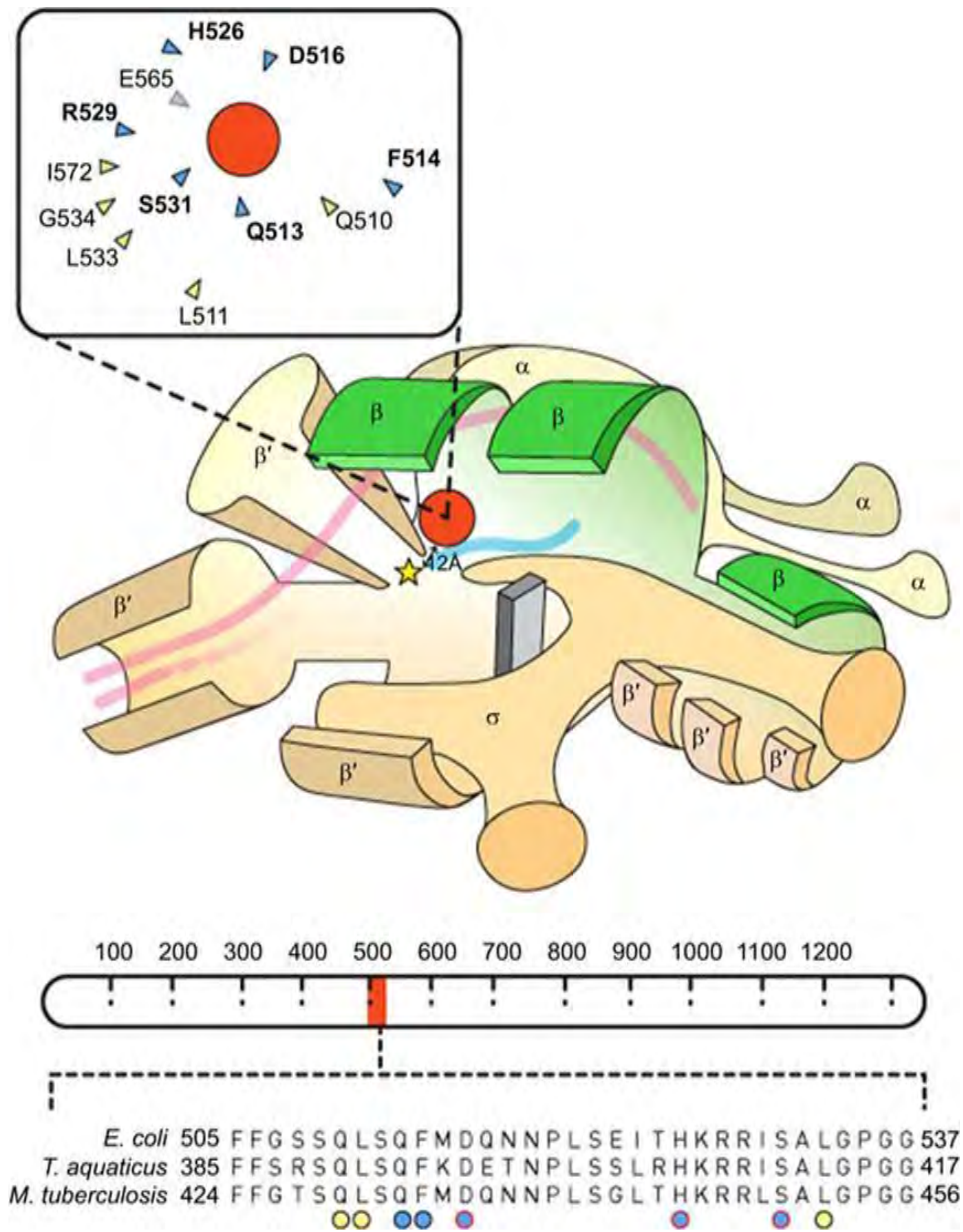


Figure 2.4: Structural elements of RNA polymerase which include the regions determining RIF resistance. The RNA polymerase active site for binding RIF molecule is 12 Å and RIF binding to it inhibits transcription (Sharma *et al.*, 2010).

The efflux pump's role in the elevation of the resistance to drugs in Mtb is now well documented (Louw *et al.*, 2009). These efflux pumps result in the reduction of the concentration of administered drugs within the cells. This reduction occurs because of the limited absorption of drugs which is influenced by the expression of these efflux pumps. This prevents the drug to reach its target and thus aid in the development of antibiotic resistance (Jin *et al.*, 2010). When exposed to antibiotics, most of the clinical isolates overexpress multiple efflux systems (Fonseca *et al*., 2015). Amino acid substitution in various drug target enzymes of Mtb along with increased expression of efflux protein in its plasma membrane are two main causes of antibiotic resistance (Piddock, 2006; Fonseca *et al*., 2015). In most cases, more than one efflux system are responsible for drug resistance, which operates together (Balganesh *et al.*, 2012). Cell membrane and efflux pumps work in a coordinated manner by altering cell membrane permeability to confer drug resistance.

A large number of lipophilic fatty acids, glycolipids, and long-chain mycolic acids are present in the outer membrane of Mtb (Swanepoel and Loots, 2014). These fatty acids are metabolized by several enzymes, which are produced in Mtb (Smith, 2003; Swanepoel and Loots, 2014). These lipids are the main source of energy, required for growth and replication, but some membrane lipids function as a virulence factor of organisms too (Swanepoel and Loots, 2014).

The cell wall of mycobacteria is unique in terms of chemical composition as compared to other bacteria (Beran *et al.*, 2006; Hett and Rubin, 2008). A variety of intercalated lipids along with high content of mycolic acid imparts low permeability (Jin *et al.*, 2010). Mtb becomes less susceptible to the action by the immune system of the host as well as different anti TB drugs because of its outer layer having low permeability (Beran *et al.*, 2006; Hett and Rubin, 2008; Jin *et al.*, 2010). The lipid metabolism of Mtb and RIF resistance was compared in two genetically distinct *rpoB* mutants strains (Ser531Leu and Ser552Leu) with the wild-type strain (du Preez and Loots, 2014). In the *rpoB* mutant strain, change in cell wall lipids was found by altering lipid metabolism, such alteration was linked to drug resistance in these Mtb strains.

There are enzymes or proteins which produce a large number of metabolites and complex lipids, called polyketides, present in the Mtb cell envelope (Swanepoel and Loots, 2014). There are multiple genes, which encode polyketide synthase enzymes, which are termed *pk*s genes. The location of *pks* genes in the Mtb genome is directly upstream of *mas* gene, whose products are phthiocerol and mycocerosic acid which are responsible for phthiocerol dimycocerosate (PDIM) production as PDIM a hydrophobic lipid act as a virulence factor in these bacilli. Proteomics, as well as metabolomics approaches, revealed that up-regulation of *drrA* (Rv2936), *ppsA-ppsE,* and polyketide synthase genes is responsible for the transport of PDIM to the outer surface in the cell envelope of mycobacteria. It occurs in the *rpoB* mutant strains of Mtb (Swanepoel and Loots, 2014).

Compensatory evolution or the presence of compensatory mutations is another concept that has not been elucidated in detail so far but its significance cannot be ignored (Poon and Chao, 2005). This concept is defined as any mutation which results in the generation of mutation in other genes that lead to drug resistance (Maisnier-Patin and Andersson, 2004; De Vos *et al.*, 2013). Another phenomenon is the reclaimed fitness which leads to the development of mutation epistatic effects in other genes (Poon and Chao, 2005) (Figure 2.5). It has been observed that these compensatory mutations are not automatically acquired after the development of resistance associated with mutations. The effect on the development of drug resistance can occur either by the certain structural compensation alone or by some synergistic relationships. In a study, the rate of growth of the RIF-resistant strains of Mtb was measured as compared to the susceptible strains. In a study, the growth rate of TB showing RIF resistance was compared with the susceptible strains (Gagneux, 2009). It was observed that the genotype of strain, as well as the nature of rpoB gene mutation, determined the fitness. It has been concluded that the fitness of strains depends on the presence or absence of compensatory mutations (Müller *et al.*, 2013).

The role of compensatory mutations in drug resistance was assessed by using a large database of RIF resistant and susceptible isolates by Koch *et al.*, in 2014. A significant ratio of Mtb strains resistant to RIF was revealed in this study, having *rpoB* gene mutation also had mutations in *rpoA* and *rpoC* gene but the same mutations were absent

from RIF susceptible isolates. Mutations in the *rpo*C gene were also reported to have a significantly high prevalence in the closely related strains isolated from South Africa. This was an indication of the relationship between the presence of mutations in the *rpo*C gene and the RIF resistance as compensatory mutations (Koch, Mizrahi and Warner, 2014). These compensatory mutations led to changes in the amino acid at positions 356 to 756 of *rpoC*, such mutations created a *rpoA-rpoC* interaction region which was responsible for increased in *vitro* fitness, which in turn effected the growth rate, virulence, and transmissibility (De Vos *et al.*, 2013; Fonseca *et al*., 2015).Another study reported that *rpoB* gene mutation in RIF resistant strains along with compensatory mutation was effected by continuous antibiotics exposure (Fonseca *et al*., 2015).

As there is a role of epistasis in drug resistance, a study was conducted to contract the web of epistasis mediated drugs resistance in Mtb plotting all known resistance genes by taking into consideration the approximate position of various genes (*rpoABC, katG, inhA, embB, gyrAB*, etc.) present in the Mtb genome. This work pointed out that there are putative epistatic interactions of resistance genes leading to the development of mechanism ancillary to drug resistance. These compensatory mutations influence various factors including cell physiology, cell permeability, lipid metabolism, purine metabolism, and transcriptional control, which acts as ancillary factors in anti TB drug resistance. A link between *rpoB, rpoC,* and *embB* genes to Rv3972 was also validated to play role in compensatory mechanisms. Overall, mutations in any of the known drug resistance genes and their type plus several mutations in these genes have a fitness cost due to other gene compensatory mutations.

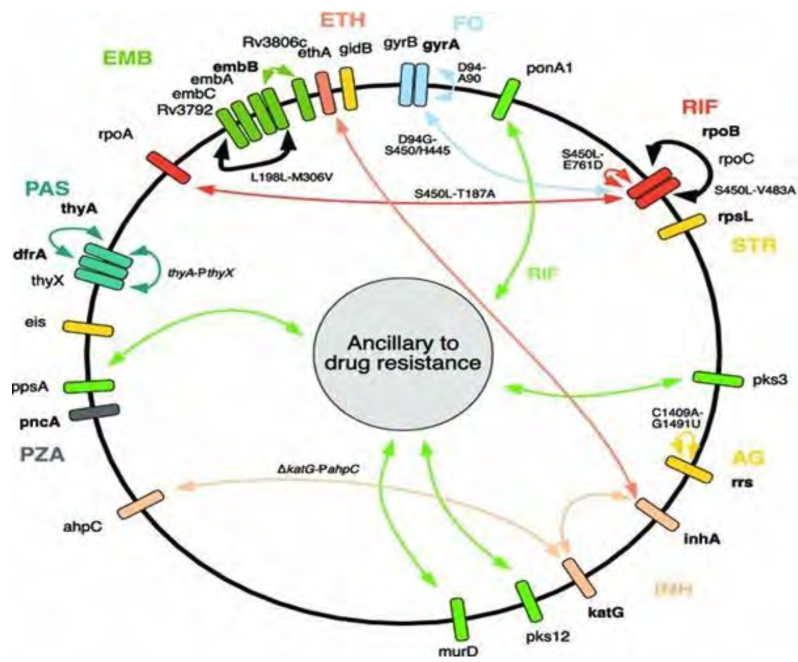


Figure 2.5: Schematic representation of epistasis-mediated drug resistance in Mtb (Trauner *et al.*, 2014)

The situation of TB is worsening due to the evolution of DR TB, MDR TB, and XDR TB across the globe due to these molecular, metabolic, and physiological mechanisms. Earlier studies showed that Mtb strains mainly develop resistance by spontaneous mutations, in SNPs in chromosomes (Mcgrath *et al.*, 2014; Nguyen, V. A. Le Berre, *et al.*, 2019). A small number of mutations have been described for each anti-TB drug in different genes and each of these mutations has a different drug resistance level. The mutation type and frequency can vary, e.g. 97% of cases of RIF resistance have been linked to *rpo*B gene mutations, where it is primarily mutation in the area of 81 bp hotspot, codon 507 to 533 (Laurenzo and Mousa, 2011), where high-level RIF resistance is mostly due to mutation in codons 526–531 of *rpoB*.

Mukinda and colleagues (2012) conducted a study in the South African Western Cape province, which showed rapidly increasing RIF mono-resistance in most HIV-positive people (Mukinda *et al.*, 2012). The development of the disease due to RIF mono-resistant Mtb in adults was characterized by variation in the RIF dosage, impairment of absorption, alteration of metabolism, and HIV infections (Dramowski *et al.*, 2012). RIF resistance is a rare form of tuberculosis and data on the prevalence of RIF resistance is also rare (Palomino and Martin, 2014). In new TB cases, its prevalence estimates in Europe are below 1%, while in Zambia 3.2%. Although relatively rare, RIF monitoring presents a major problem for TB management strategies. This form of TB has a lengthy treatment, high therapeutic costs, and a high rate of failure (Dramowski *et al.*, 2012).

Resistance to INH is acquired due to mutations in *katG, inhA* genes, and its promoter, as well as in the *ahpC, ndh,* and *furA*, genes (Laurenzo and Mousa, 2011). The most common *kat*G mutations (50-90%), occur in the codon 315 which gives a high level of INH resistance. The most frequent (47% to 62%) mutation is found in codon 306 of the *embB* gene in EMB-resistant isolates.

For second-line drugs like streptomycin resistant Mtb isolates, *rpsL* gene mutations are common in codons 43 and 88 and *rrs* codon 514 (Laurenzo and Mousa, 2011; Nguyen *et al.*, 2017). Around 60% to 70% of quinolone resisting isolates, mutations in *gyrA* genes with a frequency at codons 88, 91, 99, and 94 in the increasing order. Resistance in

fluoroquinolones occurs through mutation in *gyrA* and *gyrB* genes (Laurenzo and Mousa, 2011). The *gyrB* gene mutations rarely occur in comparison to *gyrA*.

Mutations in the *rrs* cause resistance to kanamycin, amikacin, and capreomycin injectables, which are the second-line drugs. In this case, the *rrs* A1401G mutation is very important. It is the responsible majority of capreomycin resistance, that is, 70% to 80% while 60% kanamycin resistance is also attributed to this mutation. However, in about 10% to 40% of DR TB isolates the mechanisms underlying drug resistance are not known and no mutation has been detected (Jeanes and O'Grady, 2016)

Looking at the scenario in Pakistan, a lot of research has been conducted on TB in the recent past. Recent work by Jabbar *et al*., 2021, highlight that in TB case, DR TB cases especially MDR XDR TB are present (Jabbar *et al.*, 2019). TB is alarmingly increased in the Khyber Pakhtunkhwa area in newly diagnosed patients with TB, where XDR TB was 0.4%, MDR TB 4.6%, multiple resistant 18%, mono-resistant was 16%, and 61% cases were of the pan sensitive. The female patients had a slightly high number with a ratio of 51.5% as compared to male 48.5% in the age group of 15-24 years. More or similar prevalence rates of TB, DR, and MDR TB exist in various parts of Pakistan.

According to a survey conducted by Siddiqi *et al*. (2002), in which 22/27 RIF resistant strains had a mutation in four codons (512,516, 526, and 531) of the *rpoB* gene, majority isolates (52%) had a mutation in codon 531 and in two isolates GCC insertions in codon 512 were also found (Siddiqi *et al.*, 2002). Heep *et al*. (2001), found no RRDR mutation outside of this area like V176F mutations in any of the isolates(Heep *et al.*, 2001). A study consisting of 163 isolates from Pakistan, reported 36.2% (n = 59) isolates resistant to ofloxacin, 34.36% (n = 56) to RIF, 28.8% (n = 47) to streptomycin, 19% (n = 31) to EMB and 9.82% (n = 16) resistant to PZA. These resistant isolates were randomly selected for mutation of various genes including *rpoB* gene mutation analysis in RIF resistant isolates. Mutations detected in these isolates were Pro454His, Ser450Gln, Ser450Leu, Gly455Asp, and Asp435Gly. Additionally, in 16 (34%) isolates Lys88Arg, Lys43Arg, and Lys111Ile mutations were detected in the *rspL* gene. Upon analysis of 10 (62.5%) EMB resistant isolates, various mutations (Met306Val, Ala281val, and

Met306Leu) were detected in the *embB* gene (Qazi *et al.*, 2014).

According to a study conducted by Ali *et al*., (2015), rifampicin-resistance-determining region (RRDR) in *rpoB*, as well as compensatory mutations in rpoC and rpoA, mutation at codons 531, 516, and 526, were detected in all XDR isolates in the RRDR of the *rpoB* gene. The *rpoB* codon 531 mutation was found in principal genetic group 1 (PGG1), principal genetic group 2 (PGG2), and principal genetic group 3 (PGG3) isolates, on the other hand, a mutation at codon 526 was found in one EAI3-IND isolate from the PGG1 group. In both CAS and Orphan strains, mutations at codons 515 and 516 were present simultaneously. The low fitness cost in mutation *rpoB* S531L was found in twenty-two XDR TB isolates with compensatory mutations in the *rpoA* and *rpoC* genes. Two *rpoC* SNPs were found in one isolate with the *rpoB* H526R mutation. The most prevalent SNPs were *rpoC* Ala172Val and Pro601Leu with *rpoA* SNP,

Jabbar *et al* assessed and compared 19 mutations conferring the phenotypic drug susceptibility against 11 anti TB drugs available in Pakistan. *In silico* prediction for RIF resistance showed a perfect concordance with phenotypic results. A known mutation in the *rpoB* gene at codon 450 was mainly associated with resistance. Mutation Ile491Thr was found in 7/76 strains (which were Beijing linage strains), Ser450Leu and Ile885Val mutation were present in 1/76 strains (this one was in CAS/Delhi lineage), the mutation in *rpoC* gene was present in less number of RIF resistant isolates which also had *rpoB* S450L as background mutation. In these investigated strains, Rv1482c–fabG1 intergenic region and *katG* gene (S315T) mutations were found to be responsible for INH resistance but in these isolate some additional mutations like *katG* (5/68) and *oxyR'-ahpC* (1/68) were also detected. *In silico* predictions and phenotypic results of 3 isolates showed the presence of uncharacterized frameshift mutations in the *katG* gene, which were assumed to have a role in the loss of function of the INH activation enzyme (Jabbar *et al.*, 2019).

A study by Siddiqui *et al*., (2019) found *rpoB* gene mutations responsible for RIF resistance were seen in 81/ 96 isolates. Among detected different mutations found in RR TB, the most common mutation was observed at the S531L position in 45 isolates which is 55.6% of isolates. LPA showed 71 patients (81.6%) out of the 87 persons with INH-

resistant isolates had mutations in the *katG* gene; however, in *inh*A promoter only 17.2% mutations were detected while 1.1% mutations were detected in both genes. Majority of isolates have S315T1 mutation occurring due to the substitution reaction in the *kat*G gene. It led to a high level of INH resistance. It was detected those 13 isolates representing 14.9% of the total had C15T mutation occurring in the *inh*A gene which was conferring low-level INH resistance. However, acquiring the *rpoB* mutation has a fitness cost in RIF-resistant Mtb strains, which frequently adopt compensatory mutations in the *rpoC* and *rpoA* genes to increase their development.

The challenge of drug resistance for TB therapy and eradication is affecting 10.0 million lives worldwide including Pakistan. A global increase in the prevalence of drug-resistant tuberculosis has been reported. For example, estimated new cases of MDRs resisting INH and RIF or RR treatment at least increased to almost half a million in 2019 (World Health Organization, 2018; WHO, 2020). The treatment of MDR is complex, much more costly than non-MDR TB treatment; it is linked to several side effects in TB patients. The treatment success rate decreased from 83% for new or recurring DR-free TB patients. It resulted in a 54% success rate for MDR/RR TB and 30% for high drug-resistant TB that includes resistance to at least one second-line injectable and one fluoroquinolone (WHO, 2020). There are 7.1 million people in the world receiving annual tuberculosis treatment in 2019. The access of TB cases in case of preventive tuberculosis increased to 4.1 million in 2019 from 1.0 million in 2015.

The Mtb strains are increasingly endangering the worldwide success of TB treatment particularly drug-resistant and extremely drug-resistant (Diseases, 2011). Fast and accurate laboratory diagnosis is the first obligation for the correct treatment of these patients, which is necessary to limit MDR and XDR TB transmission. Phenotypic and genotypic tests for MDR TB have been endorsed by the World Health Organization. The approved phenotypic tests include the commercial medium-based Mycobacteria growth indicator tube system (MGIT) (Becton, Dickinson) with a solid medium proportion and absolute concentration methods, the colorimeter reductase noncommercial test of nitrate (Martin *et al.*, 2008), the resazurin microtiter assay (REMA) (Palomino *et al.*, 2002), and microscopy-based (MODS) assays (Lemus *et al.*, 2004; Moore *et al.*, 2004).

The LJ medium is considered the gold standard for drug susceptibility for DR TB detection (Canetti *et al.*, 1969). The LJ culture method time taking, and it usually needs up to eight weeks for providing results. In contrast, the Middlebrook agar solid culture medium provides outcomes within two weeks (Brady *et al.*, 2008; Nguyen *et al.*, 2015). For rapid detection with great sensitivity, liquid culture methods are available too(Lawson *et al.*, 2013). For example, the BACTEC MGIT is barcoded and analyzed results automatically, which can hold (up to 960 tubes) in 10 to 30 days (Lawson *et al.*, 2013). Nonetheless, culture methods based on liquid medium have several drawbacks, including contamination that is not evident, overgrowth of undesired bacteria, and expensive instrumentation (Singhal *et al.*, 2012; Lawson *et al.*, 2013; Nguyen *et al.*, 2015). Furthermore, highly skilled staff is required along with a high level of biosafety facilities (for protection of both technicians and the environment) for performing culture-based processes. As a result, culture approaches for DR TB detection are rarely used, particularly in resources-limited countries. Phenotypic DST tests are gold standards for detecting DR TB, however, due to the long assay times, WHO has approved various molecular methods, such as MTBDRplus, Xpert MTB/RIF, and Nipro NTM+MDRTB. These molecular assays also have detection limitations, making the decision of treatment more difficult for the isolates that possess *rpoB* mutations but do not exhibit phenotypic resistance indicated as susceptible.

The Xpert MTB/RIF is a rapid molecular-based test recommended by WHO for Mtb complex identification and RIF resistance testing in clinical specimens (WHO, 2020). This test was first approved in 2010 for sputum samples used to diagnose tuberculosis in suspected adult patients. Three years later, it was approved for the detection of tuberculosis in children by WHO.

The Xpert MTB/RIF assay amplifies a sequence comprising of 81 bp hotspot domain of the *rpoB* gene which is subsequently annealed to five probes using semi-quantitative nested real-time PCR (Steingart *et al.*, 2014; Ochang *et al.*, 2016). Each probe is tagged with a fluorescent dye and recognizes a unique sequence. To avoid cross-contamination between specimens, the assay reaction is carried out in a type of mini laboratory as it is performed in a self-contained cartridge. When compared to the usual phenotypic the

sensitivity as well as specificity of culture-based DST for the specimens that smeared positive remains 100 and 99 % while it remains 67% and 99% for smear-negative specimens respectively. The Xpert MTB/RIF assay has reduced the time it takes to detect RIF resistance from several weeks to two hours. Because the Xpert MTB/RIF assay's results are accessible in a short period, it enables prompt treatment initiation, especially for MDR TB patients. Furthermore, when compared to culture-confirmed cases using smear microscopy, Xpert MTB/RIF has about 23% higher Mtb detection rate. As a result, Xpert MTB/RIF has a high TB detection accuracy and reduces Mtb and NTM misdiagnosis (Bunsow *et al.*, 2014; Steingart *et al.*, 2014; Sharma *et al.*, 2015).

Nevertheless, some false-positive results have been reported with Xpert MTB/RIF because of the silent mutations occurring in codon 514 in the *rpoB* (Bunsow *et al.*, 2014). Moreover, some researchers reported false-negative with Xpert MTB/RIF, which could be due to the inability to detection the mutations responsible for RIF resistance which occur outside the hotspot region (Sanchez-Padilla *et al.*, 2015; Theron *et al.*, 2016, 2018; Ngabonziza *et al.*, 2020). In patients from Swaziland, it was reported that more than 30% of RRTB occurred due to the 1le572Phe mutation, implying that Xpert MTB/RIF assay detection has limitations. This molecular assay does not investigate for variants in genes linked to INH resistance; instead, RIF resistance is used as a baseline for MDR TB detection (Manson *et al.*, 2017). As a result, numerous infections of INH mono-resistant tuberculosis are undiagnosed. INH resistance is reported to precede in all the geographical locations globally, linages as well as the periods according to recent whole genome sequencing research. Because RIF resistance can be distinguished only by the Xpert MTB/RIF and not INH-causing variants, it hinders the early detection of MDR TB, notably if INH mono-resistance is present.

Because the Xpert MTB/RIF assay alone does not validate all drug resistance mutations; therefore, additional tests are required. These include the DST for confirmation and identification of the resistant phenotype of the resistant Mtb isolate. Another drawback is the expensive cost of the system, which is related to the use of the complicated GeneXpert technology and single-use cartridges (Walzl *et al.*, 2018; WHO, 2020). As a result, in several high-TB-burden nations, using this assay as a foremost test for the

detection of all suspected TB patients is costly. Among 48 countries having a high burden of TB, only 15 use the GeneXpert to tests all the suspected TB patients (Forrellad *et al.*, 2013). Furthermore, the GeneXpert apparatus demands a continual power supply and it is heat and dust sensitive. As a result of these issues, several machine breakdowns, which further limits its use in developing countries (Walzl *et al.*, 2018).

Another diagnostic system, that is, the next-generation Xpert MTB/RIF Ultra system (Cepheid) is used for the processing of greater sputum amounts because of the presence of large amplification chamber amounts and has two additional targets, that is, IS1081 and IS6110 for the identification of Mtb. It has been assessed by WHO (Chakravorty *et al.*, 2017). The newer ultra-cartridge is fully compatible with the previous GeneXpert machine and costs the same as the previous one (World Health Organization, 2017). Moreover, the analytical sensitivity of this improved cartridge has been boosted more than tenfold. The sensitivity of the Mtb detection cartridge has improved to the detection limit of 16 bacilli/mL. It adds to the sensitivity of the process when compared to the 131 bacilli/mL detection limit for the prior Xpert MTB/RIF cartridge, which allows the screening of Mtb specimens having lower low bacilli counts. This next-generation cartridge has various advantages, including the ability to detect DRTB in sputum samples from children, HIV-positive patients, and complicated smear-negative pulmonary and extrapulmonary TB samples. Although having a higher sensitivity, the specificity of the Xpert Ultra for Mtb diagnosis is less than the Xpert MTB/RIF (WHO, 2020). However, the performances of both cartridges in terms of detecting RR TB are comparable. Xpert MTB/RIF Ultra has an advantage in terms of improving the existing shortcomings of the cartridge by the omission of silent *rpoB* variants, that is, Q513Q and F514F, that will be useful since certain previously latent strains are now identified by Xpert MTB/RIF (Chakravorty *et al.*, 2017). Accordingly, the WHO recommended using the Ultra cartridge recently as a primary diagnostic method for all adults and children with TB clinical symptoms. The extrapulmonary samples, like cerebral fluid, lymph nodes, and tissue biopsies, were also approved for diagnosis (World Health Organization, 2017). Another Xpert cartridge was recently developed to detect XDR tuberculosis (Cao *et al.*, 2021) but the definition of XDR TB is also changed recently as any Mtb strain which

fulfills the criteria of MDR/RR TB is also resistant to not only fluoroquinolone but also to an additional drug from group A (Bedaquiline or Linezolid) (Organization, 2020).

Overall, a review of the data has pointed towards the advantage of sequencing is the best method for analysis of the organism's genotype Whole genome sequence is a method of choice that can discriminate between the two strains was slight changes (Satta *et al.*, 2018; Bogaerts *et al.*, 2021).

Next-Generation Sequencing (NGS) was a revolutionary development in the field of molecular biology as this technique can cause a rapid sequencing of the targeted gene as well the sequencing of the whole genome (Phelan *et al.*, 2016; Dheda *et al.*, 2017; Manson *et al.*, 2017). Through sequencing, all types of mutations can be identified (i.e. insertions, deletions, synonymous and non-synonymous), species identification, the evolution of organisms and species plus the development of drug resistance. NGS-based kits for DR TB screening are commercially available. For the analysis of the full length of analysis of the Mtb genes, the development of drug resistance against first and second-line drugs was developed using a novel assay of the duration of two days assay using Ion Torrent Personal Genome Machine (Daum *et al.*, 2012). Important eight resistance genes' full-length sequence (*emb*B, *gyr*A, *katG*, *rpo*B, *rpsL, eis, and pnc*A) can be amplified by PCR-based assay and sequenced to detect new mutations. The AmpliSeq of Illumina TB Research Panel (Illumina) also detects these same genes mutations too. Besides these methods, WGS is used but it is now only valued for cultured strains as it requires a good amount and quality of DNA (WHO, 2016). In some studies, WGS was performed for detection of DR TB from direct sputum samples but a high level of contamination of the human genome was detected that why the results were very variable.

A large number of novel NGS platforms are commercially available, such as PacBio RSII (Pacific Biosciences) and Oxford Nanopore MinION (Oxford Nanopore Sequencing Technology, Oxford, United Kingdom) with advantages of long read in very short run time. But, high error rates and immense output have been the main issues until now with these applications (WHO, 2016). The Oxford Nanopore MinION is a portable small benchtop instrument that can be directly attached to a laptop via a USB port and cable,

which can generate 10-20 GB of data for a single sample. The limitation with this device is that it requires large devices for the storage of data as well as highly efficient speedy computer processors for the analysis of data. The error rate in this procedure is still 20% to 35% that is a high rate but in the future, it is expected that the minion system along with associated software will be improved (Senol Cali *et al.*, 2018). Higher genome coverage, Detection of development of new resistance mechanisms against new and existing drugs, and more information related to epidemiology are the main advantages of WGS. Due to this advantage, WGS as a detection tool for DR TB has an opportunity in clinical sceneries in the future.

In low and middle-income countries, the use of such large scale sequencing devices is very difficult because of many reasons, such as (i) Requirement of high-quality software and dedicated database tools (ii) –Highly trained professional and bioinformatics facility for data collection and analysis (iii) Highly expansive NGS tools (iv) A wet laboratory to determine whether certain mutations are the cause of antibiotics resistance or not (v) High-quality DNA for sequencing (Phelan *et al.*, 2016; Dheda *et al.*, 2017; Zignol *et al.*, 2018). Furthermore, laboratory methods for the preparation of samples and extraction of DNA need standardization as well as optimization (Zignol *et al.*, 2018).

The sequencing cost is lower than the testing of phenotypic testing of first and second-line drug resistance and it is further decreasing continuously (Dheda *et al.*, 2017; Zignol *et al.*, 2018). Many systems are under development i.e. by Foundation for Innovative Diagnostics (FIND) for targeted gene sequencing from directly sputum samples (Dolinger *et al.*, 2016).

In many studies, it is reported that WGS is very successful for targeted sequencing from primary sputum samples when processed with newly developed strategies like targeted DNA enrichment (McNerney *et al.*, 2017; Doyle *et al.*, 2018). Such protocols are becoming rapid, simpler, and affordable day by day. As it's a WHO recommendation to use NGS for detection of drug resistance-associated mutations in Mtb so, TGS and NGS are the main reliable tools for drug resistance surveillance (WHO, 2016). CRyPTIC and ReSeqTB, The two main NGS expert groups have established a massive repository of

phenotypic and genotypic data collected from Mtb strains almost all over the world. These two consortiums are the major platforms to perform automatic processing of raw data from WGS and then to organize this data into a comprehensive form of mutations lists related to drug resistance. This is easily accessible and is to use even for non-bioinformaticians.

These platforms are currently available only for the purposes of research and monitoring. It is planned that in the future they will be handed over to the WHO for short-term global TR TB monitoring and long-term clinical diagnosis of DR TB (WHO, 2016).

The progressive increase in DR TB cases highlights the critical need for accurate and rapid diagnostic tools for their detection, assessments such as DNA microarray, LPA, real-time PCR, cost-effective sequencing, quantity as well as the quality of samples and short shelf life. In addition, a new assay is developed with the ability to target currently undetected drug resistance mutations and the personnel skills necessary to run the test. In addition, affordability of diagnostic tests based on molecular biology needs to be increased for low-income countries such as Pakistan with rapid test results in which DR TB, as well as TB, are major concerns that possess multiple mutations in different genes, and as so an ideal test is needed at the same time for the detection of mutations in a single reaction. Similarly, simple diagnostic procedures are required to be increased in terms of accessibility at the level of different laboratories (Nguyen, V. A. L. Berre, *et al.*, 2019).

Recently much interest has been seen in the development of new molecular methods to measure the genetic relatedness between the different strains of Mtb and other than Mtb strains. Multiple copies of insertional sequence IS6110 are distributed throughout the genome by the processes of transposition. It is believed that this transposition is a non-random processed, tending to certain hot spot regions (McHugh and Gillespie, 1998; Warren *et al.*, 2000). IS6110-RFLP is another useful technique for the detection of TB, its outbreak, and cluster investigation, as it correctly differentiates among epidemiologically linked and unlinked clusters (Hermans *et al.*, 1990, 1991; Edlin *et al.*, 1992). Many countries are utilizing this technique to help TB control programs in differentiating the new transmission of cases. Netherland typed 24000 cases using the

RFLP technique in its first national-wide study from 1993 to 1997 conducted on 4266 strains where 1493 cases were found to be the new transmission. Thus, it was estimated that 35% of the new case burden in the Netherland was added. Similarly, results obtained from studies of other countries like the USA, Switzerland, and Denmark, also showed that despite efficient TB control programs in these countries 30%-40% of new cases were still added (Genewein *et al.*, 1993; Alland *et al.*, 1994; Small *et al.*, 1994; Yang *et al.*, 1994). The RFLP method provides insight into the phylogeny of the circulating strain in an area for epidemiological surveillance. Another method for investigation of strains in a country to assess the circulating lineages, which are Delhi/CAS, Beijing, Euroamerican, East American Indian, *etc* is MiruVNTR (variable number tandem repeats) based assay as VNTR are located in multiple variable copies at each locus that makes them a basis for the differentiating of different strains.

Spoligotyping is another widely used technique for the epidemiological studies of Mtb was developed by Kamerbeek *et al*., in 1997. Mtb genome contains variable copies of direct repeats (DR) in a specific locus. The sequence of these direct repeat is conserved and are 36 bp long. Each of the two direct repeats is separated or interspersed by a conserved sequence called a spacer, which is 34 bp to 41 bp long. Ninety-four of such spacers has been identified so far (Beggs *et al.*, 1996; Van Embden *et al.*, 2000), however, only 43 spacers are in use for the routine genotyping while the other spacers contribute to only a slight increase in information (Van Embden *et al.*, 2000; Sebban *et al.*, 2002). Different strains of Mtb vary in the number of spacers, which is the result of the presence or absence of any DR in the locus, hence this method is also called DNA finger Printing (Hermans *et al.*, 1991; Groenen *et al.*, 1993).

Spoligotyping produces similar results for phylogenetic analysis as that of restriction fragment length polymorphism, however, the level for differentiating strains is higher by RFLP. On the other hand, spoligotyping is much helpful in the characterization of strains with a low copy number of IS6110 elements which were misinterpreted by using RFLP (Goyal *et al.*, 1997). In the clinical samples, this technique is very helpful for its differentiating between Mtb and *M. bovis*. Both the species exhibit a specific pattern. *M.*

bovis is identified for its lack of the last four spacers from positions 39 to 43 (Kamerbeek *et al.*, 1997).

In Pakistan, only a few studies have been conducted for molecular epidemiology typing (Ali *et al.*, 2007, 2019). An earlier study of 2007 reported predominate lineage to be Central Asian strain 1 (CAS1) using 12 loci MIRU VNTR assay. Recent, work by Ali *et al.*, 2019 showed that the predominant lineage was again the Central Asian strain after L2/Beijing having 5.4% prevalence and L4 having 4.2% prevalence. It was reported that L3/CAS1-Delhi was the main sub-lineage among the L3/CAS family (84%). An evolutionary linkage of clinical strains with the unknown pattern was found with the L3/CAS strain. Among them 5.4% of strains remained unknown; therefore, were tentatively named as L3/CAS-KP (Khyber Pakhtunkhwa).

Only a few studies have been conducted for in silico protein prediction of rpoB protein. According to a study substitutions in *rpoB* are located within 5.0 Å resulting in either removal of direct contacts between RpoB and the drug or interactions disrupts that shape the binding site. They also pointed out that physicochemical characteristics of the amino acid substitutions nor could a stabilizing/destabilizing effect be associated with a unique phenotype (Paolo *et al*., 2018). Previously, a study calculated stabilizing and destabilizing effects on protein stability and flexibility due to identified mutations in the *rpoB* gene from Pakistani isolates, along with the effect of compensatory mutations (Khan *et al*., 2019).

TB phenotypic assays can be used to test the 1st and 2nd line drugs but there is variation in the result reliability due to the method and drug used. WHO endorsed genotypic assay GeneXpert can only be used for RIF resistance detection, while line probe assays are limited to INH, RIF, FQs, and second-line injectables resistance (Genotype MTBDRplus and MTBDR). These genotypic assays give reliable and fast results even from clinical specimens directly. On the other hand, outside the targeted region for amplification, these assays fail to detect DNA mutation and can miss the resistance for a drug. Depending on the investigated drug, the proportion of resistance missed by genotype can be measured by DST (Ramaswamy and Musser, 1998; Campbell *et al.*, 2011). The type of mutation

also affects the level of phenotypic resistance on DST, like low-level INH resistance on DST can be missed when there are mutations in *inhA* gene (Zhang and Yew, 2009). Due to the presence of occult strains in genotypic/phenotypic RIF susceptibility tests, Mtb treatment has become a significant challenge. Limited data on the prevalence of occult strains and the effect of discordance on patients' management is available.

Van Deun *et al*. (2009) investigated 19 selected Mtb strains in nine Supranational Reference Laboratories for discordances in proficiency testing. It was recorded that low level but perhaps relevant RIF clinically resistance linked to specific *rpo*B mutations was easily skipped by these automated broth-based Bactec MGIT 960 automated systems as well two agar media Middle brook 7H10 and LJ medium.

Several studies from different countries recorded that Mtb strains tested on phenotypic DST assay and molecular assay exhibit different drug susceptibility patterns. A study from Port-au-Prince (Haiti) showed that upon consecutive 153 phenotypic characterizations of clinical Mtb strains which by molecular tests were RIF-resistant, 86.9% resistant isolates were found to be both INH resistant and RIF in MGIT liquid culture-based DST, 2.6% or 4 isolates were RIF mono-resistant. The rest of the 16 RIF resistant strains by GeneXpert were found on MGIT as RIF sensitive strains, hence were an occult strain. Also in these 16 discordant strains, 5 strains had the MIC for RIF close to 1 mg/mL, the cut-off value, which is recommended for phenotypic susceptibility assays. A sub-critical MIC for RIF was found among nine strains having MIC ranging from 0.063 mg/mL to 0.5 mg/mL, among them two strains were found pan susceptible, harboring a silent mutation of the *rpoB* gene. This data indicates the presence of *rpoB* gene mutation merely is not sufficient but the sequence nature of *rpoB* mutation (synonymous, non-synonymous, etc.) is also required for precise identification of RIF resistance within Mtb. Also, the available drug's critical concentration needs to be re-evaluated using DST assays based on culture (Ocheretina *et al.*, 2014).

To determine the effect of different r*poB* RRDR mutations, 32 multidrug-resistant, 4 RIF-mono-resistant, and 5 susceptible Mtb clinical isolates were studied. These isolates' mutations were confirmed by whole genome sequencing as well as Sanger sequencing

and compared to MICs values on liquid-based MGIT. rpoB proteins with mutations Ser450Leu, His445Asp, His445Tyr, and Ser450Trp and double mutation of Asp435Ala-Arg448Gln were characterized as having high MIC for rifabutin and RIF. RIF susceptible strains had mutations in Leu430Pro, His445Leu, His445Asn, and Asp445Gly-Ser441Leu. In the case of two MDR isolates without *rpoB* RRDR mutations, the whole genome sequencing (WGS) showed V146F mutation that was outside the RRDR at 50 g/L MIC for the RIF (Jamieson *et al.*, 2014). This indicated that different mutations at the same loci can impart different drug susceptibility, such mutations also show different DST results if mutations are in genes related to other first line and second line regimen drugs are also present in the isolate.

Often phenotypic test fails to detect low-level but clinically significant resistance, which is otherwise RIF resistant on GeneXpert assay. Also, false-positive RIF resistance is seen due to silent mutations in the *rpoB* gene in GeneXpert as these mutations are outside the hotspot area of the *rpoB* gene. This points towards the need to confirm the RIF resistance detection by Xpert assay through the method of phenotypic DST, specifically for a setting where there is 15% RIF resistance prevalence and any result of discordant RIF susceptibility must be confirmed through *rpoB* gene sequencing (Mokaddas *et al.*, 2015).

A study was conducted on patients from California, where 16 out of 413 *rpoB* mutation-positive patients had molecular-phenotypic discordant RIF results. In these occult strains, 7 different mutations; Ser533Pro, His526Cys, His526Ser (TCC and AGC), His526Asn, Asp516Phe, and Leu511Pro were identified. Six isolates were resistant phenotypically to PZA and/or EMB among the 14 (88%) INH resistance. Although, this work indicated a low prevalence of RIF molecular-phenotypic discordance patients with these mutations had poor treatment outcomes (Shah, Lin, *et al.*, 2016).

Even when results of two phenotypic assays are compared, discordance is observed in some cases depending upon gene, type of mutation, other mutation's co-occurrence in the same isolate, and drug concentration. A complete agreement was observed among MGIT-DST and LJ findings for mutations situated at codons 513 (Lys or Pro) and 531 (Leu or Trp) as strains were resistant by both methods. However, for *rpoB* gene mutations

Leu511Pro, Asp516Tyr, Leu533Pro, Ile572Phe, and several different mutations in 526, MGIT, and LJ results were extremely contradictory. It was inferred that the automated MGIT 960 system may have skipped some RIF resistance-conferring mutations, on the other hand, careful DST on LJ hardly missed any these of mutations. Based on findings, it was further assumed that reconsideration is required for the gold standard method for RIF resistance detection as discrepancies in results between different phenotypic assays occurred and this can affect the treatment options by the clinician (Rigouts *et al*., 2013; Hofmann-Thiel *et al.*, 2017; Miotto *et al*., 2018).

A total of 10,209 isolates were analyzed by TB cryptic consortium of sixteen countries including Pakistan from six different continents. The smallest phenotypes proportion was predicted EMB while the largest was for RIF (9660/10,130). For the 1st and 2nd line anti-TB drug, resistance to PZA, EMB, RIF, and INH was predicted correctly with a sensitivity of 91.3% 94.6%, 97.5%, and 97.1% respectively. While susceptibility for these drugs was accurately predicted with the specificity of 96.8%, 93.6%, 98.8%, and 99.0% respectively. In 5250 out of 5865 isolates with complete genotypic predictions, 89.5% were phenotypically predicted correctly. Among the 4037 phenotypic pan-susceptible strains, 3952 were correctly predicted in the genotypic assay. For Mtb susceptibility, genotypic predictions to 1st drugs line were in line with phenotypic susceptibility results. But the substantially better performance of sequencing analysis was observed with regard to sensitivity in the case of PCR-based assays even better were results with whole genome sequencing for the identification of several missed mutations. *rpoB* 149IF mutation is an example mutation that shows susceptibility in liquid culture as sensitive but is associated with unsuccessful treatment (CRyPTIC Consortium and the 100, 2018).

In a study conducted in South Africa on 1302 RIF-resistant isolates by comparing genotypic MTBDR*plus* assay results with phenotypic assays Middlebrook 7H10 agar dilution and BACTEC MGIT 960, 4.6% isolates were found to be discordant isolates. Among these occult strains, rifabutin susceptibility was 98% while 62% were susceptible to INH. Another issue was that MICs for the RIF were near to the critical concentration

for 83% isolates which were recorded as 1 µg/mL in a range of 0.5-2 µg/mL (Mvelase *et al.*, 2019).

Recently in a study conducted in France that compared phenotypic DST results and LPA results with WGS, resistance predicted by WGS was 100% correct for susceptibility against RIF, INH, EMB while for PZA resistance was 93.8% when keeping phenotypic DST as a reference. It was recommended that WGS should be used in the future as an initial diagnostic tool for resistance prediction (Genestet *et al.*, 2020).

In a study reported from New York, 1,779 strains were tested phenotypically as well as through WGS for harboring a mutation in the *rpoB* gene over 2.5 years through routine clinical testing, and among those strains, 139 were reported to have nonsynonymous *rpoB* mutations. Among these mutations, 53 mutations were associated with RIF resistance with varying resistance levels from low to high level. In 43 (81.1%) isolates, RIF resistance was identified at 1.0 mg/mL in MGIT and 10 strains, that is 18.9% strains were reported susceptible through MGIT and those were also proven to possess a low-level RIF resistance. Full *rpoB* gene sequencing was found to be a better option as there are limitations in crucial concentration in the methods of partial gene sequencing, probe-based genotyping, and phenotyping. This work provided a complete understanding of *rpoB* type mutations, their prevalence, and their RIF resistance association through concurrent phenotypic testing with universal clinical WGS in the Mtb strains New York (Shea *et al.*, 2020). In a recent study at the Beijing Chest Hospital, 728 patients were diagnosed as rifamycin resistant among 26,826 TB suspects when tested on Xpert MTB/RIF assay. Discordance between genotypic and phenotypic assay was detected among 118 (16.2%) isolates. Among 104 successfully subcultured isolates, 86 (82.7%) harbored *rpoB* mutations and 18 (17.3%) did not harbor any. The Leu511Pro (25.0%) and Leu533Pro (17.3%) mutations were most frequently associated with discordant RIF susceptibility test results. The most prevalent discordant mutation in the study population was Leu511Pro (25.0%) followed by Leu533Pro (17.3%) (Huo *et al.*, 2020).

A retrospective analysis in a recently published study from KwaZulu-Natal south Africa has also highlighted the issue of discordance in RIF and INH between phenotypic and

genotypic methods and its impact on clinical decision making. This study compared 8273 isolates results of phenotypic DST with line probe assay and found an overall discordance of 14.6 % for RIF and 7.2% for INH (Mahomed *et al.*, 2020).

3. Material and Methods

3.1 Sample collection

All the research work and diagnostic tests were performed in the National TB Reference Laboratory, Islamabad. It is a premier laboratory facility in the country which also participates in the annual quality assurance of drug susceptibility testing (DST). This quality assurance is conducted by the Supranational TB Reference Laboratory (Antwerp, Belgium). A total of 36,339 suspected TB and MDR TB patients' samples were received at the National TB reference laboratory during the study period from January 2014 to October 2016. Among total samples cultured 6,148 yielded positive cultures. Out of these, 1986 samples were selected for study for which both MGIT and LJ DST were performed for RIF susceptibility. All the samples received from different Programmatic Management of Drug Resistant TB sites, public sector hospitals of Punjab including Islamabad capital territory (Rawalpindi Leprosy, Samli Sanatorium, Benazir Bhutto Hospital, Holy Family Hospital, Jinnah Hospital, Mayo Hospital, Gulab Devi Hospital, Sheikh Zahid Hospital, Nishtar Hospital, Allied Hospital, Victoria Hospital, District Headquarter Hospital, Military Hospital), and Azad Jammu and Kashmir (Abbas Institute of Medical Sciences) The isolation for Mtb was carried out on liquid and solid media after detection on GeneXpert MTB/ RIF assay. Sample collection was done according to the WHO guidelines from the participants of this study along with the history. The collection of samples and transport was done according to the biosafety guidelines along with their processing in the laboratory for microbiological, phenotypic, and genotypic testing.

3.2 GeneXpert MTB/RIF assay

The Gene Xpert MTB/ RIF is a test based on the amplification of nuclei acid and it is capable of detecting MTBC as well as its resistance to the RIF (mutation of *rpoB* gene), simultaneously. This assay amplifies an 81 base pair core region of the *rpoB* gene. The mutations responsible for RIF resistance as compared to the conserved wild type sequences are differentiated by using the probes present in the cartridge.

During this assay, sputum samples collected from the suspected TB patients were mixed with a reagent that is provided along with the assay kit. The sample cartridge is loaded with this mixture, and it is then placed in the GeneXpert machine according to the manufacturer's protocol (Cephid). A fully automated processing of samples occurred after this point in the machine and results in printable form were downloaded and printed. The GeneXpert MTB/RIF assay indicated whether MTBC is present in the sample and the status of RIF susceptibility. In case of invalid results, the assay was repeated.

3.3 Culture Processing of Samples

Clinical specimens collected were decontaminated using the standard *NALC*-NaOH method. Each specimen was inoculated into LJ medium and MGIT tubes for incubation. Sputum samples processing was done initially done in a centrifuge tube avoiding the risk of cross-contamination, tubes were properly labeled with sample ID. Sputa were processed in sets of more than 10 as it is a strictly time-dependent method.

3.3.1 Procedure

In the Biological Safety Cabinet class (BSC-II), specimens were transferred, and transport bags were discarded. Leakage was checked, in case of leakage, samples were not processed. The sputum sample was transferred using 50 mL sterile centrifuge tubes and the volume of the sputum sample was also checked. Then NaCl-NaOH mixture was added in the same volume, and the screw cap was tightened. If the volume was more than 5 mL, the sample proceeded for decontamination.

As it was decontaminated for 15 min, a countdown timer was used by turning that on with the addition of the first drop of NaCl-NaOH mixture to the first specimen of the specimen set. The specimen was digested and the whole tube was decontaminated by using the vortex. The cap of the tube was also decontaminated by inverting the tube. This tube was then filled with sterile PBS having pH 6.8 up to 50 mL mark after standing it at room temperature for 15 min. Then it was centrifuged at 3000 × g at 10°C for 20 min. An ID was used to label the medium. Centrifuge buckets were brought in the BSC and tubes were placed in the rack. Carefully supernatant was poured off in a discarder containing

5% phenol. The deposit was re-suspended in approximately 1 mL sterile PBS and inoculated on LJ medium (two slopes of LJ medium were used) and labeled with the ID number. Each slope was inoculated with approximately 0.2 mL (4-5 drops) with the help of a pipette. For microscopic examination, a smear was prepared using one drop on the slide and each slide was marked with the respective ID number. The contaminated material was discarded in the plastic bag. The tubes were tilted to spread the inoculum on the surface of the slope. Kept the caps loose by unscrewing them with 1 screw thread. Incubated tubes at 36 ± 1°C. The tubes were kept in a slanted position having a little loose screw cap. These were kept for at least one week to ensure the absorption as well as even distribution of the inoculum. The loosened caps were tightened after one week of incubation to prevent the drying of media by the loss of evaporation. The tubes were kept in an upright position for saving the occupied space in the incubators.

Inoculation of the decontaminated sample was also done on MGIT tubes. To unscrewed MGIT tubes aseptically 800 μL of the antibiotic working mixture was added and later inoculated. Aseptically, using an adjustable pipette, 500 μL of the well-mixed processed specimen was added as inoculum to the appropriately labeled MGIT tube by using a separate sterile barrier filter tip for each specimen. Immediately MGIT tubes were recapped tightly and mixed by inverting the tubes 3-5 times. The tubes' exterior and caps were wiped with disinfectant. The inoculated MGIT-tubes were now placed in the Bactec MGIT 960 instrument for incubation.

3. 3.2 Identification of Mtb using Ziehl Neelsen acid-fast staining

Positive culture tubes were also identified using Ziehl Neelsen acid fast staining and these were then confirmed using the BD MGIT TBc identity kit (MPT64 antigen) (Becton Dickinson, Sparks, MD) for the presence of MTBC.

3.4 Phenotypic DST on solid LJ medium

The DST was performed with slight modification in the recommended method (Canetti *et al.*, 1969; Bates *et al.*, 2013). The proportion method determines the percentage of growth (number of colonies) of a defined inoculum on a drug-free control medium versus growth

on culture media containing the critical concentration of an anti-TB drug. Following critical concentrations were used for performing DST on LJ medium, INH (0.2 µg/mL), RIF (40 µg/mL), EMB (2 µg/mL), streptomycin (4 µg/mL), kanamycin (30 µg/mL), amikacin (30 µg/mL), capreomycin (4 µg/mL) and ofloxacin (4 µg/mL). A pure culture of tubercle bacilli (test strain) in the active phase of growth (not more than 15 days after the 1st colonies appearance/growth) which was validated as a positive culture of MTBC were selected for DST.

Bacterial colonies were scraped from the culture and a loopful was used for preparing a calibrated bacterial suspension. Colonies were suspended in a sterile glass tube. The loop was shaken in the small screw caped glass tube that contains 5-6 small sterile glass beads, each having a 3-5 mm diameter. It was done by shaking the loop in containing approximately 0.1 mL of sterile distilled water (D/W). This was vortexed for 1.0 min and kept for 10 min to avoid excessive droplets when opening the tube. Aseptically, with a sterile Pasteur pipette, distilled water was added in 5.0 mL volume to each of the tubes and was left for 10 min to avoid excessive droplets when opening the tube. Aseptically, 2-3 mL of the supernatant that was already homogeneous was transferred to another tube. The tube was similar in dimensions to the McFarland turbidity standard No. 1. It was done for a visual comparison of the inoculum with the standard. This turbidity was adjusted to match the McFarland turbidity standard No.1. Starting with the calibrated bacterial suspension equivalent to McFarland No.1 which was tenfold diluted.

A bacterial suspension of 100 µL from 10^{-2} was inoculated on media without the drug as control and all drug-containing media tubes. While other growth controls media tube was inoculated on 10^{-4} suspension. The incubation temperature used for the tubes was 36 ± 1°C. The control tubes of media were also inoculated as the isolates in the drug susceptibility were also examined for contamination after 1 week of incubation and then initially lessened caps were closed tightly, for DST interpretation it was further incubated for 4-6 weeks.

The growth that was observed on the media was also compared with the control inoculated with the 10^{-4} dilution. If the growth in the strains grown on media exceeded

the growth of 10^{-4} control present on the control, the strain was concluded resistant to the tested particular drug and vice versa.

3.5 Phenotypic DST on MGIT

The susceptibility testing of Mtb isolates to Streptomycin, INH, RIF, EMB, and Pyrazinamide which are designated as S, I, R, E, and P, was performed on a liquid-based system BACTEC MGIT 960 system (Becton Dickinson Microbiology System, Sparks, NV, USA). The day the MGIT Mycobacteria Growth Indicator tube becomes positive was considered Day 0 by the instrument. These tubes were incubated for at least one more day before carrying out susceptibility testing. The undiluted inoculum was used for inoculation of MGIT DST if the culture was one or two days old. However, if culture was 2 to 5 days old 1:5 dilution was used for SIRE inoculation.

Five MGIT-tubes were labeled for each isolate isolated from the sputum sample of TB patients. One tube during the experiment was labeled as the growth control, one for INH, one for streptomycin, one for EMB, and one for RIF. 800 µL of BACTEC MGIT 960 SIRE Supplement was aseptically added to each MGIT tube, and it was followed by adding 100 µL of the properly reconstituted drug. Similarly, all drugs to be tested were added to correspondingly labeled tubes.

Later 500 µL of inoculum was added aseptically, either the broth of the positive MGIT or its dilution 1:5, depending on the number of days elapsed between the positivity and the test, into each of the tubes containing the drugs. For the growth control, 1:100 dilution of the inoculum was used. MGIT tubes were placed in a SIRE rack in the following order GC, S, I, R, E, and rack after scanning was placed in BACTEC MGIT 960 Instrument for incubation. On the instrument monitors, a susceptibility test set was entered. After the completion of the test that is with 4-13 days, the instrument indicated that the results were ready, the result was printed from the machine. The value of the drug in terms of growth units was evaluated as follows:

S = Susceptible- GU of the drug tubes < than 100.

R = Resistant-GU of the drug tube > than 100.

3.6 Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC) was determined for newly reported mutations as well as some already well-characterized mutations for comparison using the Bactec MGIT 960 system and LJ Media, as described in previous studies (Jamieson *et al.*, 2014b; Farhat *et al.*, 2019). The concentrations of the drug tested for RIF 0.125, 0.25, 0.5, 1, 2, 4, and 8 µg/mL for MGIT and 5, 0 10, 20, 40, 80, and 160 µg/mL for LJ media.

3.7 Selection of Isolates for *rpoB* Sequencing

Out of 1986 samples, 531 isolates were selected for further molecular studies of *rpoB* gene mutations detection, and sequencing was done. Out of these 531 isolates, sequencing of 516 was completed these isolates were from 95 new TB cases, 417 previously treated cases, and 4 cases whose previous treatment history was not known. Of these 516 isolates, 471 were from pulmonary TB patients and 45 from extrapulmonary TB cases. These isolates were processed for PCR based *rpoB* gene amplification and sequencing as follow:

3.7.1 Extraction of genomic DNA from Mtb isolates

CTAB Method was used for DNA extraction using 10 mg/mL lysozyme, CTAB/NaCl buffer (700 mM NaCl, 10% w/v CTAB), SDS (10% in distilled H_2O,), Proteinase K (20 mg/mL), NaCl (5M), Chloroform/Isoamyl alchohol (24:1 v/v), Isopropanol (Molecular biology grade), Ethanol (70% v/v in distilled H_2O, stored at < -20°C) and 1× Tris-EDTA (TE) solution.

A loop full of colonies was picked of Mtb isolate in a 2 ml screw-capped tube. Fifty µL of 10 mg/mL lysozyme was added and mixed gently by stirring with the pipette followed by incubation overnight in a water bath set at 37°C. After overnight incubation, 70 µL of 10% SDS and 5 µL 20 mg/mL proteinase K were added to each sample vial. It was mixed gently by stirring with the pipette tip and then incubated at 65°C for 10 min in a

water bath. After heating,100 µL of 5M NaCl was added to each vial, followed by 100 µL of pre-warmed CTAB/NaCl buffer and mixed gently with the pipette and incubate at 65°C for 10 min.

Vials were spun briefly and relocated to a fume hood where 750 µL of chloroform/isoamyl alcohol (24:1 v/v) was added. Tubes were mixed by inverting them at least 10 times. Samples were removed from the fume hood and vials were spin at 10,000 g for 5 min.

Sterile, nuclease-free 1.5 mL vials were labeled, and 450 µL of ice-cold (-20°C) isopropanol 100% was added. In the fume hood, carefully upper (aqueous) supernatant layers were transferred into nuclease-free 1.5 mL vials with ice-cold (-20°C) isopropanol. The sample was chilled at -20°C for 30 min. Samples were spun at 10,000 g for 15 min at ambient temperature, then the supernatant was removed and discarded, and washed pellets with 1 mL of ice-cold (-20°C) 70% ethanol. Vials were spun at 10,000 g for 5 min at ambient temperature. Ethanol was discarded as much as possible without disturbing the pellets. Pellets were dried by incubating with the lids open, in a heating block set at 65°C, until all the ethanol has evaporated. Pellets were rehydrated in 50 µL TE buffer overnight at 4°C and stored DNA at -20°C.

Another method was also used, where DNA was extracted by heating bacterial colonies at 90°C for 20 min in 100 microliters of water followed by sonication using ELMA E-30H Sonicator (Elma Schmidbauer GmbH) for 15 min. The DNA concentration in each of the samples was estimated by two methods detailed below to ensure the quantity and quality are sufficient for Whole Genome Sequencing.

3.7.2 Estimation of DNA Concentration Using Nanodrop

To measure nucleic acid concentration and quality of DNA extracted, the NanoDrop 2000 spectrophotometer from nanodrop technologies was used by using the 'Nucleic Acid' application module. Selected sample type 'DNA-50' for double-stranded DNA (default. Performed a blank was run before testing DNA samples). For proper working of the instrument, the pedestal was cleaned. 2 µl of molecular grade water was added onto the lower measurement pedestal. The sampling arm was closed and clicked on the 'Blank' button. When the measurement was done, the blanking buffer was wiped from

both pedestals using a laboratory wipe. All sample DNA (2 µL) was added onto the lower measurement pedestal, the 'Measure' button was clicked, repeated for other samples, wiping the pedestals in between samples. The quantity of DNA was noted for each sample.

3.7.3 Estimation of DNA Concentration Using the Qubit Fluorometer

For accurate DNA quantification, Qubit dsDNA assay kits were used Qubit 2.0 fluorometer by Invitrogen. This kit was used as it is highly selective for the determination of the accurate concentration of dsDNA. For each sample, 0.5 mL tubes were required, and standards (two tubes) were labeled. The working solution of Qubit® was prepared by the dilution of Qubit® dsDNA HS Reagent 1:200 in the Qubit® dsDNA HS Buffer using a clean plastic tube. 200 µL of the final volume was required in each tube. Then a working solution of the Qubit®, 190 µL was added in each standard tube which was followed by the addition of 10 µL Qubit® standard to the appropriate tubes. It was then mixed using vortex for 2-3 seconds. Using pipette 199 µL Qubit® working solution and 1.0 µL sample were added to individual sample tubes so that the final volume including the sample was 200 µL was made. Each sample was added to appropriate assay tubes. Then it was vortexed for 2-3 seconds using a vortex. These tubes were incubated for 2 min at room temperature. These samples were then ready for taking the reading using a fluorometer. DNA option was then pressed for taking a reading on the Qubit® Fluorometer. A dsDNA high sensitivity option was selected on the instrument in the assay type. Standards were read. A tube that contained standard # 1 was inserted into the sample chamber, the lid was closed to take the reading. After completion of reading the samples, the first standard was removed and inserted standard # 2 into the sample chamber, and pressed read again. Then the same procedure was repeated after inserting the sample tube into the sample chamber. The tube was removed after completing the reading. Results were displayed on the screen after the completion of the reading. The concentration of the assay tube was displayed in the first value. The concentration of the original sample was calculated by the stock concentration option. The selected sample volume was added to the assay tube and then the Qubit® Fluorometer calculated the

concentration of the original sample through the volume as well as the measured assay concentration. In the final step, the concentration of DNA was noted.

3.7.4 Polymerase Chain Reaction for amplification of *rpoB* gene

PCR was done using 25 µL of Qiagen HotStarTaq Master Mix containing 2.5 units HotStarTaq DNA Polymerase1 x PCR Buffer (Contains 1.5 mM $MgCl_2$) and 200 µM of each dNTP, 2 µL of each forward and reverse 10 micromolar primers, 8 microliters of DNA and 5 microliters of water with a total volume of 50 microliters in a Gene Amp PCR system 9700 (Applied Biosystem). Following cycling conditions were used; 15 min hot start, followed by 45 cycles at 94°C for 45 seconds, annealing and extension for I min 30 sec at 72°C; and a final amplification at 72°C for 10 min. Primers *rpoB*gene SA (5-GGTTCGCCGCGCTGGCGCGAAT-3) and *rpoB* gene RB (5-GACCTCCTCGATGACGCCGCTTTCT-3) were used for amplification of the 1764 bp region of the *rpoB* gene (Rigouts *et al.*, 2007). PCR amplicons were analyzed on 2% agarose gel. PCR product was purified using Qiagen purification kit as described by the manufacturer and the final volume was eluted in 30 µL of elution buffer and stored at -20°C.

3.7.5 Sequencing of *rpoB* gene

For Sanger sequencing, PCR was done using the following primers; forward 5'-GGGAGCGGATGACCACCCA-3'; and reverse, 5'-GCGGTACGGCGTYTTCGATGAAC-3' (Kapur *et al.*,1994) using BigDye™ Terminator v3.1 Cycle Sequencing Kit. PCR was done using 2 µL of the ready reaction mix, 3 µl of sequencing buffer, 3 µL of forward or reverse primer,8 µL of water, and 5 µL of the purified PCR product with a total volume of 20 microliters in a Gene Amp PCR systems 9700 (Applied Biosystem). Following cycling conditions were used; 1 min at 96°C, followed by 25 cycles at 96°C for 10 seconds, 50°C for 50 seconds and 4 min at 60°C.

An addition of 3 µL 3 mM NaCl at 4.6 pH resulted in the precipitation of 19 µL cycle product, along with 95% ethanol (62.5 µL) as well as 14.5 µL water. This mixture was

mixed using vortex and then it was centrifuged at 15000 × g at 4°C for 15 min. Then the supernatant was removed by keeping the pellet intact. 70% ethanol was added in 200 µL volume, followed by the inversion of the tube to wash the pellet. Then it centrifuged at 15000 × g at 4°C for 15 min. The supernatant was removed, and the pellet was completely dried. 15 µL of formamide was then added to each tube, and then it was loaded onto the automated plate sequencer (Applied Biosystems, Warrington, UK). For Analysis of chromatogram generated on sequencer Molecular Evolutionary Genetics Analysis Version 7 (MEGA 7) software was used in comparison to H37Rv *rpoB* gene sequence Rv0667.

3.8 Whole genome sequencing

After sequencing and analysis of the *rpoB* gene, twenty-five samples were processed for whole genome sequencing on the Illumina platform at University College London from DNA extracted using the CTAB method. These strains were selected based on genotypic and phenotypic discordance as well as confirmation of newly identified mutations in the study. WGS data was analysed using the phyResSE for analysis of *rpoB, katG, inhA, gyrA, gyrB, rrs, embABC* and *rv0678* (Feuerriegel *et al.*, 2015). Mtb strain H37Rv genome sequence (GenBank accession number, NC_000962.2) was used as a reference sequence for analysis, and WGS were deposited in NCBI.

3.9 Phylogenetic analysis

PhyResSE web tool was also used for phylogenetic lineage classification of Mtb strains for which WGS was performed. For cluster analysis minimum spanning tree was constructed by using seqspher + (ridom, Germany).

3.10 Protein structure prediction

Effect of 25 identified mutations/SNPs in *rpoB* gene on RNA polymerase structure prediction by using DynaMut web server (Rodrigues, Pires and Ascher, 2018). The server implements normal mode methods and mutation effect prediction that can be used for the analysis of the effect upon stability of proteins as well as flexibility occurring behind the vibrational entropy changes. The prediction of the mutation effect is done through graph-

based signatures as well as normal mode dynamics. The results are displayed in good resolution through this approach, and it outperforms (*p*-value < 0.001).

3.11 Statistical Analysis

For statistical analysis, the WHO EpiData analysis software (Singh, 2009) and SPSS were used. The EpiData analysis is a user-friendly software, which uses all kinds of analysis that include the cross column analysis as well plotting.

4. Results

Out of all sputum samples received, a total of 1986 culture positive isolates were processed for drugs susceptibility testing, which was performed at National TB Reference Laboratory, National of TB Control Program Pakistan from January 2014 to October 2016. These samples were received from different programmatic management of drug resistant TB sites and public sector hospitals of Punjab, Islamabad capital territory, and Azad Jammu and Kashmir located. All sputum samples were processed for DST using liquid media (BACTEC MGIT 960 system) and Egg-based solid LJ media. Only culture positive samples identified on Ziehl Neelsen acid fast staining and further confirmed using the BD MGIT TBc kit were processed for drug susceptibility testing in MGIT 960 system and LJ medium.

4.1 Gender wise distribution of study population

Out of the total study samples, 950 (48%) were from females and 1036 (52%) were from male TB patients as shown in Figure 4.1.

4.2 Age wise distribution of study population

Culture positive patients were grouped into seven age categories. The majority of the study population lay between the age group of 15 to 44 years which accounted for 72.2% of the total cases. The highest number of TB patients included in the study were found in the age range of 15- 24 years 654 (32.9%) while the lowest number was present in the age group above 65 years, which were 78 (3.9%) patients as shown in Figure 4.2.

4.3 Treatment history-based distribution of study population

Among the total TB patients in the study, 1657 (84%) patients had a previous history of TB, while 241 (12%) were newly diagnosed with TB but had yet to receive their first anti-tuberculosis treatment and 88 (4%) patients had unknown TB treatment history (Figure 4.3).

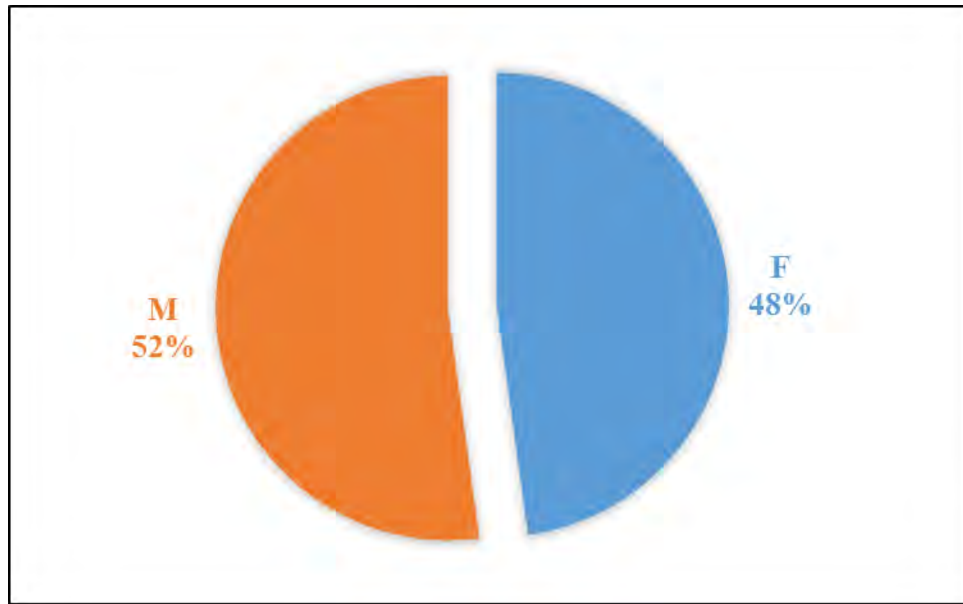


Figure 4.1: Gender wise distribution of study population from 2014-2016

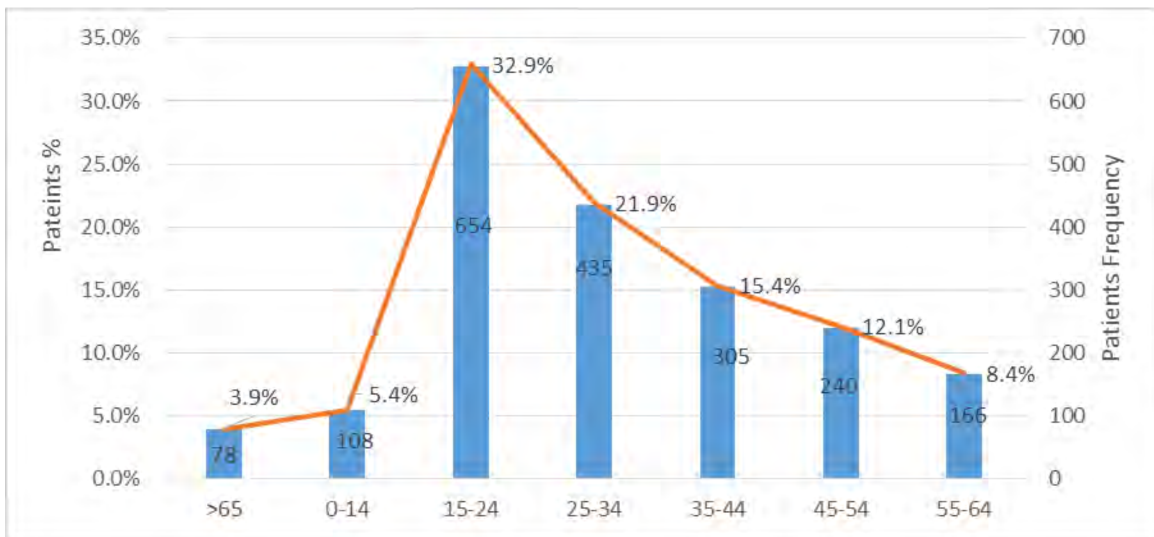


Figure 4.2: Age wise (years) distribution of study population from 2014-2016

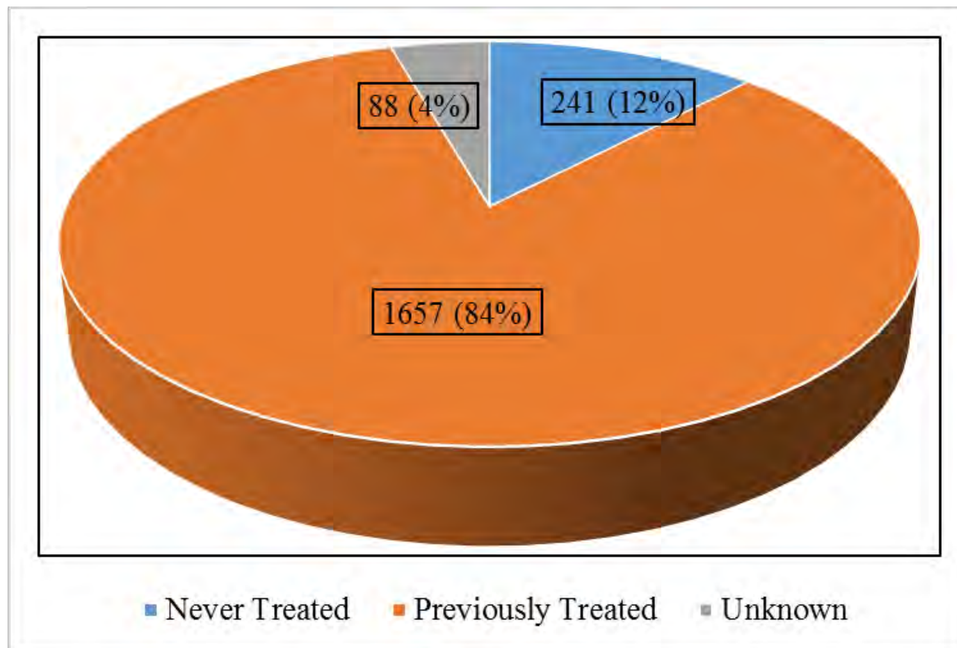


Figure 4.3: Treatment history-based distribution of study population from 2014-2016

4.4 Type of Tuberculosis based distribution of study population

Out of the total TB patients in the study, 1876 (94%) were enrolled for the diagnosis of pulmonary TB, whereas 110 (6%) were registered for the diagnosis of extra pulmonary TB as shown in Figure 4.4.

4.5 Rifampicin Susceptibility Testing result on GeneXpert MTB/RIF assay

Among 1986 TB patients in the study, rifampicin resistance was detected in 1644 (83%) while 342 (17%) were sensitive to RIF on GeneXpert MTB/RIF assay (Figure 4.5).

4.6 Rifampicin Drug Susceptibility Testing results on Lowenstein Jensen Media

Among 1986 TB patients in the study, 1551 (78%) were RIF resistant while 435 (22%) were characterized as RIF susceptible on DST performed on LJ medium as shown in Figure 4.6.

4.7 Rifampicin Drug Susceptibility Testing results on Liquid culture MGIT

Among 1986 TB patients in the study, 1463 (74%) were RIF resistant while 523 (26%) were characterized as RIF susceptible on liquid culture medium on MGIT 960 in their DST as shown in Figure 4.7.

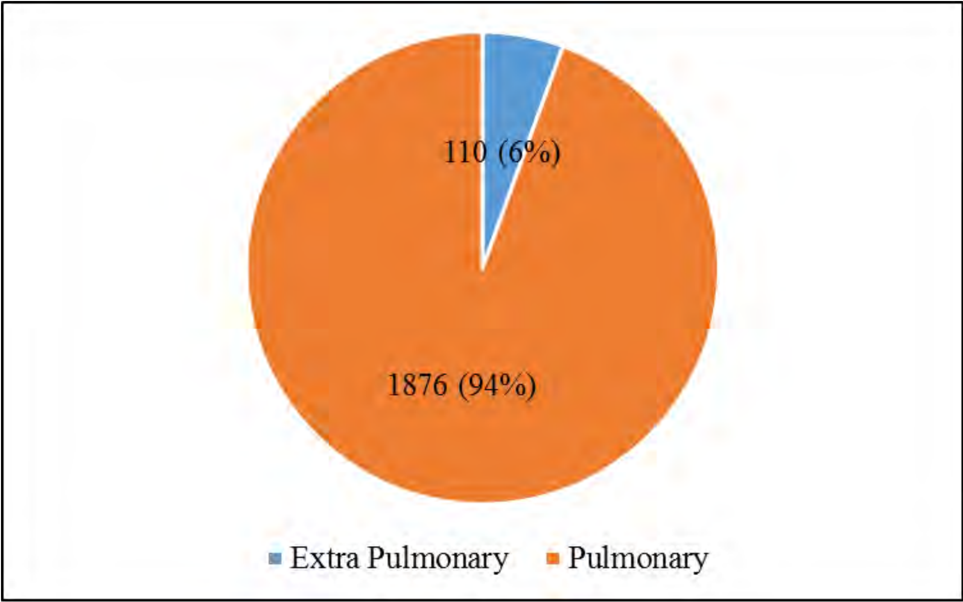


Figure 4.4: Type of tuberculosis disease-based distribution of study population

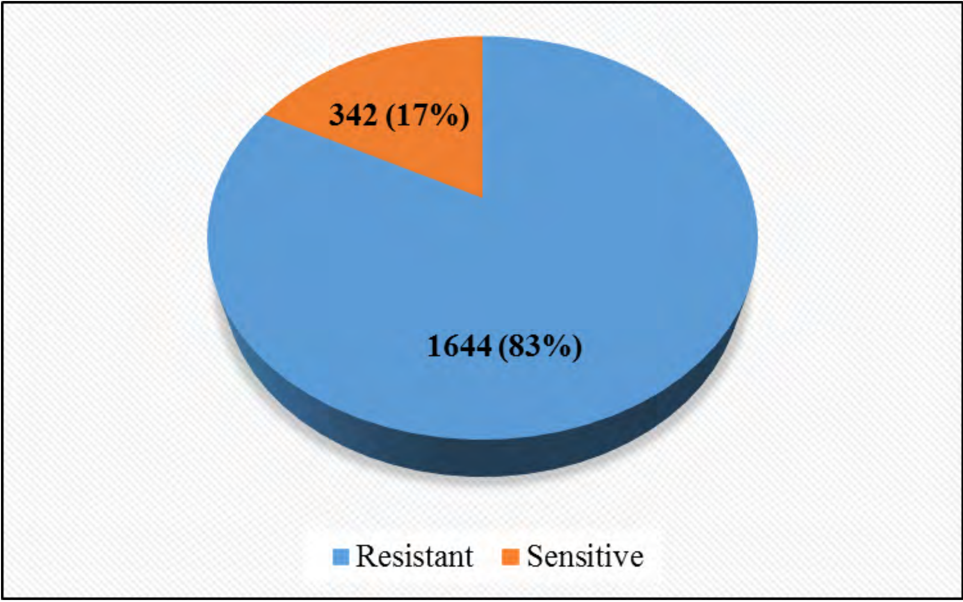


Figure 4.5: Rifampicin Drug Susceptibility Testing results on Gene Xpert MTB/RIF assay

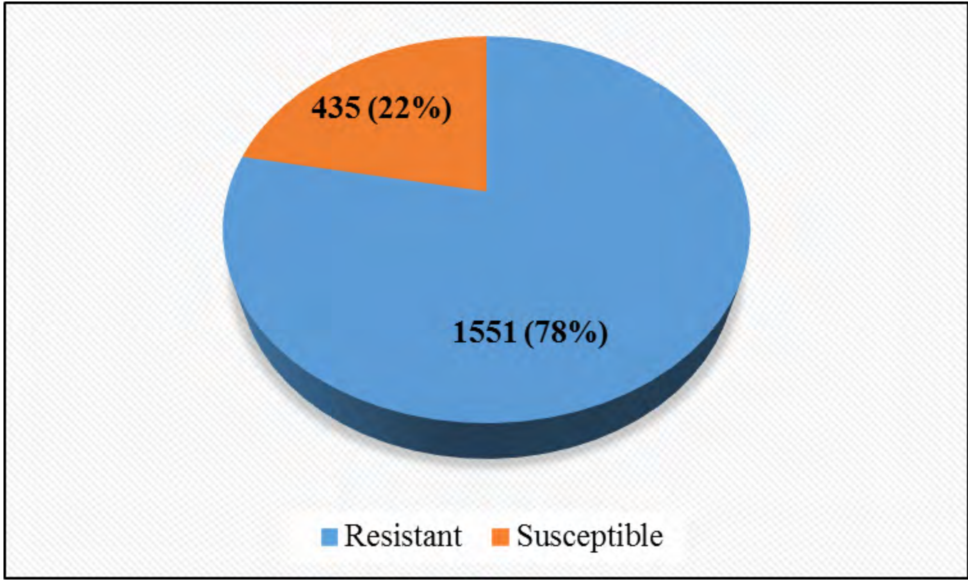


Figure 4.6: Rifampicin Drug Susceptibility Testing results on LJ Medium

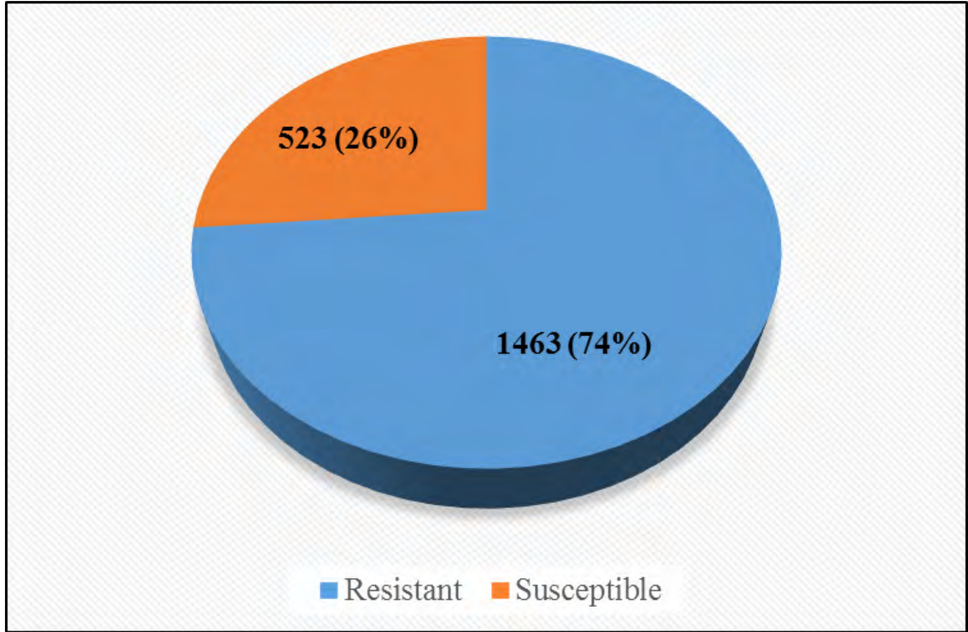


Figure 4.7: Rifampicin Drug Susceptibility Testing results on Liquid culture

4.8 Comparison of Phenotypic DST Method Lowenstein Jensen and BACTEC MGIT 960 system results with GeneXpert

All 1986 cases were tested on the three methods, *i.e.*, GeneXpert, MGIT, and LJ for detection of rifampicin resistance. As both LJ and MGIT are phenotypic methods and GeneXpert is a genotypic method, a comparison of the LJ and MGIT method was done while taking GeneXpert as the standard for comparison purposes. Among 1986, there was only 1442 resistant, and 318 cases were sensitive on all the three methods thus a total of 1760 samples were having concordant results. At the same time, 226 results were discordant, hence there were 226 occults strains.

4.8.1 Comparison of GeneXpert RIF resistant strains for their Rifampicin susceptibility results on both phenotypic methods Lowenstein Jensen media and BACTEC MGIT 960 system

Among the total 1986 cases, 1644 were resistant to GeneXpert. Out of 1644 rifampicin-resistant on GeneXpert, 1442 cases were resistant by all the three methods while in 202 cases discordance with GeneXpert results was observed. Among these 202 cases, which were resistant by GeneXpert, 117 were found to be sensitive by both LJ and MGIT DST while 85 cases were seen to be sensitive on MGIT DST only but were resistant on LJ DST (Table 4.1). Thus, LJ missed 117 resistant cases while MGIT missed 202 cases in total which were resistant by GeneXpert (Table 4.1).

4.8.2 Comparison of GeneXpert RIF sensitive strains for their Rifampicin susceptibility results on both phenotypic methods Lowenstein Jensen media and BACTEC MGIT 960 system

Among the total 1986 isolates in this study, 342 were found sensitive GeneXpert. Out of these 318 sensitive cases, all samples were sensitive on all three methods except for 24 cases that were not detected resistant by GeneXpert but were resistant by DST on LJ and MGIT as shown in Table 4.2. Among these 24 sensitive by GeneXpert cases, 21 were found resistant by both LJ and MGIT while 3 cases were seen to be sensitive on MGIT but resistant on LJ DST. Thus, GeneXpert missed 21 cases that were given resistant by

both LJ and MGIT method while 3 cases were given resistant by LJ which were missed by both MGIT and GeneXpert system (Table 4.2).

Table 4.1: Comparison of GeneXpert RIF resistant strains for their Rifampicin susceptibility results of LJ and MGIT

Rifampicin Resistant on GX		**LJ DST**		**Total**
		R	S	
MGIT DST	R	1442	0	1442
	S	85	117	202
Total		1527	117	1644

Table 4.2: Comparison of GeneXpert RIF sensitive strains for their rifampicin susceptibility results on LJ and MGIT

Rifampicin Sensitive on GX		**LJ DST**		**Total**
		R	S	
MGIT DST	R	21	0	21
	S	3	318	321
Total		24	318	342

4.8.3 Comparison of Rifampicin Susceptibility Results on GeneXpert, Lowenstein Jensen Media and MGIT with rpoB Gene Sequencing

A comparison was done on 1986 patients' samples, which were tested for rifampicin susceptibility on GeneXpert, LJ, and MGIT DST. Among these 1644 were resistant to GeneXpert and 342 were GeneXpert sensitive to rifampicin. In 1644 GeneXpert resistant samples, 1527 and 1442 samples were resistant on rifampicin DST on LJ and MGIT, respectively from which *rpoB* gene sequencing was performed on 415 isolates. Among 342 rifampicin sensitive isolates on GeneXpert, 24 were resistant on LJ and 21 were resistant on MGIT DST, while 318 and 321 were susceptible on LJ and MGIT DST, respectively. *rpoB* gene sequencing was performed on these 101 samples as shown in Table 4.3.

4.9 Phenotypic Drugs Susceptibility Pattern of TB Isolates Against Isoniazid, Rifampicin, Kanamycin and Ofloxacin with Previous Treatment History

Phenotypic drugs susceptibility testing was performed for all 1986 Mtb isolates for Isoniazid (INH), rifampicin (RIF), kanamycin (KAN), and ofloxacin (OFX). As shown in Table 4.4, DST results showed different susceptibility patterns in study isolates. Out of total 1986, 14.7% were susceptible to isoniazid, rifampicin, kanamycin, and ofloxacin (HRKO) while 85.3% were monoresistance to at least one of these drugs. The most prevalent resistance pattern was a combination of isoniazid, rifampicin, and ofloxacin (HRO) in 36.7% isolates followed by a resistance pattern of a combination of isoniazid and rifampicin (HR). Among these isolates, nonresistance to isoniazid was present in the highest number of isolates, however, no isolate was mono resistant to the second line of injectable kanamycin. In ninety isolates, susceptibility pattern showed resistance to all these four drugs HRKO, which classify these isolates as XDR TB which make up 5.4% of studied Mtb. Among all isolates, 745 were characterized as pre-XDR as they were resistant to rifampicin and isoniazid along with resistance fluoroquinolone or second-line injectable in the study population during the study period.

Table 4.3: Comparison of Rifampicin Susceptibility Results on GeneXpert, LJ and MGIT DST with *rpoB* Sequencing

GeneXpert RIF Result	Total	LJ-RIF Results		MGIT-RIF Results		*rpoB* sequencing
		R	S	R	S	
R	1644	1527	117	1442	202	415
S	342	24	318	21	321	101
Total	1986	1551	435	1463	523	516

Table 4.4: Phenotypic Drugs Susceptibility Pattern against isoniazid, rifampicin, kanamycin and ofloxacin of Mtb isolates with previous treatment history

Resistance pattern	History of previous anti TB treatment			
	Never Treated n = 241	**Previously Treated** n = 1657	**Unknown** n = 88	**Total (%)** n = 1986
INH, RIF, KAN, OFX-Susceptible	114	170	8	292 (14.7)
INH	23	43	4	70 (3.5)
RIF	2	31	4	37 (1.8)
OFX	12	32	0	44 (2.2)
INH, OFX	4	20	0	24 (1.2)
INH, RIF	30	610	29	669 (33.6)
INH, RIF, KAN,	2	10	0	12 (0.6)
INH, RIF, KAN, OFX	5	82	3	90 (4.5)
INH, RIF, OFX	48	641	39	728(36.7)
INH, KAN, OFX	0	5	0	5 (0.3)
RIF, OFX	1	10	1	12 (0.6)
RIF, KAN	0	2	0	2 (0.1)
RIF, KAN, OFX	0	1	0	1 (0.05)

4.10 Identification of *rpoB* Gene Mutation using sanger sequencing

Among the total 516 strains sequenced, upon analysis for mutations/SNP, 77% (396/516) strains showed mutations in the *rpoB* gene whereas 23% (120/516) strains had no mutation. Among these 396-mutation positive resistant strains, 368 were identified with a SNP which resulted in codon changes, hence these were predicted to have either an insertion or deletion of amino acid as shown in Table 4.5, while in 28 isolates, there were two more than two codon deletion/insertion with change in amino acids (Table 4.6). Nonsynonymous mutations due to mono or polymutations were found due to deletion, insertion, and substitution of nucleotides.

The most frequent mutations which were 58%, 14%, and 11% were detected at codon 450, 445, and 435 of the *rpoB* gene, respectively. The highly prevalent mutation was substitution where Serine → Leucine due to TCG → TTG at codon 450 followed by Histidine → Asparagine amino acid substitution due to change of CAC→AAC at codon 445 (Table 4.5). Other mutations with their corresponding codon changes are listed in Table 4.5. A high number of mutations (362) were detected in the 81-bp core region of the *rpoB* gene, whereas 6 mutations were detected outside the core region including one mutation at position 170 and 5 mutations at codon 491 respectively. Out of the 28-poly mutation listed in Table 4.6, the most common poly mutation was found at codon 434 which was in combination with either Asp435Val or His445Asn.

Table 4.5: Single mutations detected in *rpoB* gene of various *M. tuberculosis* isolates

Codon No.	Mutated Codon	Amino acid change	Total	Percentage
170	GTC→TTC	Val170Phe	1	0.27
428	AGC→ATC	Ser428Ile	1	0.27
430	CTG→CCG	Leu430Pro	20	5.43
	CTG→CGG	Leu430Arg	2	0.54
431	AGC→GGC	Ser431Gly	1	0.27
432	CAA→AAA	Gln432Lys	1	0.27
	CAA→CCA	Gln432Pro	1	0.27
	CAA→CTA	Gln432Leu	1	0.27
	Ins CGC →1294	Arg at 432	1	0.27
433	Ins of TTC →1297	Phe at 433	3	0.82
434	ATG→ATA	Met434Ile	1	0.27
435	GAC→TAC	Asp435Tyr	21	5.71
	GAC→GTC	Asp435Val	19	5.16
	GAC→GGC	Asp435Gly	1	0.27
436	AGAA→A AT	Gln436His	1	0.27
438	DEL		1	0.27
	AAC→AAG	Asn438Lys	1	0.27
444	ACC→GCC	Tyr444Ala	1	0.27
445	CAC→CTC	His445Lys	9	2.45
	CAC→GAC	His445Asp	8	2.17
	CAC→TAC	His445Lys	8	2.17
	CAC→TGC	His445Cys	6	1.63
	CAC→CGC	His445Arg	1	0.27
	CAC→GGC	His445Gly	1	0.27
	CAC→AAC	His445Asn	18	4.89
450	TCG→TTG	Ser450Leu	199	54.08
	TCG→TGG	Ser450Trp	15	4.08
452	CTG→CCG	Leu452Pro	20	5.43
491	ATC→TTC	Ile491Phe	5	1.36

Table 4.6: Double and triple mutations detected in *rpoB* gene of various *M. tuberculosis* isolates

Amino acid change	427(del)	Leu430Arg	Asp435Val	437	His445Asn	Lys446Gln	Ser450Trp	Ala451Val	Leu452Pro	Total
Ser428Ile		1	1		1*					3
Gln429His					1					1
Leu430Arg			3		1					4
Gln432Lys					1					1
Met434Ile			5**		3					8
Asp435Val	1***			1				2		4
His445Asn						1	2		2	5
Total	**1**	**1**	**9**	**1**	**7**	**1**	**2**	**2**	**2**	**26**

* + Leu452Pro ** +Ala451Val ***+Ile491Leu
Two deletions were at positions 427-430 and 435-439 not mentioned in Table 4.6.

4.11 Previous Anti TB Treatment History of Sequenced Mtb Strains

Among these 516 isolates sequenced for the *rpoB* gene, 417 patients were previously treated and 95 were newly treated while 4 patients who had an unknown history of treatment were either new cases or previously treated (Figure 4.8). In the previously treated category, 292 cases were previously treated with category I regimen while 110 were treated with category II regimen and 15 were unknown with treatment regimen category. In patients treated with category, I regimen 164 had failure outcome, while 102 were cured and 17 lost to follow up and 9 were with unknown treatment outcome. In patients treated with category II regimen 73 had failure outcomes, while 21 were cured and 8 were lost to follow up while 8 had unknown treatment outcomes.

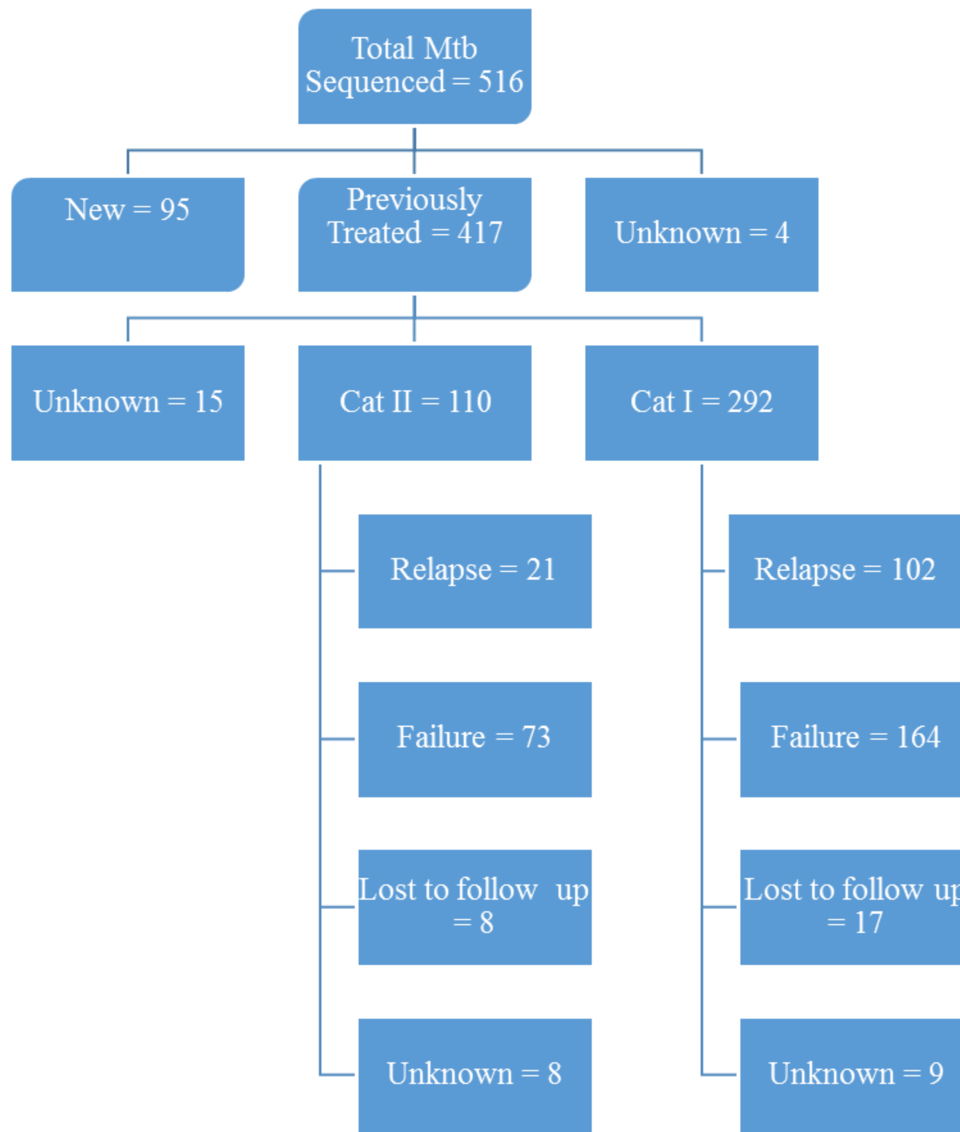


Figure 4.8: Distribution of sequenced *M. tuberculosis* isolates and previous TB treatment outcomes of TB patients

4.12 Predication of Change in Amino Acid due to Mutations and Single Nucleotide Polymorphism

The previous treatment outcome of the patient was compared with amino acid change predicted due to mutations/SNPs, which were detected at least twice in the 516 sequenced strains, were 364 (Table 4.7). Out of these 364 mutations detected, 198 were present in strains isolated from treatment failure cases, 94 were from relapse cases, 19 from patients lost to follow-up cases while 53 were detected in isolates from patients with unknown previous treatment history. Mutants whether it was concordant or discordant on phenotypic DST, were mostly from the treatment failure category and then from the relapse category.

4.13 Novel mutations detected in *rpoB* gene among RIF resistant Mtb isolates

In the RRDR region of one isolate, the mutation detected was due to the insertion at codon (CGC1294) at position 432 of *rpoB*, it was predicted to change in amino acid to arginine (Arg). In another isolate, deletion of GAA codon at position 1308-10 resulted in the substitution of Gln436His and deletion of amino acid asparagine (Asn) at codon 437. Another mutation was detected in another isolate due to deletion of sequence CACCAGCCAGCTG/C at position 1281-1294 resulting in deletion of 4 amino acids threonine (Thr), serine (Ser), glutamine (Glu), and leucine (Leu). Phenotypic genotypic results, MICs, genotypes, DST pattern, and GenBank accession numbers of newly reported mutations are summarized in Table 4.8.

Table 4.7: Predicated amino acid change identified in Mtb strains isolated from TB patients with previous treatment history

***rpoB* amino acid Change**	**Previous treatment outcome**				**Total**
	Failure	Relapse	Lost to follow-up	Unknown	
Ser450Leu	109	52	12	26	199
Asp435Tyr	11	5		5	21
Leu430Pro	5	7		8	20
Leu452Pro	14	4		2	20
Asp435Val	9	9		1	19
His445Asn	8	5	2	2	17
Ser450Trp	10	1	3	1	15
His445Leu	8	1			9
His445Asp	5	2		1	8
His445Tyr	7	1			8
His445Cys	2	2		2	6
Ile491Phe	2	1		2	5
Met434Val, His445Asn	2		2	1	5
Ins of Phe at 434	3				3
Leu430Arg, Asp435Tyr	1	2			3
His445Asn, Ser450Trp	1	1			2
Leu430Arg	1	1			2
Met434ILe, His445Asn				2	2
Total	198	94	19	53	364

Table 4.8: Novel mutations detected in *rpoB* among RIF resistant Mtb isolates

#Codon change	Amino acid change Mtb Numbering	Amino acid change *E. coli* Numbering	GeneXpert Result	LJ MIC in µg /mL	MGIT MIC in µg /ml	Type of mutation	Genotype	*Res-Type	Gene Bank accession numbers
Ins of cgc at 1294	Insertion of Arg at 432	Arg513ins (inframe)	R	>80	2	Insertion	Delhi⁄CAS	MDR	MT411895
Caccagccagctg/ C from 1279-1290	deletion of AA TSQL at 427-30	Del Tyr508-Ser509-Gln510-Leu511 (inframe)	R	>80	4	Deletion	Euro-American Super Linage	RIF-R	MT411896
CAGAAC/CAC at 1308-10	Gln436His + Asn437del	Gln517His+ Asn518del	R	80	0.5	Substitution+ deletion	Delhi⁄CAS	PRE-XDR	MT411897

*Type of resistance. MDR; Multidrug resistance, XDR; Extensively drug resistance, #Codon change in *rpoB* gene. GX; GeneXpert, LJ; Lowenstein Jensen media

4.14 Categorization of GeneXpert, LJ, and MGIT Drug Susceptibility Testing in Comparison to Sequencing

Among 516 Sanger sequenced isolates for the *rpoB* gene, there was concordance among GeneXpert, LJ, and MGIT DST with sequencing in 396 isolates while discordance in 140 isolates. Among discordance results in 52 isolates, GeneXpert, LJ DST and sequencing detected rifampicin resistance while rifampicin resistance was missed on MGIT DST. In 42 isolates, GeneXpert and sequencing detected rifampicin resistance while rifampicin resistance was missed on LJ and MGIT DST. In 8 isolates LJ, MGIT, and sequencing detected rifampicin resistance while rifampicin resistance was missed on GeneXpert. Similarly in 32 samples initially, resistance to rifampicin was detected, however, no resistance was detected upon sequencing, LJ, and MGIT DST. In 5 isolates, in which mutation was detected outside RRDR in sequencing, all were resistant on LJ DST, however, 1 was sensitive on MGIT DST.

4.14.1 Comparison of Rifampicin Susceptibility of results on GeneXpert with rpoB gene sequencing result

GeneXpert MTB/RIF assay results were compared with sequencing results of 516 *rpoB* sequenced strains for the detection of mutations. In 13 (3.2%) strains, mutations in *the rpoB* gene were detected, hence, were classified as resistant upon *rpoB* sequencing, however, these strains on GeneXpert were not detected as resistant isolates for rifampicin. Similarly, 32 (7.7%) strains had no mutation in the *rpoB* gene, hence were wild-type but were found resistant on GeneXpert as shown in Table 4.9.

4.14.2 Comparison of Rifampicin Susceptibility results of MGIT with rpoB gene sequencing results

MGIT DST results were analyzed with gene sequencing results, mutations were found in 94 (23.7%) strains in the *rpoB* gene, hence were classified as resistant, however, these isolates were RIF sensitive on MGIT DST. Similarly, 1 (0.3%) strain which was resistant

upon MGIT DST, was found to be wild type on *rpoB* sequencing as no mutation was detected in it (Table 4.10).

4.14.3 Comparison of Rifampicin Susceptibility results of LJ with rpoB gene sequencing results

Lowenstein Jensen DST results with sequencing results were compared in 516 sequenced strains. In *rpoB* sequencing, 42 (10.6%) strains were detected with a mutation in the *rpoB* gene and were classified as resistant, but these were found to be sensitive on LJ DST. Similarly, 1 (0.3%) strain which was classified by LJ DST as resistant was wild type on *rpoB* sequencing (Table 4.11).

Table 4.9: Comparison of Rifampicin susceptibility results on GeneXpert with *rpoB* gene sequencing results

***rpoB* gene sequencing results**	**GeneXpert rifampicin result**				
	Resistant	Sensitive	*Total*	X^2	*p-value*
Any Mutation identified	383	13	396	287.1	<0.00001
Wild Type (no mutation)	32	88	120		
Total	415	101	516		

X^2 = Chi square, p-value (<0.05) = significant

Table 4.10: Comparison of Rifampicin Susceptibility results of MGIT with *rpoB* gene sequencing results

***rpoB* gene sequencing results**	**MGIT rifampicin DST result**				
	Resistant	Sensitive	Total	X^2	*p-value*
Any Mutation identified	302	94	396	212.7	<0.00001
Wild Type (no mutation)	1	119	120		
Total	301	215	516		

X^2 = Chi square, p-value (<0.05) = significant

Table 4.11: Comparison of Rifampicin Susceptibility results of LJ with *rpoB* gene sequencing result

***rpoB* gene sequencing results**	**LJ rifampicin DST result**				
	Resistant	Sensitive	Total	X^2	*p-value*
Any mutation identified	354	42	396	336.5	<0.00001
Wild Type (no mutation)	1	119	120		
Total	415	101	516		

X^2 = Chi square, p-value (<0.05) = significant

4.14.4 Rifampicin concordance and discordance in *M. tuberculosis* isolates for LJ and MGIT DST sensitivity after stratification based on *rpoB* mutation type

Sanger sequencing of *rpoB* was performed on 516 strains, which included 140 discordant and 376 concordant strains. Sequencing results are presented in Table 4.8 based on the Mtb and *Escherichia coli rpoB* codon numbering system. The sensitivity was calculated (at 95% confidence interval) for each mutation identified in this study to check the level of sensitivity on LJ and MGIT DST (Table 4.1). These results were compared with Miatto Paolo's comprehensive review as a reference standard as it is now part of the WHO guideline "The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical guide" (Miotto *et al*. 2017). Among mutations identified in 396 strains in *rpoB* sequencing analysis, overall, 42/396 strains were identified as susceptible in LJ DST, while 94/396 strains were identified as susceptible in MGIT DST.

Mutation at position S450L was detected in the highest number (199) of the total isolates and fully concordant results were observed between phenotypic DST on LJ and MGIT with sequencing. Similarly, fully concordant results were detected for mutation Gln435Val (19/19), Ser450Trp (15/15), His445Gln (8/8), His445Lys (8/8), and insertion of Phe at position 433 (3/3). For mutation Ile491Phe all (5/5) isolates were resistant on LJ DST, however, one strain was susceptible on MGIT DST. In the case of His445Leu mutation, all strains (9/9) were detected as resistant on LJ DST, however, only one isolate (1/9) was resistant on MGIT DST. Mutations detected in isolates at position His445Cys were detected as resistant in LJ (5/6) and MGIT DST (4/6) in most of the strains, however, only 1/6 on LJ and 2/6 on MGIT DST were found to be susceptible. In the case of mutation Asp435Lys, 16/21 isolates on LJ and 4/21 isolates on MGIT were detected as resistant, however, 5/21 on LJ and 17/21 on MGIT DST were seen to be susceptible. In the case of mutation Leu452Pro, 15/20 isolates on LJ and 3/20 isolates on MGIT were found as resistant, however, 5/20 on LJ and 17/20 on MGIT DST were susceptible. In case of mutation, Leu430Pro 6/20 isolates on LJ and 1/20 isolates on MGIT were detected as resistant, however, 14 on LJ and 19 on MGIT DST were phenotypically

susceptible. In case of mutation His445, 7/18 isolates on LJ were resistant as well as 3/18 isolates on MGIT, however, 11/18 on LJ and 15/18 on MGIT DST were susceptible. Two strains with a mutation at Leu430Arg were identified, which were susceptible to LJ and MGIT DST. Insertion of F at codon 433 was observed in three isolates, which were rifampicin-resistant on LJ and MGIT DST.

The double mutations detected at codon 434 and 445 (Met434Val + His445Asn) were present in all five isolates resistant on LJ but in 4/5 isolates on MGIT, however, only 1/5 isolate on MGIT DST was detected as susceptible. In the case of double mutations detected at Leu430Arg + Asp435Lys, all three isolates on LJ and 2/3 isolates on MGIT were found to be resistant, however, one on MGIT DST was detected as susceptible. In the case of double mutations detected at codon His445Asn + Ser450Trp in 2 isolates, both isolates were resistant to LJ and MGIT. In case of deletion of five amino acids, Asp-Gln-Asn-Asn-Pro from codon 435-439 and deletion of four amino acid Tye-Ser-Gln-Leu from 427-430, deletion of single amino acid Asn at 438, all isolates with this deletion were found to be resistant on LJ and MGIT DST. Four SNPs (Ser431Gly, Asp435Gly, Asn438Tyr, and Ser431Gly) were detected in one isolate each; these isolates were identified phenotypically susceptible in both LJ and MGIT DST.

SNP detected at Ser428I and His445Gly, double nucleotide polymorphism detected at Ser428Arg + Leu430Pro, Met434Leu + His445Asn, and Asp435Gly + Ala451Val, and triple nucleotide polymorphism detected in Met434I + Asp435Gly + Ala451 in single isolates were detected as resistant on LJ while susceptible on MGIT DST. In another isolate detected with deletion of Gly-Ala-Ala codon at position 1308-10, which resulted in substitution GlnAsn436His and deletion of amino acid asparagine at codon 437 was resistant on LJ while susceptible on MGIT DST. Insertion of R at 432 and SNP in Val170Phe, Gln432Pro, Gln432Leu, Gln432Tyr, Met434Ile, and His445Asp were each detected in a single isolate and were found as resistant on both LJ and MGIT DST.

Double nucleotide polymorphism detected at Ser428Thr + Asp435Gly, Ser428Asp + His445Lys, Gln429His + His445Arg, Leu430Pro + His445Asn, Gln432Lys + His445Asp, His445Asn + Leu452Gln and His445Pro + Lys446Gln. Two triple

nucleotide polymorphisms were detected in Thr427Ile + Asp435Tyr + I491Leu and Ser428Gly + His445Asn + Leu452Pro, all these isolates were phenotypically resistant on both LJ and MGIT DST. The rest of the 120 isolates were susceptible on both LJ and MGIT DST.

Table 4.12: Summary of rifampicin concordance and discordance on LJ and MGIT DST, sensitivity, stratified by *rpoB* mutation type for *M. tuberculosis* isolates

***rpoB* sequencing Codon Change, Amino Acid Change Mtb numbering (Amino Acid Change *E. coli* numbering)**	**Rifampicin DST results on LJ**			**Rifampicin DST results on MGIT**			**Total**	**Our Study HC = >80% MC = >60-80% LC = >40-60 MIC >20-40 NC = 0-20%**
	R (%)	**S (%)**	**Sensitivity 95% CI**	**R (%)**	**S (%)**	**Sensitivity 95% CI**		
tcg→ttg, Ser450Leu (Ser531Leu)	199(100)	0(0)	98.16% to 100.00%	199(100)	0(0)	98.16% to 100.00%	199	High confidence LJ high confidence MGIT
gac→tac, Asp435Tyr (Asp516Tyr)	16(76.2)	5(23.8)	52.83% to 91.78%	4(19)	17(80.9)	5.45% to 41.91%	21	Moderate confidence LJ no confidence MGIT
ctg→ccg, Leu430Pro (Leu511Pro)	6(30)	14(70)	11.89% to 54.28%	1(5)	19(95)	0.13% to 24.87%	20	Minimal confidence LJ no confidence MGIT
ctg→ccg, Leu452Pro (Leu533Pro)	15(75)	5(25)	50.90% to 91.34%	3(15)	17(85)	3.21% to 37.89%	20	Moderate confidence LJ no confidence MGIT
gac→gtc, Asp435Val (Asp516Val)	19(100)	0(0)	82.35% to 100.00%	19(100)	0(0)	82.35% to 100.00%	19	High confidence LJ high confidence MGIT
cac→aac, His445Asn (His526Asn)	7(38.9)	11(61.1)	17.30% to 64.25%	3(16.6)	15(83.3)	3.58% to 41.42%	18	Minimal confidence LJ no confidence MGIT
tcg→tgg, Ser450Trp (Ser531Trp)	15(100)	0(0)	78.20% to 100.00%	15(100)	0(0)	78.20% to 100.00%	15	High confidence LJ high confidence MGIT

cac→ctc, His445Leu (His526Leu)	9(100)	0(0)	66.37% to 100.00%	1(11.1)	8(88.8)	0.28% to 48.25%	9	High confidence LJ No confidence MGIT
cac→gac, His445Asp (His526Asp)	8(100)	0(0)	63.06% to 100.00%	8(100)	0(0)	63.06% to 100.00%	8	High confidence LJ high confidence MGIT
cac→tac, His445Tyr (His526Tyr)	8(100)	0(0)	63.06% to 100.00%	8(100)	0(0)	63.06% to 100.00%	8	High confidence LJ high confidence MGIT
cac→tgc, His445Cys (His526Cys)	5(83.3)	1(16.6)	35.88% to 99.58%	4(66.6)	2(33.3)	22.28% to 95.67%	6	Minimal confidence LJ moderate confidence MGIT
atc→ttc, Ile491Phe (Ile572Phe)	5(100)	0(0)	47.82% to 100.00%	4(80)	1(20)	28.36% to 99.49%	5	Minimal confidence LJ no confidence MGIT
ins of ttc →1297, Ins of Phe at 433 (Phe514ins (inframe)	3(100)	0(0)	29.24% to 100.00%	3(100)	0(0)	29.24% to 100.00%	3	High confidence LJ high confidence MGIT
caa→aaa, Gln432Lys (Gln513Lys)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ high confidence MGIT
caa→cca, Gln432Pro (Gln513Pro)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ high confidence MGIT
caa→cta, Gln432Leu (Gln513Leu)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ high confidence MGIT
cac→cgc, His445Arg (His526Arg)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ high confidence MGIT
cac→ggc, His445Gly (His526Gly)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
gac→ggc, Asp435Gly (Asp516Gly)	0(0)	1(100)	0.00% to 97.50%	0(0)	1(100)	0.00% to 97.50%	1	No confidence LJ no confidence MGIT

atg→gtg , cac→aac, Met434Val + His445Asn (Met515Val + His526Asn)	5(100)	0(0)	47.82% to 100.00%	4(80)	1(20)	28.36% to 99.49%	5	High confidence LJ moderate confidence MGIT
ctg→cgg , gac→tac, Leu430Arg + Asp435Tys (Leu511Arg + Asp516Tyr)	3(100)	0(0)	29.24% to 100.00%	2(66.6)	1(33.3)	9.43% to 99.16%	3	High confidence LJ moderate confidence MGIT
atg→ata , cac→aac, Met434Ile + His445Asn (Met515Ile + His526Asn)	2(100)	0(0)	15.81% to 100.00%	2(100)	0(0)	15.81% to 100.00%	2	High confidence LJ High confidence MGIT
cac→aac , tcg→tgg, His445Asn + Ser450Trp (His526Asn + Ser531Trp)	2(100)	0(0)	15.81% to 100.00%	1(50)	1(50)	1.26% to 98.74%	2	High confidence LJ Low confidence MGIT
ctg→cgg, Leu430Arg (Leu511Arg)	0(0)	2(100)	0.00% to 84.19%	0(0)	2(100)	0.00% to 84.19%	2	No confidence LJ No confidence MGIT
Ggaccagaacaacccg/G , Del of AA Asp-Gln-Asn-Asn-Pro from 435-439 (inframe) (Del of Asp-Gln-Asn-Asn-Pro from 516-520 (inframe)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT

Caccagccagctg/C , Del of AA Thr-Ser-Gln-Leu from 427-30 (inframe) (del of Thr-Ser-Gln-Leu from 508-511 (inframe)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
Aaac/A at 1312-1314, del of Asn at 438 (Del of Asn at 519)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
aac→aag, Asn438Lys (Asn519Lys)	0(0)	1(100)	0.00% to 97.50%	0(0)	1(100)	0.00% to 97.50%	1	No confidence LJ No confidence MGIT
acc→acg , cac→ccc , aag→cag, His445Pro + Lys446Gln (His526Pro + Lys527Gln)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
acc→atc , gac→tac , atc→ctc, Thr427Ile + Asp435Tyr + Ile491Leu (Thr508Ile + Asp516Tyr + Ile572Leu)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
acc→gcc, Thr444Ala (Thr515Ala)	0(0)	1(100)	0.00% to 97.50%	0(0)	1(100)	0.00% to 97.50%	1	No confidence LJ No confidence MGIT

Agaa/A at 1308-10, Gln-Asn436His (Gln-Asn517His)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
agc→acc , gac→ggc, Ser428Thr + Asp435Gly (Ser509Thr + Asp516Gly)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
agc→agg , cac→tac, Ser428Arg + His445Tyr (Ser509Arg + His526Tyr)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
agc→agg , ctg→ccg, Ser428Arg + Leu430Pro (Ser509Arg + Leu511Pro)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
agc→atc, Ser428Ile (Ser509Ile)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
agc→ggc, Ser431Gly (Ser512Gly)	0(0)	1(100)	0.00% to 97.50%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
agc→ggc , cac→aac , ctg→ccg, Ser428Gly + His445Asn + Leu452Pro (Ser509Gly + His526Asn + Leu533Pro)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT

atg→ata, Met434Ile (Met515Ile)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
atg→ata , gac→ggc , gcg→gtg, Met434Ile + Asp435Gly + Ala451Val (Met515Ile + Asp516Gly + Ala532Val)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
atg→ctg , cac→aac, Met434Leu + His445Asn (Met515Leu + His526Asn)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
caa→aaa , cac→gac, Gln432Lys + His445Asp (Gln513Lys + His526Asp)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
cac→aac , ctg→cag , His445Asn + Leu452Gln (His526Asn + Leu533Gln)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
cag→cac , cac→cgc, Gln429His + His445Arg (Gln510His +	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT

His526Arg)								
ctg→ccg , cac→aac, Leu430Pro + His445Asn (Leu511Pro + His526Asn)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
gac→ggc , gcg→gtg, Asp435Gly + Ala451Val (Asp516Gly + Ala532Val)	1(100)	0(0)	2.50% to 100.00%	0(0)	1(100)	0.00% to 97.50%	1	High confidence LJ No confidence MGIT
gac→tac , aac→cac, Asp435Tyr + AsnAsn437His (Asp516Tyr + Asn518His), Asp435Tyr + Asn437His	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
gtc→ttc, Val170Phe (Val251Phe)	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
ins cgc 1294, Ins of Arg at 432 (Arg513ins (inframe))	1(100)	0(0)	2.50% to 100.00%	1(100)	0(0)	2.50% to 100.00%	1	High confidence LJ High confidence MGIT
WT	0(0)	120(100)		0(0)	120(100)		120	

4.14.5 Isoniazid Phenotypic Resistant Isolates' rifampicin susceptibility on GeneXpert verses *rpoB* gene Sequencing

There were a total of 391 strains that were INH resistant in the study on phenotypic DST. Out of 391 INH, resistant 367 revealed any mutation on sequencing. Comparison of GeneXpert results with sequencing revealed 13 Strains were misclassified on GeneXpert as rifampicin sensitive which was also resistant to Isoniazid. So, a total of 13((3.54%) MDR TB cases were misdiagnosed as isoniazid-resistant rifampicin sensitive if only GeneXpert will be performed as shown in (Table 4.13).

4.14.6 Isoniazid Phenotypic Resistant Isolates' rifampicin susceptibility on MGIT vs *rpoB gene* Sequencing

There was a total of 391 strains that were INH resistant in study on phenotypic DST. Out of 391 INH, resistant 367 revealed any mutation on sequencing. Comparison of MGIT DST results with sequencing revealed 87 strains were misclassified on MGIT DST as rifampicin sensitive which was also resistant to Isoniazid. So, a total of 87 (23.7%) MDR TB cases will be misclassified as isoniazid-resistant rifampicin sensitive if only MGIT DST will be performed as shown in (Table 4.14).

4.14.7 Isoniazid Phenotypic Resistant Isolate's RIF susceptibility on Lowenstein Jensen *verses rpoB* gene Sequencing

Among 391 strains that were INH resistant in the study on phenotypic DST. Out of 391 INH, resistant 367 revealed any mutation on sequencing. Comparison of LJ DST results with sequencing revealed 35 strains were misclassified on LJ DST as rifampicin sensitive which was also resistant to Isoniazid. So, a total of 35(9.5%) MDR TB cases will be classified as isoniazid-resistant rifampicin sensitive if only LJ DST will be performed as shown in (Table 4.15).

Table 4.13: Isoniazid Phenotypic Resistant isolate's RIF susceptibility on GeneXpert and *rpoB* gene Sequencing

***rpoB* gene sequencing results**	**GeneXpert rifampicin result**				
	Resistant	Sensitive	Total	X^2	*p-value*
Any mutation identified	354	13	367	130.7	<0.00001
Wild Type (no mutation)	8	16	24		
Total	362	29	391		

X^2 = Chi square, p-Value (<.05) = significant

Table 4.14: Isoniazid Phenotypic Resistant RIF susceptibility on MGIT DST verses *rpoB* gene Sequencing

***rpoB* gene sequencing results**	**GeneXpert rifampicin result**				
	Resistant	Sensitive	Total	X^2	*p-value*
Any mutation identified	280	87	367	57.9	<0.00001
Wild Type (no mutation)	1	23	24		
Total	281	110	391		

X^2 = Chi square, p-Value (<.05) = significant

Table 4.15: Isoniazid Phenotypic Resistant RIF susceptibility on LJ DST verses *rpoB* gene Sequencing

***rpoB* sequencing results**	**LJ rifampicin DST result**				
	Resistant	Sensitive	Total	X^2	*p-value*
Any mutation identified	332	35	367	132.7	<0.00001
Wild Type (no mutation)	1	23	24		
Total	333	58	391		

X^2 = Chi square, p-Value (<.05) = significant

4.15 Mutational analysis of *rpoB* using Whole Genome Sequencing

To have a deep look beside Sanger sequences, whole genome sequencing (WGS) of 25 selected isolates was also performed. The DNA sequence of the *rpoB* gene, which is 3519 bp long, was analyzed on these 25 isolates, and sequences were compared to the reference Mtb strain (H37Rv). The majority of mutations were observed in the RRDR region of the *rpoB* between positions 426 and 452.

To compare the differential behavior of isolates having a single mutation on different DST media, whole genome sequencing was also done. The most prevalent discordant mutation harboring isolates were sequenced using WGS (Figure 4.9 and Table 4.16). In two occult isolates which were identified on Sanger sequencing to have mutation Leu430Pro, but upon WGS analysis it was found that only one isolate (Leu430Pro) had this mutation which was found to be sensitive on both LJ and MGIT DST. An isolate that had mutation Cys701Trp in both sequencing methods was also resistant on both LJ and MGIT DST. Similarly, in two other strains with Asp435Tyr mutation in Sanger sequencing, only one isolate was positive for Asp435Tyr mutation in WGS which was sensitive on MGIT DST. An isolate resistant on both LJ and MGIT DST had confirmed mutation Pro358Leu in WGS too.

His445Asn mutation in *rpoB* gene containing isolates after Sanger sequencing, on further WGS, 2 had His445Asn in WGS analysis but only one of these isolates was sensitive on both LJ and MGIT DST, while other was resistant on both LJ and MGIT DST. Two isolates were identified to carry mutation His445Asn by Sanger sequencing but in WGS analysis only isolate had His445Asn mutation which was sensitive on both LJ and MGIT DST. Isolates with Met434Val along with His445Asn in WGS were found to be resistant on both LJ and MGIT DST.

Similarly, two isolates in which Leu452Pro was identified on Sanger sequencing when WGS analysis was performed it was found that in one isolate Leu452Pro mutation was present which was sensitive on MGIT DST, however, in the second isolate another mutation His445Tyr was also present which was resistant on both LJ and MGIT DST.

One isolate with amino acid change Asn438Lys was sequenced and later confirmed on WGS analysis too, was sensitive on LJ and MGIT DST. Another strain having codon change leading to amino acid change Leu430Arg which was sensitive on LJ and MGIT DST, both on WGS no other mutation besides Leu430Arg mutation was identified in it.

An isolate sensitive on LJ and MGIT DST having amino acid change Tyr435Gly when analyzed on WGS detected no other mutation besides this mutation (Tyr435Gly). The same condition was found in another isolate (Thr444Ala) sensitive on LJ and MGIT DST but no mutation was identified in WGS. A strain sensitive on MGIT DST with a His445Leu mutation on WGS analysis did not harbor amino acid changing mutation. Five strains carrying different insertion and deletions, which included deletion of amino acid 428 to 430, deletion of amino acid 435 to 439, insertion of phenylalanine at 434, Ins of Arginine at position 432 and Gln436His + Asn437 deletion were also sequenced using WGS to check any other mutation. However, no other mutation was identified in these isolates. All insertions and deletions carrying Mtb were resistant on both LJ and MGIT DST except for the one with Gln436His + Asn437 deletion, which was sensitive on MGIT DST.

The mutation Ser450Leu was identified in the majority of isolates, to check any difference in compensatory mutations associated with this mutation, it was analyzed by WGS too. Isolates with this mutation were never missed on both LJ and MGIT DST. Four isolates were identified as wild type upon sequencing but 3 were sensitive on LJ and MGIT DST, except one isolate which had no mutation on WGS but was found resistant on LJ and MGIT.

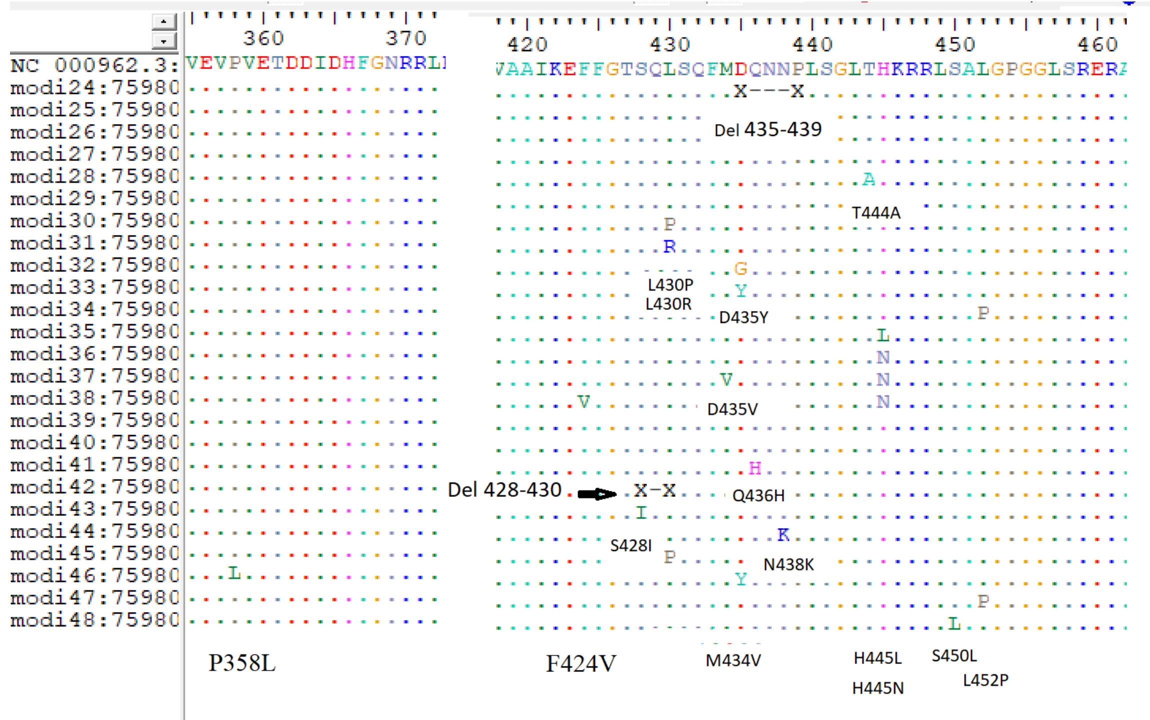


Figure 4.9: Resistance Conferring Mutations to Rifampicin identified in *rpoB* gene of Mtb after aligned with H37Rv reference strain

Table 4.16: Nucleotide change, amino acid change identified in *rpoB* of Mtb due to mutation, RIF susceptibility on LJ and MGIT DST of the whole genome sequenced Isolates

WGS final id	***rpoB*_nt_wgs**	***rpoB* _aa_wgs**	Sequencing RIF result	LJ- RIF result	MGIT- RIF result
stand 24	Indel 1305: TGGACCAGAACAACCC	InDel 435-439	R	R	R
stand 28	c759746t + a761136g + t762434g + t763031c	Thr444Ala + Gly876Gly + Ala1075Ala	R	S	S
stand 30	c759746t + a761095g + t762434g + t763031c	Leu430Pro + Gly876Gly + Ala1075Ala	R	S	S
stand 45	t761095c + t761909g + t762434g + t763031c	Leu430Pro + Cys701Trp + Gly876Gly + Ala1075Ala	R	R	R
stand 31	t761095g + t763031c	Leu430Arg + Ala1075Ala	R	S	S
stand 32	c759746t + a761110g + t762434g + t763031c	Asp435Gly + Gly876Gly + Ala1075Ala	R	S	S
stand 33	c759746t + a761109g + t762434g +	Asp435Tyr +	R	R	S

	t763031c	Gly876Gly + Ala1075Ala			
stand 46	c760879t + a761109g + t762434g + t763031c	Pro358Leu + Asp435Tyr + Gly876Gly + Ala1075Ala	R	R	R
stand 35	c759746t + a761140t + t762434g + t763031c	His445Leu + Gly876Gly + Ala1075Ala	R	R	S
stand 36	c759746t + c761139a + t762434g + t763031c	His445Asn + Gly876Gly + Ala1075Ala	R	S	S
stand 37	c759746t + a761106g + c761139a + t762434g + t763031c	Met(s)434Val(s) + His445Asn + Gly876Gly + Ala1075Ala	R	R	R
stand 38	c759746t + t761076g + t761127g + c761139a + g762194c + t762434g + g762989c + t763031c	Phe424Val + Ser441Ala + His445Asn + leu796Leu + Gly876Gly + Arg1061Arg +	R	R	R

		Ala1075Ala			
stand 39	ins 761102: ttc	Del of Phe at 434	R	R	R
stand 40	c759746t + Ins 761097: gcc + t762434g + t763031c	Ins of Arg at 432 + Gly876Gly + Ala1075Ala	R	R	R
stand 41	c759746t del 761112: aga + t762434g + t763031c	Indel of aga Gly876Gly + Ala1075Ala	R	R	S
stand 42	Del of accagccagctg at 761084	Indel 427-430	R	R	R
stand 43	g761089t + t762434 + t763031c	Ser428Ile + Gly876Gly + Ala1075Ala	R	R	S
stand 44	c761120g + t762434 + t763031c	Asn438Lys + Gly876Gly + Ala1075Ala	R	S	S
stand 34	c759746t + t761161c + t762434g + t763031c	Leu452Pro + Gly876Gly + Ala1075Ala	R	R	S
stand 47	c761139t + t761161c + t762434g + t763031c	His445Tyr + Leu452Pro + Gly876Gly +	R	R	R

		Ala1075Ala			
stand 48	c761155t + Gly876Gly + Ala1075Ala	Ser450Leu(s) + Gly876Gly + Ala1075Ala	R	R	R
stand 25	t763031c	Ala1075Ala	S	S	S
stand 26	c759746t + t762434g + t763031c	Gly876Gly + Ala1075Ala	S	R	R
stand 27	WT	WT	S	S	S
stand 29	c759746t + t762434g + t763031c	Gly876Gly + Ala1075Ala	S	S	S

4.16 Bioinformatic Analysis for other Anti TB Drug resistance Gene Mutations through Whole Genome Sequencing

4.16.1 Resistance to Isoniazid and mutations in *katG* gene

Out of the total 25 isolates, alignment against the reference genome revealed 3 different mutations in the *katG* gene which were Ser315Thr, Arg463Leu, and Thr475Pro (Figure 4.10). Twelve mutations detected in 25 isolates were at position 315 of the *katG* gene. In the reference position, S was substituted after mutation to the new amino acid Thr. All Ser315Thr mutations were in combinations with other mutations and it was noted that no mutation occurred alone at 315 positions. The 11 mutations in Ser315Thr were in combination with Arg463Leu and all isolates having this mutation were phenotypically resistant to the drug.

The second most prevalent mutation was Arg463Leu in 10 isolates. It was observed that 8 isolates having this mutation were phenotypically resistant while 02 samples having Arg463Leu mutation were sensitive to the INH drug. Of the total isolates, no mutation was detected in three isolates that were sensitive to INH drug (Table 4.17).

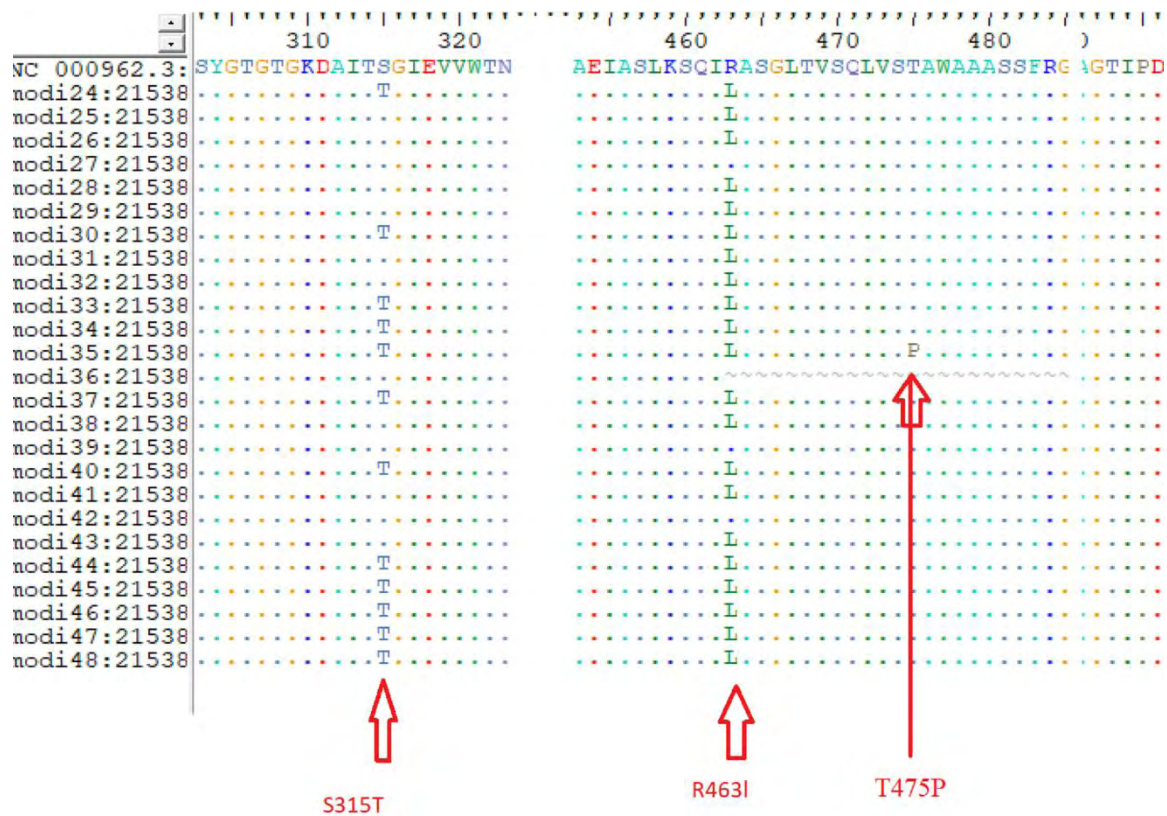


Figure 4.10: Resistance Conferring Mutations to isoniazid identified in *katG* gene of Mtb after aligned with H37Rv reference strain

Table 4.17: Phenotypic resistance to Isoniazid and mutations in *katG* gene identified through WGS

Mutation	Phenotypic resistant	Phenotypic sensitive	Total
Ser315Thr	0		0
Arg463Leu	8	2	10
Ser315Thr + Arg463Leu	11		11
Thr475Pro + Ser315Thr+ Arg463Leu	1		1
WT		3	3
Total	20	5	25

4.16.2 Resistance to Isoniazid and mutations in inhA gene

In 5/25 isolates, mutations in the promotor region of the *inhA* gene were detected. In the 5 mutations detected in the intergenic region of the *inhA* gene, 4 mutations were at genomic position 1673425 where Cytosine was substituted by Thymine and one mutation was detected at genomic position 1673432 where Thymine was substituted by Guanine. All these 5 mutations had resistance in phenotypic DST (Table 4.18).

14.16.3 Resistance conferring mutations in KatG and inhA gene verses phenotypic DST

Out of the total 25 samples, 20 were phenotypically resistant in the conventional DST method. To correlate the mutations in *katG* and *inhA* genes, 6 mutations identified in the *inhA* gene were associated with phenotypic resistance while there was no mutation in *the inhA* gene in 14 phenotypic resistance isolates. Out of the 6 mutations in the *inhA* gene, 4 intergenic mutations of *inhA* were Arg463Leu mutation while one intergenic mutation was in strain already having a double mutation of Ser315Th + Arg463Leu in *katG* gene. All INH resistant (20) strains had a mutation in the *katG* gene (Table 4.19).

4.16.4 Pyrazinamide resistance and mutations in pncA gene

Five different mutations were detected in the *pncA* gene as shown in Figure 4.11, one mutation at each position Pro62Thr, Lys96Asn, Glu144Asp, and Val155Gly was detected while one deletion Ser (TCC) at genomic position 2288807 was detected (Table 4.20). Comparing mutations with phenotypic resistance, all mutations mentioned below were conferring resistance.

Table 4.18: Phenotypic resistance to Isoniazid and mutations in *inhA* gene identified through WGS

Mutation	Phenotypic resistant	Phenotypic sensitive	Total
Intergeneic	5		5
WT	15	5	18
Total	20	5	25

Table 4.19: *katG gene* vs *inhA* gene mutations identified in Isolated through WGS

	InhA gene			
katG Gene	**intergenic**	**Leu203Leu**	**WT**	**Total**
Arg463Leu	4		3	7
ins of 2154405 + Arg463Leu			1	1
Ser315Th + Arg463Leu	1	1	9	11
Ser315Th + Arg463Leu + Thr475Pro			1	1
Grand Total	5	1	14	20

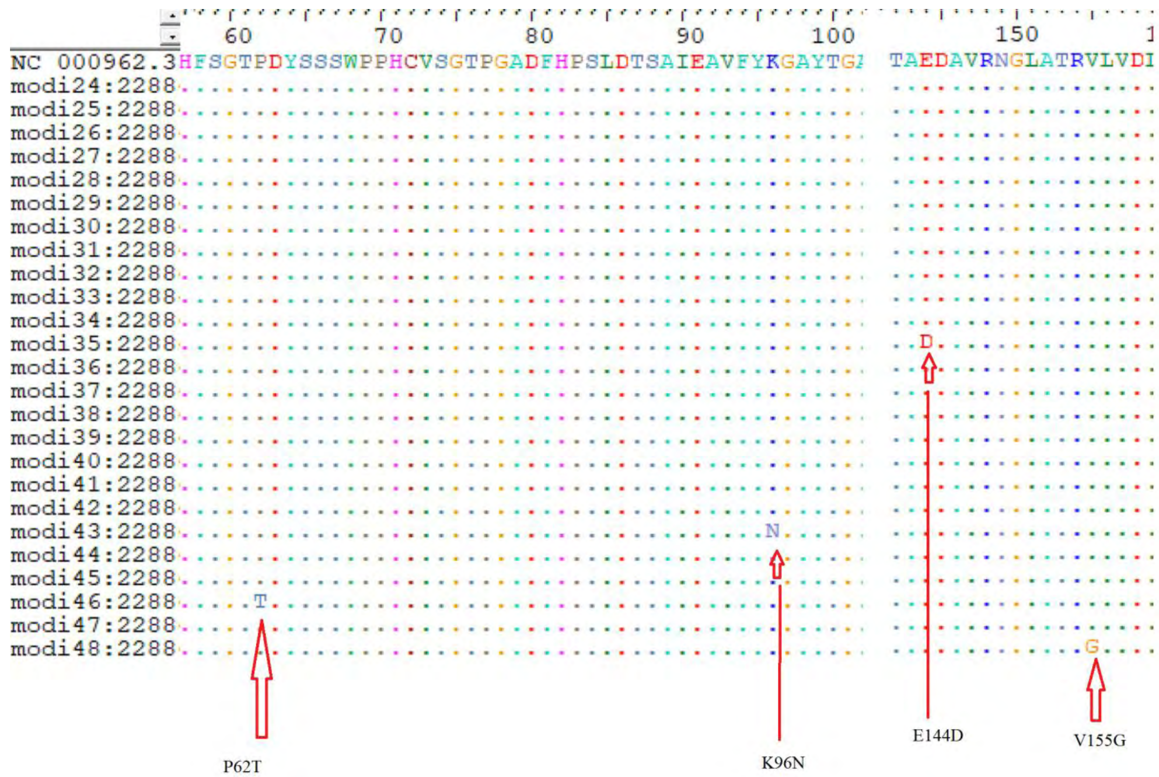


Figure 4.11: Resistance Conferring Mutations to pyrazinamide identified in *pncA* gene of Mtb after aligned with H37Rv reference strain.

Table 4.20: Pyrazinamide resistance and mutations in *pncA* gene through WGS

Mutation	Phenotypic resistant	Phenotypic sensitive	Total
Pro62Thr	1		1
Lys96Asn	1		1
Glu144Asp	1		1
Val155Gly	1		1
Deletion of TCC at 2288807	1		1
WT	5	20	20
			25

14.16.5 Mutations in rrs and resistance to streptomycin, amikacin, and kanamycin drugs though WGS

Overall, 53 different mutations in *rrs* were identified, however except for one mutation (A1473246G) all were not significant and did not confer any resistance to streptomycin, amikacin, or kanamycin drugs (Table 4.21).

14.16.6 Fluoroquinolone resistance and mutation in gyrA gene identified through WGS

Analysis of the *gyrA* gene revealed mutations in 24 isolates while one was wild type. The isolate with mutations (Ser95Thr + Gly668Asp + Ala90Val + Asp94Gly) was also resistant on phenotypic DST to ofloxacin (Table 4.22). In another isolate with mutations Ser95Thr + Gly668Asp + Gly668Asp + Asp89Asn was also resistant on phenotypic DST to ofloxacin. Of the 5 strains having mutations Ser95Thr + Asp94Gly, four were resistant on phenotypic DST to ofloxacin. Another Ser95Thr + Gly668Asp + Asp94Tyr harboring isolate, phenotypic DST showed ofloxacin resistance. Two ofloxacin susceptible on phenotypic DST had three mutations Ser95Thr + Gly668Asp + Ala288Asp. The 10/12 isolates with mutations Ser95Thr + Gly668Asp were susceptible on phenotypic DST to ofloxacin, however, the rest were phenotypic resistant on DST. One phenotypic ofloxacin sensitive isolate was identified with mutation Ser95Thr + Gly668Asp + Ala384Val Ala456Val. Ser95Thr+ Gly668Asp +Pro17Thr + Ser91Pro + Asp94Gly mutations carrying isolate were also resistant on phenotypic DST to ofloxacin.

Table 4.21: Resistance to amikacin, kanamycin, and capreomycin and mutations in *rrs* identified through WGS

Genomic position	REF	ALT	Mutation	Frequency	Phenotypic DST result
1471896	T	C	T1471896C	1	S
1471900	C	T	C1471900T	1	S
1472106	G	A	G1472106A	1	S
1472127	C	T	C1472127T	1	S
1472129	G	C	G1472129C	1	S
1472137	G	A	G1472137A	1	S
1472150	T	A	T1472150A	2	S
1472172	T	C	T1472172C	1	S
1472203	G	A	G1472203A	1	S
1472210	A	T	A1472210T	1	S
1472213	G	C	G1472213C	1	S
1472222	G	A	G1472222A	1	S
1472225	C	A	C1472225A	1	S
1472229	C	T	C1472229T	1	S
1472236	C	G	C1472236G	1	S
1472240	G	A	G1472240A	1	S
1472251	G	A	G1472251A	1	S
1472344	C	T	C1472344T	1	S
1472359	A	T	A1472359T	1	S
1472362	C	T	C1472362T	1	S
1472378	G	T	G1472378T	1	S
1472379	T	C,G	T1472379C,G	3	S
1472382	G	A	G1472382A	1	S
1472396	A	T	A1472396T	1	S
1472400	C	T	C1472400T	2	S
1472530	G	A	G1472530A	1	S
1472557	G	A	G1472557A	1	S

1472571	G	C	G1472571C	1	S
1472573	C	T	C1472573T	1	S
1472579	G	T	G1472579T	1	S
1472581	A	T	A1472581T	2	S
1472598	A	C	A1472598C	1	S
1472616	G	A	G1472616A	1	S
1472697	T	C	T1472697C	1	S
1472708	T	A	T1472708A	1	S
1472713	T	C	T1472713C	1	S
1472716	C	T	C1472716T	1	S
1472733	G	C	G1472733C	1	S
1472734	C	T	C1472734T	1	S
1472741	G	A	G1472741A	1	S
1473246	A	G	A1473246G	A	R

Table 4.22: Resistance to Fluoroquinolone and mutations in *gyrA* gene identified through WGS

Mutations in g*yrA gene*	**PhneoR**	**PhneoS**	**Total**
Ser95Thr + Gly668Asp + Ala90Val + Asp94Gly	1		1
Ser95Thr + Gly668Asp + Gly668Asp + Asp89Asn	1		1
Ser95Thr + Asp94Gly	4	1	5
Ser95Thr + Gly668Asp + Asp94Tyr	1		1
Ser95Thr + Gly668Asp + Ala288Asp		2	2
Ser95Thr + Gly668Asp	2	10	12
Ser95Thr + Gly668Asp + Ala384Val Ala456Val		1	1
Ser95Thr+ Gly668Asp +Pro17Thr + Ser91Pro + Asp94Gly	1		1
WT		1	1
Total	10	15	25

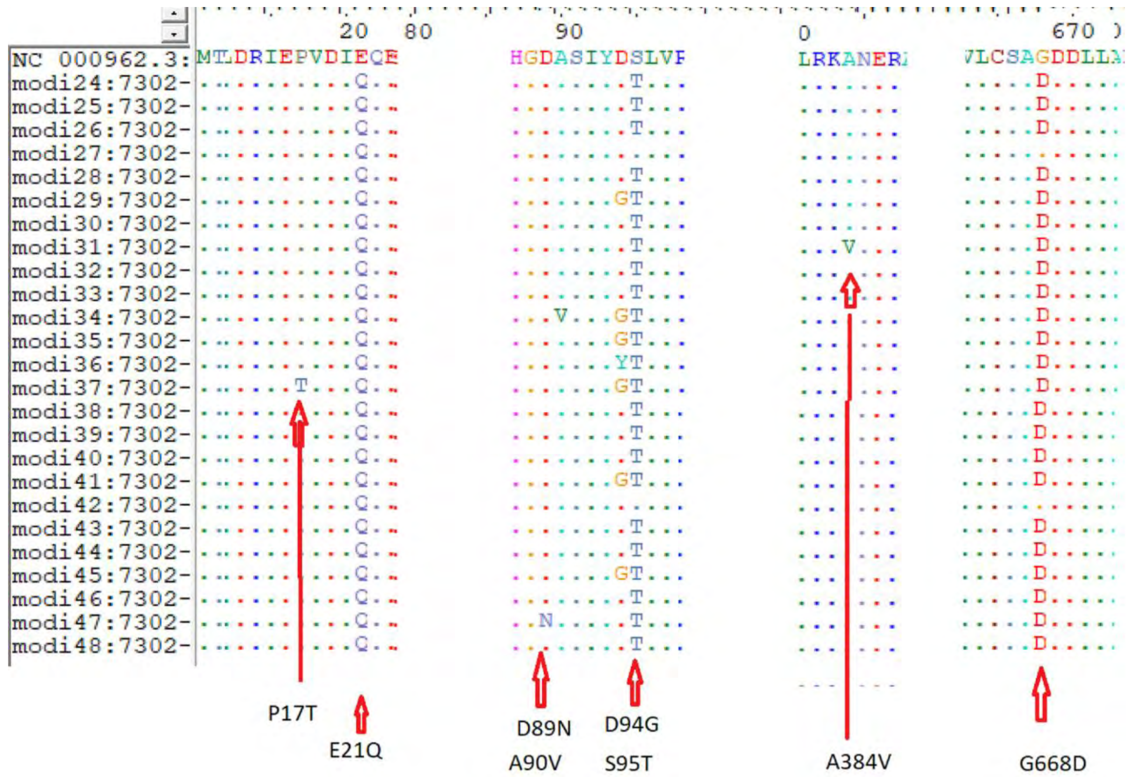


Figure 4.12: Resistance Conferring Mutations to Fluoroquinolone identified in *gyrA* gene of Mtb after aligned with H37Rv reference strain

14.16.7 Fluoroquinolone resistance and mutation in gyrB gene

Out of 5 mutations in the *gyrB* gene, one mutation was at each position Glu21Gln, Met (s) 291Ile, and Thr500Pro while one double mutation Arg429Ser + Arg446Leu was also detected (Figure 4.5, Table 4.23). Upon comparing mutations with phenotypic resistance, mutation Glu501Gln and Thr500Pro were associated with phenotypic resistant isolates.

14.16.8 Resistance to ethambutol and Mutations in embB gene

Out of the total of 25 WGS isolates, mutations were detected 13 isolates, while the remaining were wild-type on WGS analysis of the *embB* gene. Phenotypic resistance to ethambutol was detected in 9 strains, in which some had mutations. Similarly, 6 strains with mutations were sensitive on phenotypic DST. The Met306Ile was the most common mutation, which was present in 4 strains alone while in 2 isolates in combination with Ser651Thr and Val39Gly which were phenotypic resistant (Figure 4.14 and Table 4.24). In an isolate with a mutation at Ala637Val, there was phenotypic resistance to ethambutol. On the other hand, 2 isolates with a mutation at Asp1024Asn but only one of these was resistant on phenotypic DST while the other was sensitive to ethambutol. In an isolate, mutation Gln497Arg was detected which was also resistant to ethambutol phenotypically. Mutations detected at Glu378Ala, Gly406Asp, Ser174Arg, and Val88Val were all sensitive to ethambutol on phenotypic DST.

Table 4.23: Resistance to fluoroquinolone and mutations in *gyrB* gene identified through WGS

Mutatio in gyrB	Phenotypic R	Phenotypic S	Total
Arg429Ser + Arg446Leu		1	1
Glu21Gln		1	1
Glu501Gln	1		1
Met(s)291Ile		1	1
Thr500Pro	1		1
WT	8	12	20
Total	10	15	25

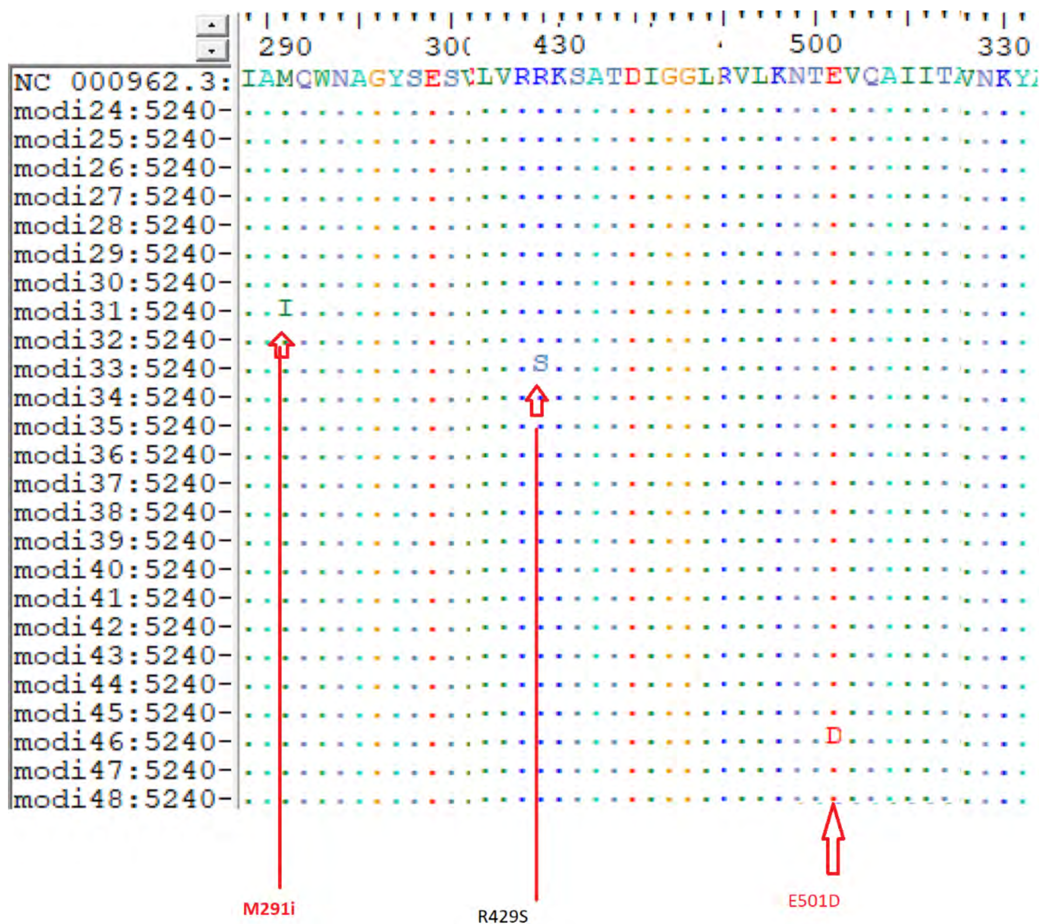


Figure 4.13: Resistance Conferring Mutations to Fluoroquinolone identified in *gyrB* gene of Mtb and aligned with H37Rv reference strain

Table 4.24: Resistance to ethambutol and mutations in *embB* gene identified through WGS

	Phenotypic DST		
embB gene mutations	Phenotypic DST R	Phenotypic DST S	
Ala637Val(s)	1		1
Asp1024Asn	1	1	2
Gln497Arg	1		1
Glu378Ala		1	1
Gly406Asp		1	1
Met(s)306Ile	2		2
Met(s)306Ile + Ser651Thr	1		1
Ser174Arg		1	1
Thr642Ala		1	1
Val(s)39Gly + Met(s)306Ile	1		1
Val(s)88Val		1	1
WT	2	10	12
Total	9	16	25

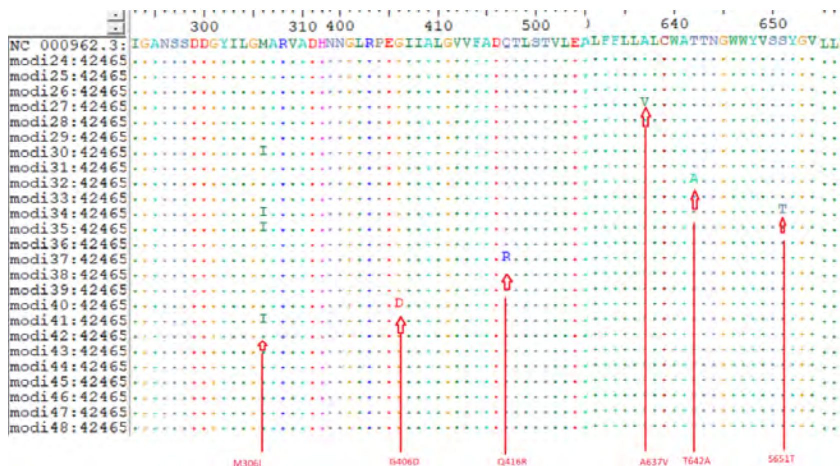


Figure 4.14: Resistance Conferring Mutations to ethambutol identified in *embB* gene of Mtb and aligned with H37Rv reference strain

14.16.9 Bedaquiline and clofazimine resistance conferring mutations identified through WGS

For bedaquiline and clofazimine resistance, the Rv0678 gene was analyzed for resistance conferring mutations. Out of these 25 isolates, one mutation A84S and another Leu117Arg were detected in one isolate each as shown in Figure 4.15. Phenotypic DST results are not available for these drugs.

14.16.10 DNA dependent RNA polymerase structure prediction of rifampicin resistance rpoB mutants

To understand molecular consequences of mutations, 25 different mutations identified in the *rpoB* gene were used to predict RNA polymerase protein structure using free online available DynaMut web server (Table 4.25). Protein dynamics and the prediction of the effect of mutations using elastic network atom contact model (ENCoM) and normal mode analysis (NMA) predictions, and vibrational entropy energy between wild type and mutant were computed. The binding affinity change caused by a mutation (i.e., ΔΔG) was lowered in predicting outcome in case of most frequently prevalent discordant mutations Leu430Pro, His445Asn and Leu452Pro except for Asp435Tyr. NMA based prediction showed that these mutations were destabilizing the protein except for Val170Phe which was stabilizing the protein. Vibrational entropy energy between wild type and mutants resulted in an increase of molecule flexibility in case of discordant mutations Leu430Pro, His445Asn, and Leu452Pro except for Asp435Tyr, however, a decrease in molecular flexibility was observed in most concordant mutations like Ser450Leu and Ser450Try.

Figure 4.15: Resistance Conferring Mutations to Bedaquiline and clofazimine identified in Rv0678 gene of Mtb after aligned with H37Rv reference strain

Table 4.25: Structure guided prediction of RNA polymerase of various rifampicin resistance mutations identified in *rpoB* gene of isolates

S. No	Wild-type amino acid	Position of amino acid	Mutant amino acid	Chain	Prediction Outcome ΔΔG ENCoM	NMA Based Predictions ΔΔG ENCoM	Δ Vibrational Entropy Energy Between Wild-Type and Mutant ΔΔSVib ENCoM: in(kcal.mol-1.K-1)
1	Gln	432	LEU	C	1.322 kcal/mol (Stabilizing)	0.089 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.111)
2	Gln	432	PRO	C	-0.138 kcal/mol (Destabilizing)	0.028 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.035)
3	Gln	432	Lys	C	0.338 kcal/mol (Stabilizing)	0.087 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.109)
4	Ser	431	Gly	C	-0.687 kcal/mol (Destabilizing)	-0.445 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.557)
5	His	445	Leu	C	0.120 kcal/mol (Stabilizing)	-0.164 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.205)
6	Asp	435	Val	C	1.401 kcal/mol (Stabilizing)	-0.225 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.281)
7	Val	170	Phe	C	-0.876 kcal/mol (Destabilizing)	0.884 kcal/mol (Stabilizing)	Decrease of molecule flexibility (-1.105)

8	Met	434	Ile	C	-1.029 kcal/mol (Destabilizing)	0.039 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.04
9	His	445	Asn	C	-1.509 kcal/mol (Destabilizing)	-0.563 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.703)
10	Leu	452	Pro	C	-0.550 kcal/mol (Destabilizing)	-0.628 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.785)
11	Ile	491	Phe	C	1.581 kcal/mol (Stabilizing)	0.445 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.557)
12	Ser	450	Leu	C	1.622 kcal/mol (Stabilizing)	0.291 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.363)
13	Ser	450	Trp	C	0.859 kcal/mol (Stabilizing)	0.372 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.465)
14	His	445	Gly	C	-1.066 kcal/mol (Destabilizing)	-0.803 kcal/mol (Destabilizing)	Increase of molecule flexibility (1.004)
15	His	445	Arg	C	-0.637 kcal/mol (Destabilizing)	-0.031 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.039)
16	Asp	435	Tyr	C	0.879 kcal/mol (Stabilizing)	0.313 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.391)
17	His	445	Asp	C	-0.296 kcal/mol (Destabilizing)	-0.455 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.568)

18	His	445	Cys	C	-0.557 kcal/mol (Destabilizing)	-0.294 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.367)
19	His	445	Tyr	C	1.116 kcal/mol (Stabilizing)	0.184 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.231)
20	Thr	444	Ala	C	0.803 kcal/mol (Stabilizing)	-0.089 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.111)
21	Asn	438	Lys	C	0.203 kcal/mol (Stabilizing)	0.191 kcal/mol (Destabilizing)	Decrease of molecule flexibility (-0.238)
22	Gln	436	His	C	-0.197 kcal/mol (Destabilizing)	-0.308 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.385)
23	Asp	435	Gly	C	0.636 kcal/mol (Stabilizing)	-0.470 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.588)
24	Leu	430	Pro	C	-0.600 kcal/mol (Destabilizing)	-0.588 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.734)
25	Leu	430	Arg	C	-0.533 kcal/mol (Destabilizing)	-0.094 kcal/mol (Destabilizing)	Increase of molecule flexibility (0.117)

4.17 Structures Predication of RNA polymerase of high confidence mutants

4.17.1 Serine 450 Leucine

Mutation Ser450Leu was a high confidence mutation and never get missed on any media, hence it was considered a strong mutant. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was 1.622 kcal/mol and was stabilizing. NMA Based Predictions ΔΔG ENCoM was 0.291 kcal/mol and it was destabilizing with vibrational entropy energy between wild type and mutant was -0.363 $kcal.mol^{-1}.K^{-1}$ which resulted in a decrease of molecule flexibility. The predicted structure change due to this mutation is shown in Figure 4.16.

4.17.2 Serine 450 Tryptophan

Another high confidence mutation Ser450T is considered a strong mutant and not missed on any media. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was 0.859 kcal/mol and was stabilizing. NMA Based Predictions ΔΔG ENCoM was 0.372 kcal/mol and destabilizing. Also, vibrational entropy energy between wild type and mutant was -0.465 $kcal.mol^{-1}.K^{-1}$ which decreased the flexibility of the molecule (Figure 4.17).

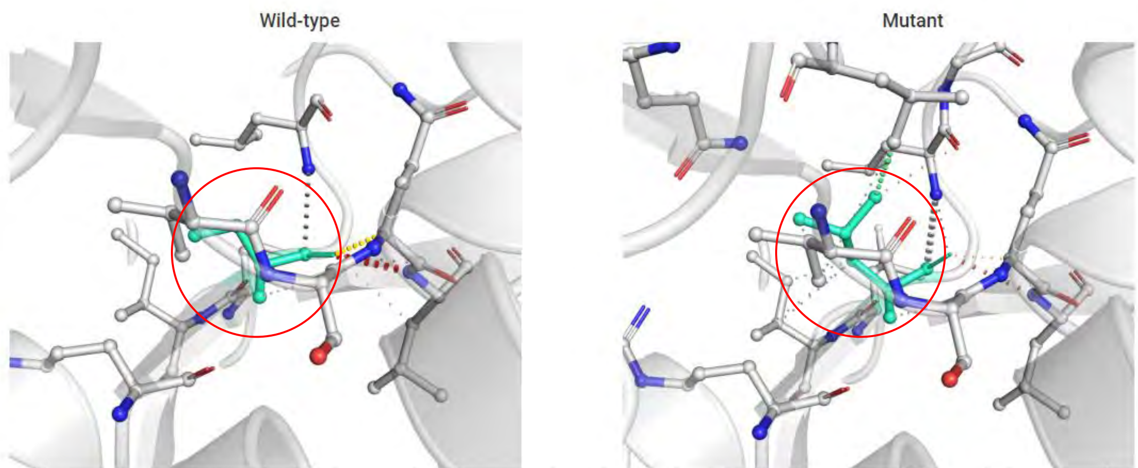


Figure 4.16: Protein structure change due to mutation Ser450Leu

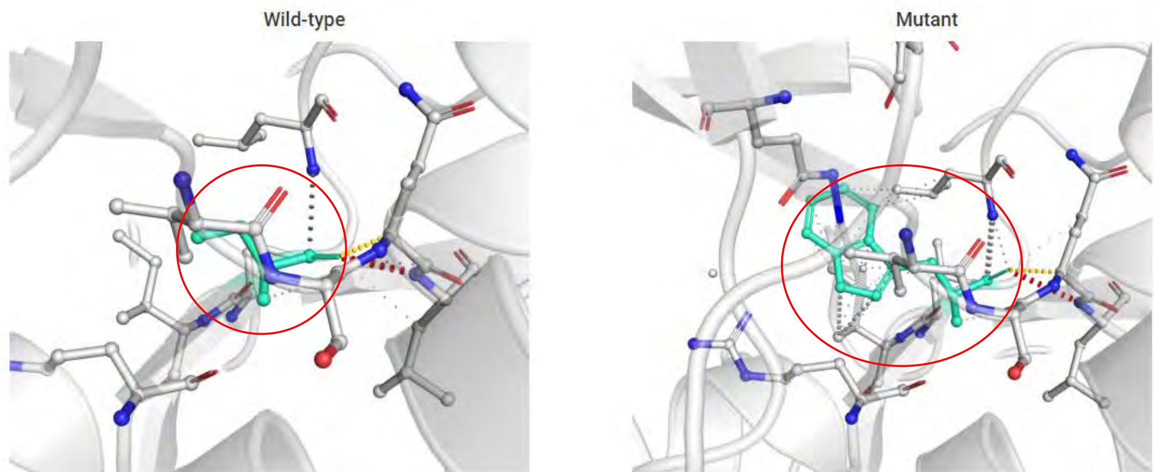


Figure 4.17: Protein structure change due to mutation Ser450Try

4.17.3 Aspartic acid 435 Valine

Mutant Asp435Val strong mutant having a high confidence mutation and it is detected on all media. Prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was stabilizing (1.401 kcal/mol). NMA Based Predictions ΔΔG ENCoM was -0.225 kcal/mol, which was destabilizing. The vibrational entropy energy between wild type and mutant was 0.281 $kcal.mol^{-1}.K^{-1}$ making it a more flexible protein (Figure 4.18).

4.17.4 Histidine 445 Aspartic acid

A strong Mutant His445Asp was also having high confidence mutation. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was -0.296 kcal/mol and destabilizing. NMA Based Predictions ΔΔG ENCoM was -0.455 kcal/mol and destabilizing. This mutant which was detected on all media had vibrational entropy energy between wild type and mutant as -0.568 $kcal.mol^{-1}.K^{-1}$ with increasing its flexibility. The structure change due to this mutation is shown in Figure 4.19.

4.17.5 Histadine 445 Leucine (A high confidence on LJ but no confidence on MGIT)

Mutant His445Leu is a high confidence mutation on LJ DST, however, has no confidence when DST is performed on MGIT. Prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB was* 0.120 kcal/mol and is stabilizing. NMA Based Predictions ΔΔG ENCoM is -0.164 kcal/mol which destabilizes it (Figure 4.20). There was an increase in flexibility of this mutant is due to the vibrational entropy energy difference between wild-type and mutant (-0.205 $kcal.mol^{-1}.K^{-1}$).

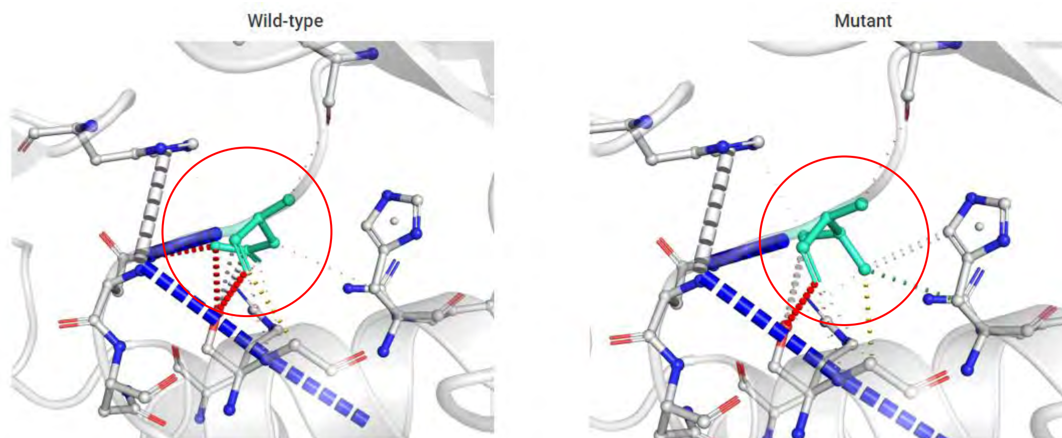


Figure 4.18: Protein structure change due to mutation Asp435Val

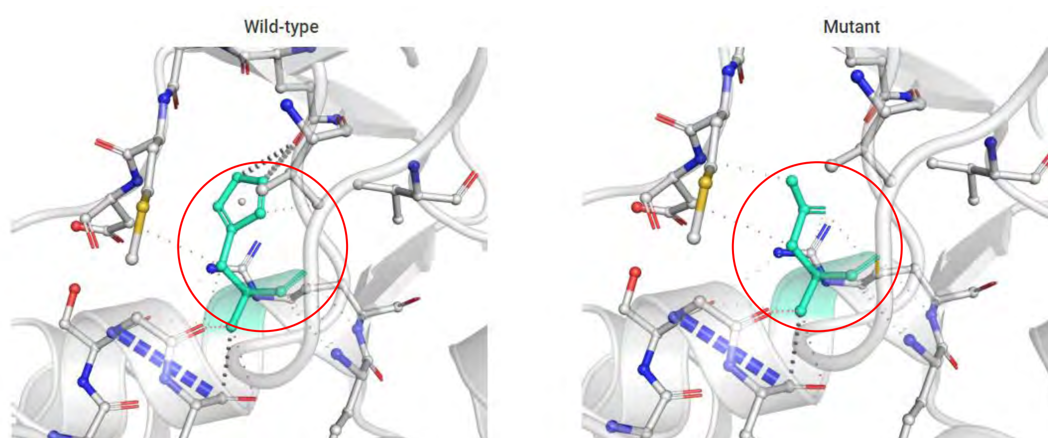


Figure 4.19: Protein structure change due to mutation His445Asp

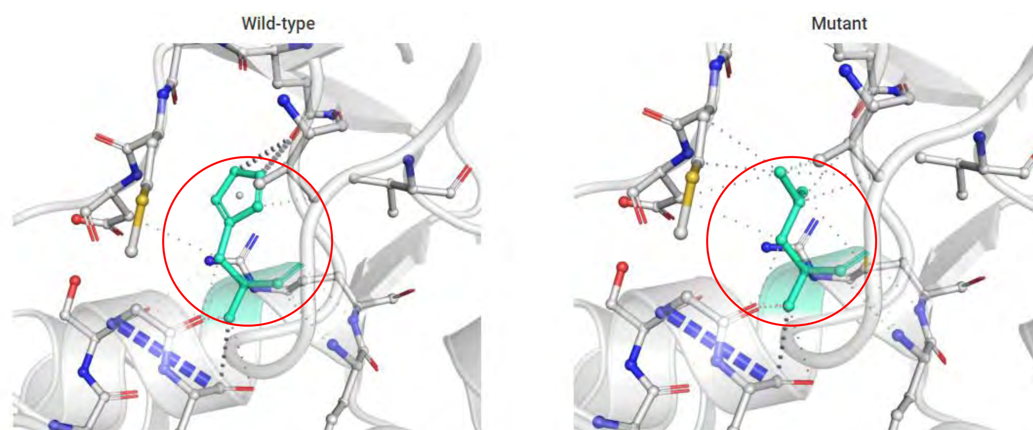


Figure 4.20: Protein structure change due to mutation His445Leu

4.18 Structures of protein with Moderate or minimal confidence

4.18.1 Leu452Pro (Moderate confidence on LJ but no confidence on MGIT)

Mutant Leu452Pro is a moderate confidence mutation on LJ DST, however, has no confidence on MGIT DST. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was -0.550 kcal/mol and was destabilizing. NMA Based Predictions ΔΔG ENCoM was -0.628 kcal/mol and it was destabilizing. The vibrational entropy energy between wild-type and mutant was -0.785 $kcal.mol^{-1}.K^{-1}$ which could increase its flexibility (Figure 4.21).

4.18.2 Asp435Tyr (Moderate confidence LJ but no confidence on MGIT)

Mutant Asp435Tyr is a moderate confidence mutation on LJ DST, however, has no confidence when DST was performed on MGIT. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was 0.879 kcal/mol, which indicates to make this protein more stable. NMA Based Predictions ΔΔG ENCoM was 0.313 kcal/mol, which indicates that it is destabilizing The vibrational entropy energy between wild-type and mutant is -0.391 $kcal.mol^{-1}.K^{-1}$, causing decrease in molecule's flexibility. The structure change due to this mutation is shown in Figure 4.22.

4.18.3 His445Asn (Minimal confidence LJ but no confidence MGIT)

Mutant His445Asn is a minimal confidence mutation on LJ DST, however, have no confidence on MGIT. Prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was -1.509 kcal/mol) and it is destabilizing. NMA Based Predictions ΔΔG ENCoM was -0.563 kcal/mol and was destabilizing. The increased flexibility is due to the difference in vibrational entropy energy between wild-type and mutant is 0.703 $kcal.mol^{-1}.K^{-1}$ was predicted (Figure 4.23).

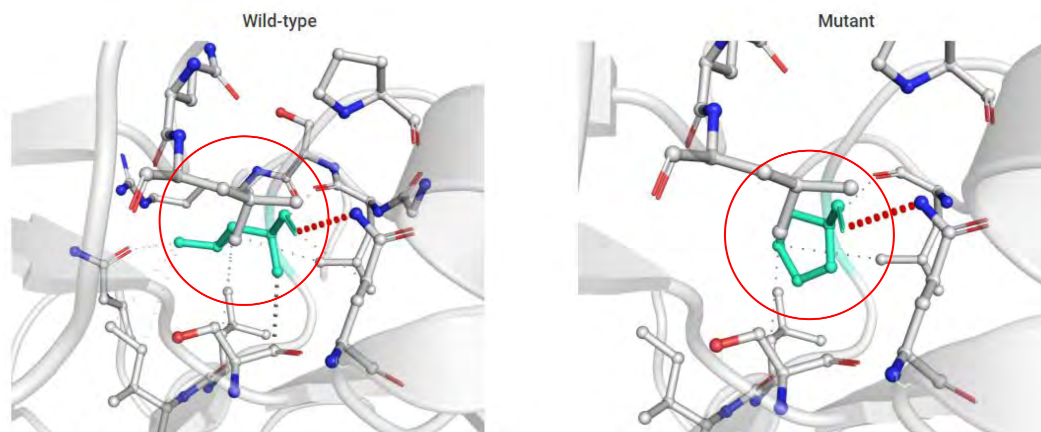


Figure 4.21: Protein structure change due to mutation Leu452Pro.

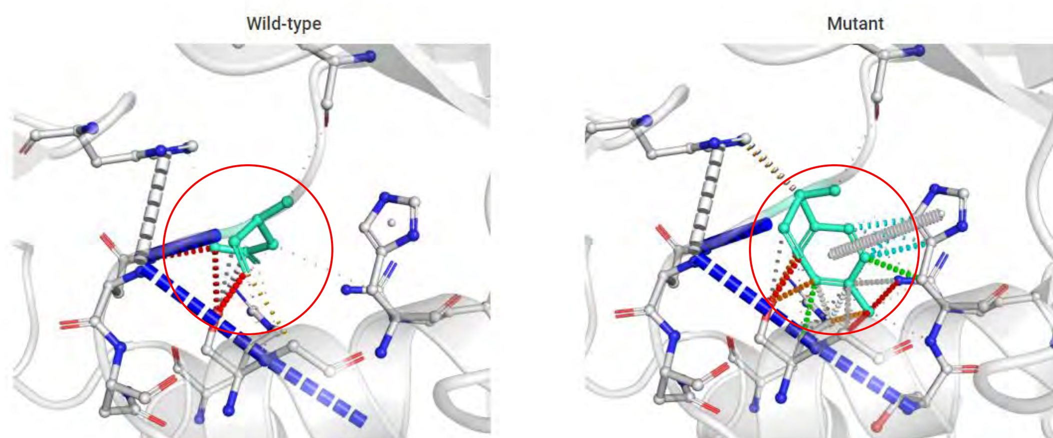


Figure 4.22: Protein structure change due to mutation Asp435Tyr

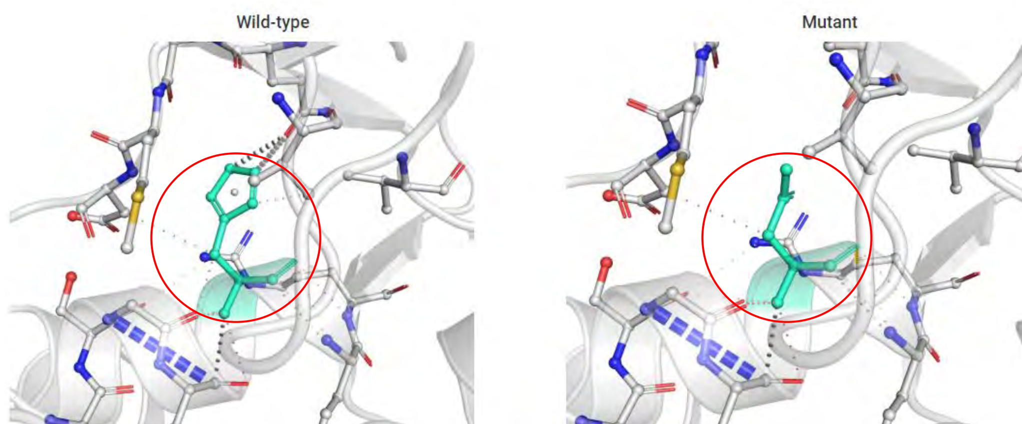


Figure 4.23: Protein structure change due to mutation His445Asn.

4.18.4 Leu430Pro (Minimal confidence LJ but no confidence on MGIT)

Mutant Leu430Pro is a no confidence mutation on MGIT DST but minimal confidence mutation on LJ DST. The prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was - 0.600 kcal/mol and was destabilizing. NMA Based Predictions ΔΔG ENCoM was -0.588 kcal/mol and was destabilizing with the vibrational entropy energy difference between wild-type and mutant 0.734 $kcal.mol^{-1}.K^{-1}$ increasing its flexibility (Figure 4.24).

4.18.5 Leu430Arg (Minimal confidence LJ but no confidence on MGIT)

Mutant Leu430Arg has no confidence when DST is performed on LJ and MGIT. Prediction outcome (ΔΔG ENCoM) due to this mutation in *rpoB* was - 0.533 kcal/mol and was destabilizing. NMA Based Predictions ΔΔG ENCoM was -0.094 kcal/mol and was destabilizing whereas vibrational entropy energy between wild-type and mutant was 0.117 $kcal.mol^{-1}.K^{-1}$ increases the molecule's flexibility. The structure change due to this mutation is shown in Figure 4.25.

4.19 Phylogenetic Analysis

Phylogenetic analysis was performed for 25 selected isolates based on WGS. Mean coverage of mapped read, Lineage identified, *rpoB* mutation, and GeneXpert, LJ, and MGIT DST results are presented in Table 4.26. Out of 25 isolates, 20 strains were of Delhi ⁄CAS lineage, 3 Euro-American Superlinage whereas the rest Beijing and East African Indian lineage each. For cluster analysis minimum spanning tree was constructed (Figure 4.26), there was no single cluster identified in the tree as all strains were having a distance greater than the minimum requirement for cluster makeup. Add linage of discordant and concordant strain.

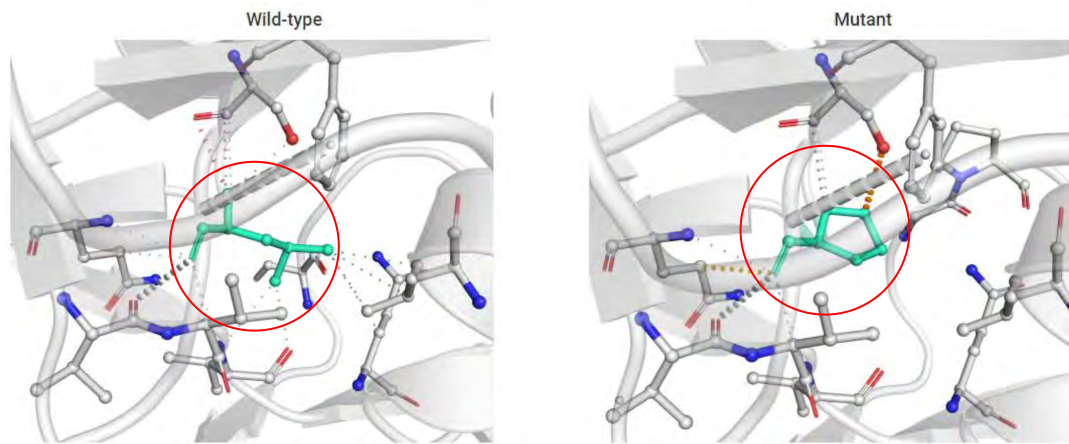


Figure 4.24: Protein structure change due to mutation Leu430Pro.

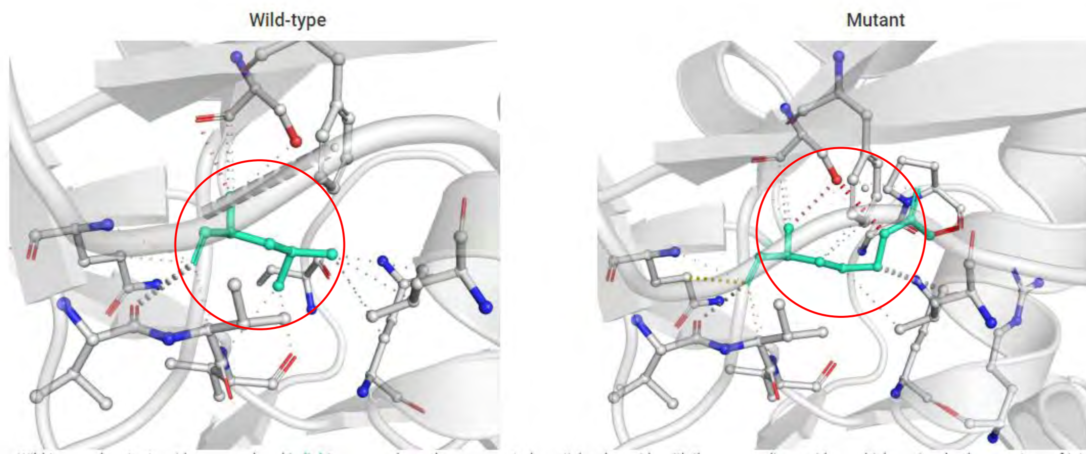


Figure 4.25: Protein structure change due to mutation Leu430Arg.

Table 4.26: Distribution of discordant and concordant WGS sequenced Mtb isolates in various lineages with their GeneXpert, LJ and MGIT rifampicin resistance results

ID code	WGS ID	Mean coverage	Lineage	Amino acid change *rpoB* if any	GeneXpert-LJ-MGIT DST results
MQ-01	stand_024	123.7 ± 47.52	Delhi⁄CAS	del of AA 435-439 AspGlnAsnAsnPro	GX-R-LJ-R-MGIT-R
MQ-02	stand_025	267.43 ± 67.67	Beijing	WT	GX-S-LJ-S-MGIT-S
MQ-03	stand_026	471.04 ± 97.36	Delhi⁄CAS	WT	GX-S-LJ-R-MGIT-R
MQ-04	stand_027	310.3 ± 66.27	Euro-American Superlinage	WT	GX-S-LJ-S-MGIT-S
MQ-05	stand_028	156.83 ± 34	Delhi⁄CAS	Thr444Ala	GX-R-LJ-S-MGIT-S
MQ-06	stand_029	423.6 ± 86.44	Delhi⁄CAS	Wt	GX-S-LJ-S-MGIT-S
MQ-07	stand_030	388.74 ± 82.13	Delhi⁄CAS	Leu430Pro	GX-R-LJ-S-MGIT-S
MQ-08	stand_031	215.94 ± 57.76	East African Indian	Leu430Arg	GX-R-LJ-S-MGIT-S
MQ-09	stand_032	167.69 ± 36.8	Delhi⁄CAS	Asp435Gly	GX-R-LJ-S-MGIT-S
MQ-10	stand_033	318.58 ± 83.83	Delhi⁄CAS	Asp435Tyr	GX-R-LJ-R-MGIT-S
MQ-11	stand_034	173.74 ± 47.28	Delhi⁄CAS	Leu452Pro	GX-R-LJ-R-MGIT-S
MQ-12	stand_035	159.65 ± 41	Delhi⁄CAS	His445Leu	GX-R-LJ-R-MGIT-S
MQ-13	stand_036	183.66 ± 43.19	Delhi⁄CAS	His445Asn	GX-R-LJ-S-MGIT-S
MQ-14	stand_037	162.99 ± 36.07	Delhi⁄CAS	Met434Val, His445Asn	GX-R-LJ-R-MGIT-R

MQ-15	stand_038	143.52 ± 32.33	Delhi/CAS	His445Asn	GX-R-LJ-R-MGIT-R
MQ-16	stand_039	298.8 ± 73.81	Euro-American Superlinage	Ins of Phe at 434	GX-R-LJ-R-MGIT-R
MQ-17	stand_040	159.96 ± 46.32	Delhi/CAS	Ins of Arg at 432	GX-R-LJ-R-MGIT-R
MQ-18	stand_041	146.85 ± 37.59	Delhi/CAS	Gln436His + Asn 437 deletion	GX-R-LJ-R-MGIT-S
MQ-19	stand_042	404.58 ± 111.41	Euro-American Superlinage	del of AAs ThrSerGlnLeu at 427-30	GX-R-LJ-R-MGIT-R
MQ-20	stand_043	122.15 ± 26.84	Delhi/CAS	Ser428Ile	GX-R-LJ-R-MGIT-S
MQ-21	stand_044	140.99 ± 29.65	Delhi/CAS	Asn438Lys	GX-R-LJ-S-MGIT-S
MQ-22	stand_045	167.16 ± 39.7	Delhi/CAS	Leu430Pro	GX-R-LJ-R-MGIT-R
MQ-23	stand_046	157.48 ± 36.56	Delhi/CAS	Asp435Tyr	GX-R-LJ-R-MGIT-R
MQ-24	stand_047	178.57 ± 49.25	Delhi/CAS	Leu452Pro	GX-R-LJ-R-MGIT-R
MQ-25	stand_048	141 ± 47.2	Delhi/CAS	Ser450Leu	GX-R-LJ-R-MGIT-R

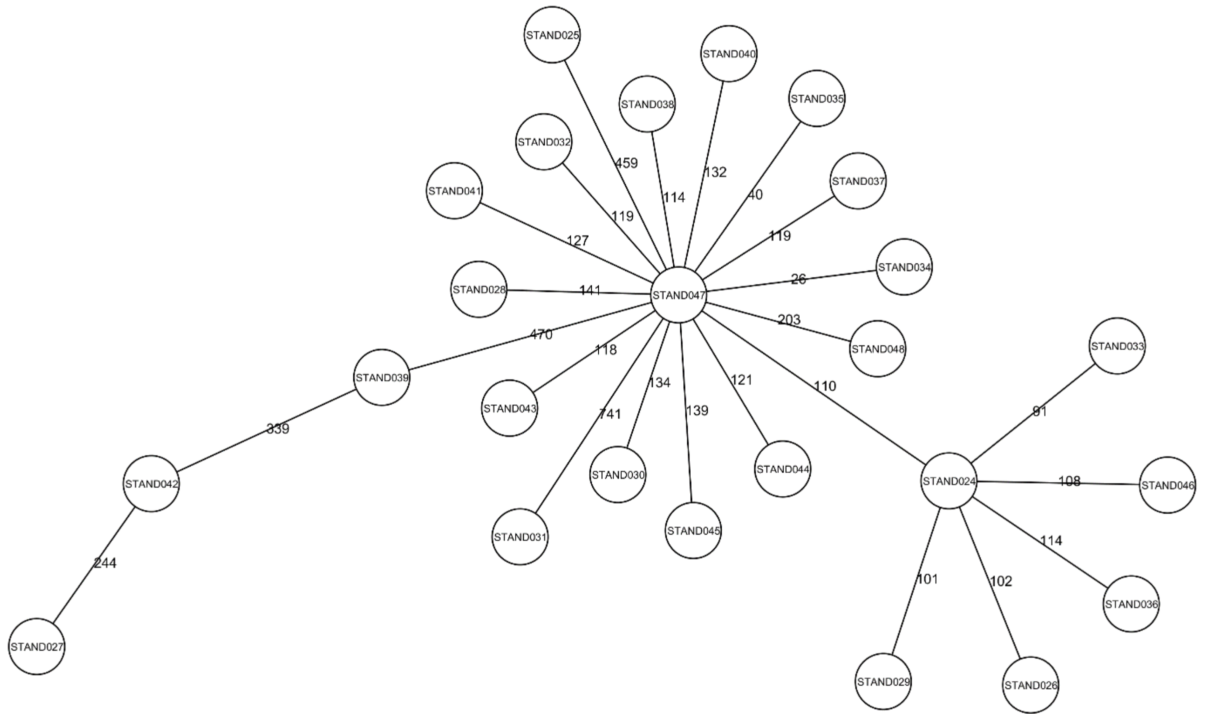


Figure 4.26: Minimum spinning tree of isolates based on WGS showing different lineages with predominate lineage being Dehli/CAS lineage

5. Discussion

The rise of drug resistant TB is not directly associated with the high prevalence of TB, which itself is a dangerous global health issue, rather the improper diagnosis and prognosis. Despite vaccination and availability of the first and second line of anti TB drug regimens, there is a continuous increase in the emergence of resistance against these anti-TB drugs, which has compelled scientists around the world to investigate and understand the molecular basis of drug resistance development. In an era of antibiotic resistance escalation, drug resistant microbes, especially drug resistance in *Mycobacterium tuberculosis* are of great concern due to difficulty in patients' treatment, who remain infected for even months and years with high mortality rates. DR TB can be caused either by direct mutations in different genes, as well as due to compensatory mutations, or even by epigenetic regulation of various genes along with drug concentration, dosage, *etc*. Not only is there a need to understand the basis of drug resistance in TB, but enhancement in sensitivity and specificity of TB diagnosis methods by the identification of novel targets, which can be used for the development of genotypic drug resistance detection assays. Up to now, DR TB cases are misdiagnosed on both phenotypic and genotypic WHO recommended assays, which is one of the major concerns for treatment failure and reoccurrence of TB globally including Pakistan.

In the current study, 1036 (52%) TB patients were males, and 96% had a known treatment history of either as the first line of drug regimen or second line of anti TB drug regimen. The Majority of TB cases (94%) had Pulmonary TB disease while 6% had extra pulmonary TB. In a previous study from Pakistan in which 1,075 Mtb positive patients from 17 districts of Khyber Pakhtunkhwa were studied, but contrary to present work female TB patients were more prevalent (54.6%) as compared to males (45.3%) (Khan *et al*., 2018). Faisal *et al*., (2018) reported that among 22,399 TB suspected patients visiting health camps to assess that chest cramps and their association with TB in various cities of Pakistan, found that more males were TB positive. A work on MDR TB cases and their first line of anti TB treatment from Sindh province found that patients' first line of anti TB receiving treatment 53% were men and 47% females (Khan *et al*., 2019).

In the drug resistance survey of Pakistan of the 1972 eligible patients recorded, 1078 (54.7%) were male patients and 894 (45.3%) were female patients, but in contrast, there were 1675 newly diagnosed TB cases while 277 were previously treated TB cases (Tahseen *et al*., 2016). However, the high prevalence of pulmonary cases was reported earlier is in line with current work as there were 1,023 cases of pulmonary TB type with only 52 were extra pulmonary (Khan *et al*., 2018). A study by Tahseen *et al*., (2020) reported 38,302/54,092 pulmonary TB and 15,790/54,092 extra-pulmonary TB cases which were roughly one third of the cases (29.2%) registered in 2016, which is a higher prevalence compared to present work. A retrospective study (2013-2017) was conducted to determine the prevalence of DR TB in Karachi, where MDR TB was found in 64.1% cases followed by 27.9% Rif resistant with 1% poly-drug resistant cases even low level (1.6%) of XDR was seen. Age and gender were found to play a role in MDR, but drug resistance TB patients were found to be increasing which pointed towards the need for a proper diagnosis for treatment and control of disease transmission (Masood *et al*., 2019). In this study, most (72.2%) of the TB patients were of age group 15 to 44 years. TB is prevalent in this younger age group in Pakistan where the median age for males is 37 years and females is 30 years as documented by Tahseen *et al*., 2016.

This study was designed to determine the limitations of various techniques used for the diagnosis of TB, DR TB, and MDR TB in the Pakistani population, which are WHO-recommended and adapted by Pakistan's National TB Control Program nationwide. Despite rigorous diagnostic facilities and availability of free anti TB treatment in Pakistan, still there is a high burden of DR TB, MDR TB, and now even XDR TB. Many factors have been found to play role in contributing to the TB burden (Khurram *et al*., 2012, Khan *et al*., 2019c), mainly patients' non-compliance to anti TB regimens due to long therapy, and another main factor is believed to be the lack of rapid, high specificity and sensitivity assay for diagnosis of DR TB. Different countries are recently reporting that WHO endorsed drug susceptibility assays are unable to detect resistance in Mtb strains and there is a disparity in drug susceptibility results of genotypic assays with phenotypic DST (Rigouts *et al*., 2013; Grace Lin, *et al.*, 2016; Tehseen *et al*., 2016; Miotto *et al.*, 2017; Torrea *et al.*, 2019; Shea *et al.*, 2020). Hence, there is the presence of

occult TB strains (strains with discordant results on phenotypic DST assays in comparison with genotypic DST on GeneXpert assay) globally in different regions. It is believed that these discordant Mtb strains are circulating in the Pakistani TB population too.

No study has been carried out to assess the prevalence of the occult strains in the local TB population and decipher the molecular basis for non-detection on existing assays. For this purpose, work was carried out for diagnosis of drug susceptibility of Mtb, initially on genotypic GeneXpert for RIF susceptibility which is a surrogate marker followed by phenotypic LJ and MGIT DST. Results were compared for rifampicin susceptibility displayed by local Mtb isolates on GeneXpert, LJ, and MGIT DST. Resistance to rifampicin on GeneXpert was detected in overall 1644 samples, while 342 isolates were sensitive to rifampicin.

GeneXpert resistant and GeneXpert sensitive isolates were compared for their RIF sensitivity results on LJ and MGIT DST. A total of 226 isolates with discordance results were identified between all the three assays for rifampicin susceptibility in either assay, which was an overall 11.4% discordance among 1986 isolates.

Different studies reported from different geographical areas with different frequencies of discordance results between genotypic and phenotypic assays. In a study conducted in Iraq discordance result between GeneXpert and phenotypic DST was detected in 17(4%) isolates (Ali and Alsudani, 2016). In a recent study from China, 118 (16.2%) GeneXpert resistant isolates showed discordance results on phenotypic DST. Upon sequencing of 104 of these isolates, 86 (82.7%) isolates were found with mutations in the RRDR region of the *rpoB* gene (Huo *et al*., 2020). In another recent study from Indonesia, discordance between GeneXpert and phenotypic DST was found in 22% of their isolates (Kusmiati *et al*., 2021).

In this current study, rifampicin resistance-causing mutations were identified using sanger sequencing and were compared to resistance detected in phenotypic DST assays (LJ and MGIT) and genotypic GeneXpert MTB/RIF assay. A total of 516 isolates were Sanger sequenced for the *rpoB* gene, which included 376 concordant strains on all three assays GeneXpert, LJ, MGIT, and, 140 discordant strains on either assay were sequenced for detection of mutations in the *rpoB* gene.

The phenotypic DST is still considered to be the gold standard method to infer the drug resistance, but some studies have reported that among these phenotypic liquid culture systems (e.g. LJ, Middle Brook, 7H10 agar, MGIT platform) often fail to detect low levels of phenotypic resistance of RR TB due to RRDR mutations (Rigouts *et al*., 2013; Van Deun *et al*., 2009, 2013; Yakrus *et al*., 2014). These phenotypic low-level resistance and RRDR region mutations have been reported in treatment failure or relapse cases as well (Shah *et al*., 2016; Van Deun *et al*., 2015; van Ingen *et al*., 2011). The role of these detected mutations in the *rpoB* gene can be investigated using *in silico* protein structural analysis for encoding their respective protein (Tunstall *et al*., 2020), as previous studies have shown that mutations can affect the structural dynamics of proteins and also decrease affinity for the target site for the drug, hence resulting in drug resistance (Khan, 2019; Khan *et al*., 2019; Muhammad Tahir Khan *et al*., 2018; Shivakumar *et al*., 2014).

The most prevalent mutations identified in the *rpoB* gene were in codon 450, 445, and 435 respectively in the current study, which was previously reported from Pakistan and even globally in different studies (Jabbar *et al*., 2019; Javed *et al*., 2017; Noor *et al*., 2015; Qazi *et al*., 2014; Siddiqi *et al*., 2002). Most common mutation (C→T) identified in this study, upon translation into amino acid, change in amino acid Ser450Leu was seen, which was already reported for rifampicin resistance throughout the world in TB cases (Madania *et al*., 2012; Qazi *et al*., 2014; Sanchez-Padilla *et al*., 2015; Shea *et al*., 2020; Yakrus *et al*., 2014; Yue *et al*., 2003).

A total of 368 SNPs in the *rpoB* gene were detected by Sanger sequencing in the current work, including deletion or insertion causing a change in single amino acid, whereas 28 genetic variants had double, and triple nucleotide polymorphisms resulted in change/deletion of even more than one amino acid.

Most mutations identified in *rpoB* were in its hot spot region except for 6 isolates, which included 5 mutations at codon 491 and 1 at codon 170 and were detected outside the *rpoB* hotspot region which is not covered by GeneXpert. A lower percentage of mutations in the present work was due to screening of all samples received from various TB hospitals of Pakistan, which was done initially on the GeneXpert in which mutations outside the RRDR are missed.

Various countries are reporting mutations outside the RRDR, which are missed on GeneXpert. Swaziland reported 30% of RR cases with mutations outside the RRDR (Sanchez-Padilla *et al*., 2015). Similarly, in Uganda, 18% (8/45) of the RIF-resistant isolates again had mutations, not detected as were outside RRDR (Kigozi *et al*., 2018). In China, five novel mutations in RIF resistant isolates were detected outside the RRDR region (Yue *et al*., 2003). In Syria, out of 69 RIF-resistant isolates, 62 harbored mutations in RRDR while one only mutation was detected outside the RRDR (Madania *et al*., 2012). In a previous study conducted in Pakistan (Qazi *et al*., 2014), out of 63 isolates, 24 (38%) were resistant to RIF harboring a single mutation in the RRDR region while five isolates did not harbor any mutation in the RRDR of the *rpoB* region on GeneXpert. The current work had found only a very low prevalence of mutation outside RRDR compared to earlier Pakistani studies and also studies from other countries cited.

Besides earlier reported mutations, three novel mutations were detected in this work. In these, two novel mutations were translated to deletion of four amino acids Thr, Ser, Gln, and Leu from 427-430 position and insertion of amino acid Arg at position 432 in RIF-resistant by GeneXpert as well as these isolates were resistant on a phenotypic method using Liquid MGIT DST and Solid LJ DST, hence were concordant strains. MIC of this newly reported mutation was above critical concentration on both MGIT and LJ media and was detected resistant on all three assays. However, the third novel mutation (CAG→CAC) resulted in amino acid change Gln436His with simultaneous deletion of Asn437 was detected as resistant on GeneXpert and LJ DST but was found to be susceptible on Liquid MGIT 960. MIC was determined for this strain, and it was 0.5 µg /mL on MGIT 960, which is below the critical concentration of 1 µg /mL and will be reported as susceptible. These novel mutations highlight that those critical concentrations might have a role in varied phenotypic susceptibility results and might be missed due to the higher concentration used in DST. These newly identified mutations are very important as drug susceptibility testing is moving from phenotypic to genotypic method especially whole-genome sequencing. This data is now submitted to Gene Bank and is made available for future identification and classification of resistance-causing mutations. This data will also be helpful as a lot of new diagnostics tools are in the process of development as well as getting upgraded. The sequences of the newly

identified mutations are available in Gene Bank for consideration by various commercial platforms to design automated assays even for a specific population. A study conducted in Zimbabwe detected novel mutations in the *rpoB* gene that highlighted the molecular assay kits variations associated with the geographic specificity of the target genes (Takawira *et al*., 2017).

The prevalence of occult strains generally missed by rapid phenotypic DST methods even by genotypic assay is largely unknown, now few studies have started to address this question. In the case of GeneXpert, several mutations outside and within the RRDR are also misclassified. Therefore, 516 strains were Sanger sequenced, results of these two molecular assays (*rpoB* gene vs. GeneXpert) were compared in the current study, it revealed that 10.9% of cases were misdiagnosed for susceptibility. There were 13 (3.2%) isolates, which had the *rpoB* gene mutation, among these six strains having mutations outside the RRDR region were not detected on GeneXpert. Similarly, 7.7% of cases were wild-type on Sanger sequencing but were earlier classified by GeneXpert as rifampicin-resistant which is a significant number. Different studies have reported different percentages of discordances, which include discordance outside the RRDR region as well as discordance within the RRDR region which includes silent mutations to mutations at the extreme end like Ser450 to Leu452 (Huo *et al*., 2020; Kusmiati *et al*., 2021; Tahseen *et al*., 2016). Similarly, other resistance mechanisms, including efflux pumps and drug-modifying enzymes, can also play a role in the development of resistance, in such cases, no mutation will be observed in genes responsible for the development of resistance (Dheda *et al.*, 2018).

Comparing MGIT RIF DST result with sequencing result in the current study revealed that 24% cases had discordance in results, which included 23.7% isolates harboring changes in *rpoB* gene in Sanger sequencing but were classified as RIF susceptible on MGIT DST which is a significant number of discordances. However, compared to GeneXpert results, a very few wild-type isolates were classified as RIF resistant which means that drug-susceptible TB has little chance to be misdiagnosed compared to drug-resistant TB when performing MGIT DST.

Comparing LJ RIF DST result with sequencing result in our study revealed that 10.9% cases were with discordance which included 10.6% isolates harboring changes in *rpoB* gene were classified as RIF susceptible on LJ DST which is also a significant number of discordances but was much less than MGIT RIF DST. However, compared to GeneXpert results, a very few wild-type isolates were classified as RIF resistant which means that drug susceptible TB have very little chances to be misdiagnosed as drug resistant TB when performing LJ DST. About 14% of all the *rpoB* mutations are causing discordance according to previous studies (Van Deun *et al*., 2013). Thus, if only the conventional phenotype-based drug susceptibility testing method is used, some Mtb isolates with discordant mutations would be classified as RIF-susceptible and such patients will be treated as drug susceptible TB (Van Deun *et al*., 2015; Williamson *et al*., 2012). Many reports have shown that outcome of treatment for such patients is poor if treated with rifampicin containing regimen, in such a situation relapses occurs (Shah *et al*., 2016; Van Deun *et al*., 2013; Williamson *et al*., 2012). Also, the critical concentration used for MGIT DST is required to be revised and needs to be lowered as low as 0.0625 μg/mL to 0.5 μg/mL (Gumbo, 2010; Shah *et al*., 2016; World Health Organization, 2021). Though, earlier studies showed that performing MGIT DST at a critical concentration of 0.25 μg/mL failed to address the problem as some discordant isolates are still missed, however, some susceptible isolates were missed and were classified as resistant (Mvelase *et al*., 2019).

In the current study 19/20 (95%) isolates with a mutation at Leu433Pro were detected susceptible to RIF on MGIT DST at a critical concentration of 1 μg/mL and 14/20 (70%) were detected susceptible on LJ DST at a critical concentration of 40 μg/mL. This mutation is not associated with resistance by Miotto *et al* if MGIT DST was only performed, while it gave minimal confidence grading on solid DST (Miotto *et al*., 2017). Rigouts *et al* detected this mutation in 6 strains and missed resistance to RIF completely on MGIT DST, however, detected as resistant to RIF on LJ DST in all strains (Rigouts *et al*., 2013). Similarly, other studies have also reported that this mutation causes discordant results in their studies (Ho *et al*., 2013; Miotto *et al*., 2017; Mvelase *et al*., 2019; Rigouts *et al*., 2013; Shea *et al*., 2020; Torrea *et al*., 2019; Van Deun *et al*., 2009).

In the current study, 17/21 (80.9%) isolates with mutation Asp435Tyr were detected as susceptible to RIF on MGIT DST at a critical concentration of 1 μg/mL and 5/21 (23.5%) were susceptible to RIF on LJ DST at a critical concentration of 40 μg/mL. This mutation's confidence grading is not available on MGIT DST, while gave moderate confidence grading on solid DST in a study by (Miotto *et al*., 2017). Leen rigouts reported this mutation in 6 strains was missed completely on MGIT DST, however, was detected as RIF resistance causing on LJ DST in all these strains (Rigouts *et al*., 2013). Similarly, other studies have also reported this mutation as giving discordant results (Miotto *et al*., 2017; Mvelase *et al*., 2019; Rigouts *et al*., 2013; Shea *et al*., 2020; Torrea *et al*., 2019; Van Deun *et al*., 2009).

In the current study, 15/18 (83.3%) isolates with a mutation at position His445Asn were found to be susceptible to RIF on MGIT DST at a critical concentration of 1 μg/mL and 11/18 (61.1%) were susceptible on LJ DST at a critical concentration of 40 μg/mL. This mutation's confidence grading is not available on MGIT DST, while minimal confidence grading on solid DST was given by Miotto Paolo (Miotto *et al.*, 2017). In the rigouts *study,* this mutation was found in 5 strains and detected resistance in 1 (20%) strain on MGIT DST, however, on LJ DST all strains were resistant. Similarly, This mutation is reported as discordant in different studies (Jamieson *et al*., 2014; Miotto *et al*., 2017; Rigouts *et al*., 2013; Shah *et al*., 2016).

Also isolates found with mutation His445Leu in this work, 8/9 (88.8%) were susceptible to RIF on MGIT DST at a critical concentration of 1 μg/mL. However, all of these nine isolates were resistant to LJ DST. This mutation's confidence grading is not available on MGIT DST, while high confidence grading on solid DST was given by Paolo *et al*. This mutation was reported in earlier work in 10 strains and 6/10 strains were detected as resistance on MGIT DST, however, all were resistance to RIF on LJ DST (Rigouts *et al*., 2013). The results regarding this mutation to be present in occult strains have been documented in other work (Jamieson *et al*., 2014; Miotto *et al*., 2017; Mvelase *et al*., 2019; Rigouts *et al*., 2013; Van Deun *et al*., 2009).

The 20 isolates were identified with mutation Leu452Pro in this study. In which 17//20 (85%) were susceptible to RIF on MGIT DST at a critical concentration of 1 µg/mL and 5/20 (25%) and also susceptible to LJ DST at a critical concentration of 40 µg/mL. This mutation is not associated with RIF resistance but when DST is performed on MGIT no susceptibility is seen, it gives moderate confidence grading on LJ. Rigouts *et al* reported that 14 strains with mutation Leu452Pro were missed completely on MGIT DST, however, were detected as resistant on LJ DST in all of these 14 strains. The discordant results due to this mutation (Ho *et al*., 2013; Miotto *et al*., 2017; Rigouts *et al*., 2013; Shah *et al*., 2016; Shea *et al*., 2020; Torrea *et al*., 2019; Van Deun *et al*., 2009).

A mutation His445Cys was identified in six isolates from the local population, in which 2/6 (33.3%) were susceptible to RIF on MGIT DST at critical concentration and 1/6(16.6%) was susceptible on LJ DST at critical concentration. It is another mutation, which is giving discordance and is reported by various studies (Miotto *et al*., 2017; Shah *et al*., 2016). But according to present work findings, this mutation confidence grading on MGIT DST should be revisited as resistance in 66.6% strains in the present study was missed.

Some of the mutations give discordant results, which are reported earlier. In five isolates detected with mutation Ile491Phe, 1 (20%) was susceptible to RIF on MGIT DST at a critical concentration of 1 µg/mL and all were resistant on LJ DST at critical concentration. This mutation gave minimal confidence grading on solid DST (Miotto *et al*., 2017). Resistance to RIF due to mutation Leu430Arg in two isolates, Ser431Gly, Asn438Lys, and Thr444Ala in one isolate in the present study was missed on both liquid (MGIT) and solid (LJ) DST. These mutations are causing discordant rifampicin results and were not previously reported as a discordant mutation in any of the earlier studies. Mutation due to Gln436His+437Asn deletion and Ser428Ile were detected as susceptible to RIF on MGIT DST at a critical concentration of 1 µg/mL and were resistant on LJ DST at a critical concentration of 40 µg/mL, hence are classified as discordant mutations. It is only gives discordant results on MGIT DST, which is not previously reported as discordant mutation.

In the case of double and triple mutations, results from various assays were analyzed to assess if these more than one mutation in the *rpoB* gene have a role in imparting discordance in resistance pattern. All of these mutations identified in isolates which were co-occurring (Met434Val + His445Asn, Leu430Arg + Asp435Tyr, His445Asn + Ser450Trp, Ser428Arg + Leu430Pro, Met434Leu + His445Asn, Asp435Gly + Ala451Val, Met434Ile + Asp435Gly + Ala451Val) also demonstrated discordant results but on MGIT DST only, however, all these strains were found resistant on LJ media. Mutation Asp435Gly was detected with other isolates in combination as a double or triple mutant but in one isolate each, it was associated with high-level resistance on LJ but was not resistant on MGIT in previous studies. However, this mutation showed discordance on both liquids (at a critical concentration of 1 μg/mL) and solid DST (critical concentration of 40 μg/mL on LJ).

Mutations leading to single amino acid change at position Ser450Leu, Asp435Val, Ser450Trp, His445Asp, and His445Tyr were all detected resistant to RIF on MGIT and LJ in the current study as well as by another study (Rigouts *et al.*, 2013). These mutations were given as high confidence grading on liquid and solid DST by (Miotto *et al.*, 2017). Also, previous studies have reported these mutations to be associated with high-level resistance(Miotto *et al*., 2017; Mvelase *et al*., 2019; Rigouts *et al*., 2013; Shea *et al*., 2020; Torrea *et al*., 2019; Van Deun *et al*., 2009). Mutations Phe433ins, Gln432Lys, Gln432Pro, Gln432Leu, His445Arg, and His445Gly were detected in a few isolates in this study, which were associated with high-level resistance which is in agreement with a previous study (Miotto *et al.*, 2017).

According to our study both LJ and MGIT DST, are prone to miss RIF resistance, however, percentages of discordance in MGIT are more as compared to LJ. This work showed that in Pakistani study population has Mtb with a substantial number of common and less common mutations leading to a change in their sensitivity on LJ and MGIT DST. Rigouts *et al*., 2013 showed that the automated MGIT DST was more prône to misdiagnose RIF-susceptible strains in the presence of discordant *rpoB* mutations as compared with LJ, and the same was observed in this current investigation in our region. Similarly, supra-national TB Reference laboratories network of the WHO have shown

that more RIF susceptible results were observed in liquid DST as compared to solid LJ DST and 7H10 agar in samples with discordant strains with *rpoB* mutations (Van Deun *et al*., 2009). It was hypothesized that discordant mutations lower the rifampicin binding affinity, but do not completely terminate the function of *rpoB* gene coding polymerase. Some other studies have also shown that these mutations and SNPs can cause loss of fitness and hence some strains even grow slowly (Gagneux, 2009; Rifat *et al*., 2017). Considering the proven relevance of Leu430Pro, Asp435Tyr, His445Cys/Leu/Asn, and Leu452Pro mutations in determining clinical resistance, genotypic drug susceptibility testing should be used to replace phenotypic results when such mutations are found (Miotto *et al*., 2018).

As RIF resistance is a surrogate marker for other drug resistance too, the drug resistance genes besides rifampicin for the first and second line of anti TB treatment were analyzed for identification of mutation through WGS. For resistance to isoniazid, mutations in the *katG* gene were screened in total for these 25 WGS sequenced isolates. In WGS, 3 different mutations in the *katG* gene were S315T, R463L, and T475P. In a previous study (Ahmad *et al*., 2002), S315T mutation was detected in 18 (64%) of INH-resistant Mtb from Dubai, however, in another study *katG,* S315T mutation was highly prevalent as was detected in 94% of INH-resistant Mtb isolates in a study conducted in Russia (Mokrousov *et al*., 2002). Some other studies have also reported that high level of INH drug resistance (50% to 95% of INH resistance) due to the *katG* gene mutations, in which 75% to 90% of these isolates had mutations at the 315th codon of the *katG* gene, it was due to either Ser315Thr1 or Ser315Thr2 mutations (Tessema *et al*., 2012).

The mechanism of action of isoniazid involves inhibition of the synthesis of mycolic acids by acting through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by *inhA* (Palomino and Martin, 2014). Isoniazid resistance, therefore, involves *katG* and *inhA* genes, but also other loci *(oxyS-ahpC, ahpD, and ahpE*) (Bakonyte *et al*., 2003; Palomino and Martin, 2014). In the current study, five mutations in the *inhA* gene were identified of which 4 were present at the genomic position of 1673425. All of these isolates with this mutation were also found to be phenotypic resistant. It is also known that *inhA* with *ahpC* and *ahpE* genes can mitigate the initial

fitness cost caused by *katG* mutations (Brandis *et al*., 2012; Comas *et al*., 2011), and *ahpC-oxyR* mutations are directly linked to resistance (Rinder *et al*., 1998). Recent work has shown that overexpression of *ahpC* and *inhA* may be not involved in resistance to isoniazid (Heym *et al*., 1997; Xu *et al*., 2018). When sequencing results for rifampicin overrule the results of GeneXpert, LJ, and MGIT DST in phenotypic isoniazid-resistant strains 13 (3.3%) on GeneXpert, 87 (22.2%) on MGIT DST and 35(8.9%) on LJ DST will be declared as MDR cases which were misclassified if we take results of only one assay. As treatment management of drug resistant TB is different from drug susceptible TB, if such cases will not be diagnosed properly will result in the proliferation and transmission of occult strains. So, to tackle such discrepancies, Clinicians involved in TB patients' treatment should be well trained to design proper treatment regimens to avoid further transmission of occult strains in the population.

A total of 5 different mutations were detected in the *pncA* gene, one mutation at each position P62T, K96N, E144D, V155G, and one deletion Ser (TCC). In a previous study, these mutations K96T and S179G were detected and reported in the number of Mtb isolates from Pakistan (Khan *et al*., 2019), which were conferring resistance to PZA. The resistance against KAN, AMK, and CAP is believed to emerge due to mutations in the 16SrRNA (*rrs*) gene at region 1400, particularly at positions 1401, 1402, and 1484 (Georghiou *et al*., 2012). In the case of second-line injectables there is also the development of resistance, a total of 53 different types of mutation in *rrs* were detected through WGS in these local strains. Among these reported three mutations, the common *rrs* mutation is 1401A→ G, which is also detected in one of the local isolates. Other detected *rrs* mutations (52) in these *Mycobacterium tuberculosis* isolates were not found in the literature to be associated with DR TB, however, their mutations are needed in the future to be investigated for a possible link to amikacin, capreomycin, and kanamycin resistance.

Similarly, mutations in the *gyrA* gene were identified in 24 WGS sequenced strains. Five strains had mutations Ser95Thr + Asp94Gly, four were resistant on phenotypic DST to ofloxacin due to Asp94Gly previously reported as mutation conferring drug resistance. Similarly isolates with previously reported amino acid changes Asp94Tyr, Ala90Val,

Asp89Asn and Ser91Pro were resistant to phenotypic DST. Isolates harboring Ser95Thr and Gly668Asp in 12 isolates were ofloxacin susceptible in 10 isolates which are in line with previous studies that these changes have no role in resistance to fluoroquinolone. However resistance in two isolates was detected due to mutations in the *gyrB* gene due to Glu501Gln and Thr500Pro (Li *et al*., 2019; Laurenzo and Mousa, 2011; Lau *et al*., 2011; Maruri *et al*., 2012).

Among ethambutol resistant isolates which were sequenced on WGS, M306I was the most common mutation in *embB* was detected which was present in 4 isolates alone, while in 2 isolates was in combination with mutations S651T and V39G. Similar to the previous study, a mutation in the *embB* gene was detected in seven EMB resistant isolates, in which substitution of amino acid in codon 306 was found (Ramazanzadeh *et al*., 2016). Among the bedaquiline and clofazimine resistance conferring mutations, two mutations were detected in the Rv0678 gene where one local isolate had A84S mutations and other L117R mutations. Recently, Bedaquiline drug is approved to be included in the treatment regimen for patients with MDR TB and XDR TB, this highlights a serious concern that there is intrinsic resistance to these newly approved drugs is already present in Pakistani studied isolates despite these strains were isolated from TB patients from the year 2014-16. Bedaquiline and clofazimine were introduced in anti TB regimens after 2019, studied isolates were not assessed for susceptibility to these drugs on phenotypic DST, so their phenotypic profile is not known.

Protein dynamics and the prediction of the effect of mutations on RNA polymerase due to identified mutations in indigenous Mtb strain, protein predication was done using elastic network atom contact model and normal mode analysis predictions. Also, vibrational entropy energy between wild type and mutant was computed in this work for high confidence, moderate confidence, minimal confidence, and mutations with no confidence on LJ and MGIT DST. The binding affinity change caused by a mutation (i.e., $\Delta\Delta G$) was lowered in predicting outcome in the case of most commonly prevalent discordant mutations, which included Leu430Pro, His445Asn, and Leu452Pro except Asp435Tyr. NMA based prediction showed that all mutations destabilized predicted protein except Val170Phe which was stabilizing. Vibrational entropy energy between wild type and

mutants resulted in increased molecule flexibility in case of discordant mutations Leu430Pro, His445Asn, and Leu452Pro except for Asp435Tyr, however, a decrease in molecular flexibility was observed in most concordant mutations like Ser450Leu and Ser450Try. However, to precisely conclude the effect of these mutations on protein there is a need for wet lab expressional studies using these mutants. Previously, a study calculated stabilizing and destabilizing effects on protein stability and flexibility due to identified mutations in the *rpoB* gene from Pakistani isolates, however, the mutations (Arg552Leu, Ser874Tyr, and Lys 891Glu) used for protein structure prediction were different from mutations used for RNA polymerase structure prediction in the current study. In a study conducted by Paolo *et al*., 2018 in silico analysis of protein could not predict any significant difference between disputed and undisputed substitutions and concluded that all mutations in the rpoB gene affect the binding site of rifampicin.

Phylogenetic analysis revealed that Delhi⁄CAS lineage was yet the predominant lineage in local Mtb strains which also correlates with previous studies from the region (Ali *et al*., 2015, 2019). There was not a single cluster found to be present in Pakistan, as Delhi⁄CAS lineage, Euro-American Superlinage, Beijing, and East African Indian lineage are co-existing. Out of 25 strains sequenced on WGS, 12 were discordance strains and 11/12 of these were categorized as Delhi/Cas and one was East African Indian lineage. As the majority of these isolates belonged to Delhi/CAS, hence it cannot be inferred that discordance is more in this lineage. There is a need for larger WSG and phylogenetic analysis needed to decipher the role of lineage in discordance on various diagnostic methods.

After stratification of data, concordance results for the prevalent TB patients age group (15-24 years) were analyzed in 177 patients of this age group, where in all four assays 74.5% concordance in results was found. There were 25% discordance strains present in female TB patients while 29% were male patients. In the case of age and gender similar trend in discordance was present. With reference to previous treatment history, discordant strains were 27%, while 26% were from patients which were never treated before. From this, it can be inferred that a significant number of the isolates show discordance upon the phenotypic and genotypic assay. The roughly same percentage of discordance in both

newly diagnosed and previously treated TB cases was observed, indicating that the chances of discordance in results remain the same in either situation. The discordance in the result of various assays can be due to the possibility of less specificity for detection of mutations in GeneXpert and in the case of phenotypic assays the critical concentration remains the issue. Comparison of WGS results with PZA phenotypic results correctly identified all phenotypic resistance isolates on WGS.

In the case of WGS isolates, 16 were RIF resistant phenotypically and 9 were RIF sensitive. Among these 16 phenotypic RIF resistant strains, 6 were MDR, 6 were Pre-XDR, and 1 XDR along with 3 RIF monoresistant. Mtb isolates were analyzed through WGS to detect if these isolates were also genotypic MDR, Pre-XDR, and XDR. For phenotypic isoniazid isolates were results correctly identified all phenotypic resistance as well as on WGS. Among 7/9 INH resistant strains were having mutations in the *rpoB* gene, so were classified as RIF sensitive on phenotypic results but were resistant for RIF. So, such strains are diagnosed as RIF sensitive but are MDR which can cause treatment failure. This shows that phenotypic DST of isoniazid is not creating discordance results for INH but discordance for RIF.

The 6/25 phenotypic Pre-XDR isolates were compared for genotypic susceptibility on WGS, ofloxacin phenotypic resistance was in line with WGS. In addition, one isolate which was phenotypic ofloxacin sensitive, however, had resistance causing mutation making it ofloxacin resistant. This shows that phenotypic DST of ofloxacin may have some discordance between genotypic and phenotypic results, in this case, WGS is a better option for diagnosis of Pre- and XDR cases to avoid mistreatment and chances of relapse.

Based on the data findings of this study, it can be summed up that LJ DST is a better choice among phenotypic DST assays compared to liquid automated MGIT for rifampicin susceptibility testing. Recently, WHO has revised rifampicin critical concentration from 1 µg/mL to 0.5 µg/mL on MGIT DST to reduce this issue (World Health Organization, 2021) but based on current work local isolates in large numbers will remain discordant. The currently endorsed genotypic assays were found to be less efficient in the detection of mutations, especially outside the RRDR, and contributed to

the misdiagnosis of DR TB. However, GeneXpert still has advantages over the conventional DST methods as it is rapid, cost-effective, with no requirement of specialized infrastructure and highly trained manpower. All these methods (LJ, MGIT, and GeneXpert) had limitations as a high percentage of discordance is observed not only due to already reported mutations responsible for RIF resistance as detected in Sanger sequencing but some new and novel mutations of *rpoB* were identified to be causative for misdiagnosing. Phenotypic DST has its importance in place as currently endorsed genotypic assays cannot detect susceptibility patterns of important TB drugs. The inconsistency caused by the limitations of the detection method in actuality can be addressed by taking sequencing as the gold standard. This should be in guideline if such occult strain come across phenotypically or genotypically final decision should be made after sequencing.

The gold standard assay endorsed by WHO (phenotypic DST), when DST results were compared with the sequencing and WGS results, better detection of drug resistance was observed with sequencing, there were reduction discordance results. Based on findings in this work, it can be proposed that the gold standard for DR TB worldwide as well as in Pakistan should be revised and sequencing should be endorsed especially in the diagnosis of rifampicin resistance. The study has important findings for health practitioners, researchers, and the National TB control program in Pakistan to devise and revise the evidence-based assay for detection of DR TB to avoid discordant results and misdiagnosis.

6 Conclusion

Tuberculosis is a major health issue in Pakistan, which is among the top five high TB burden countries of the world. The dilemma of drug resistance is hampering the successful treatment of both the first and second line of anti TB regimens in both DR TB and MDR TB. This situation is further aggravated by the discordances in the drug susceptibility testing assays routinely used. In this context, this work was carried out to determine the true prevalence of occult strains, their molecular basis of resistance as well as discordance. The main findings of the research work based on the objectives of the study are:

- The prevalence of occult strains was recorded at 11.4% in 1986 tested Mtb isolates and for the first-time discordant isolates are reported from Pakistan to our knowledge. Discordance results were identified most commonly in isolates due to non-synonyms mutations detected through Sanger sequencing, which resulted in codon change leading to single amino acid change, were Leu430Pro, Leu452Pro, Asp435Tyr, His445Asn, and His445Leu.
- A high discordance in results among phenotypic assays (MGIT, LJ) versus genotypic assay GeneXpert within RRDR and outside RRDR was detected, however, LJ DST showed better results than MGIT as fewer discordance results were found on LJ DST but still missed several mutations.
- A large number of mutations (49 mutations) due to insertions, deletions, and single double and triple nucleotide polymorphisms were detected in the *rpoB* gene including three novel mutations were detected in the *rpoB* gene through Sanger sequencing.
- Bedaquiline and clofazimine resistance conferring mutations in the Rv0678 gene were detected in Whole Genome Sequencing.
- Four lineages were identified, Delhi/CAS lineage (major lineage) followed by Euro-American Super linage with one isolate from each Beijing and East African Indian lineage.
- *In silico* structure prediction of DNA dependent RNA polymerase due to mutations identified in the *rpoB* gene revealed that RNA polymerase structure

might have changed in its stability and flexibility, even there is a possibility for change binding affinity to the drug.

- Discordance results among phenotypic DST should be overruled with genotypic results using different assays, especially GeneXpert Ultra and sequencing.

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Original Article

Profiling and identification of novel *rpoB* mutations in rifampicin-resistant *Mycobacterium tuberculosis* clinical isolates from Pakistan

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ABSTRACT

Introduction: Rifampicin (RIF) is one of the most effective anti-tuberculosis first line drugs prescribed along with isoniazid. However, the emergence of RIF resistance *Mycobacterium tuberculosis* (MTB) isolates is a major issue towards tuberculosis (TB) control program in high MDR TB-burdened countries including Pakistan. Molecular data behind phenotypic resistance is essential for better management of RIF resistance which has been linked with mutations in *rpoB* gene. Since molecular studies on RIF resistance is limited in Pakistan, the current study was aimed to investigate the molecular data of mutations in *rpoB* gene behind phenotypic RIF resistance isolates in Pakistan.
Method: A total of 322 phenotypically RIF-resistant isolates were randomly selected from National TB Reference Laboratory, Pakistan for sequencing while 380 RIF resistance whole genome sequencing (WGS) of Pakistani isolates (BioProject PRJEB25972), were also analyzed for *rpoB* mutations.
Result: Among the 702 RIF resistance samples, 675 (96.1%) isolates harbored mutations in *rpoB* in which 663 (94.4%) were detected within the Rifampicin Resistance Determining Region (RRDR) also known as a mutation hot spot region, including three novel. Among these mutations, 657 (97.3%) were substitutions including 603 (89.3%) single nucleotide polymorphism, 49 (7.25%) double and five (0.8%) triple. About 94.4% of Phenotypic RIF resistance strains, exhibited mutations in RRDR, which were also detectable by GeneXpert.
Conclusion: Mutations in the RRDR region of *rpoB* is a major mechanism of RIF resistance in MTB circulating isolates in Pakistan. Molecular detection of drug resistance is a faster and better approach than phenotypic drug susceptibility testing to reduce the time for transmission of RIF resistance strains in population. Such insights will inform the deployment of anti-TB drug regimens and disease control tools and strategies in high burden settings, such as Pakistan.

1. Introduction

Tuberculosis is among the top ten leading causes of death worldwide and, according to WHO estimates, ten million people developed TB in 2017 globally. The emergence of first-line drug-resistant tuberculosis (TB) [1,2], especially multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, poses formidable challenges to controlling TB in high burden countries (WHO 2020, https://www.who.int/publications/i/item/9789240013131). In 2017, 558000 of global TB cases were resistant to rifampicin (RIF) [3]. RIF is one of the most effective anti-TB drugs and resistance to it serves as a valuable surrogate marker for diagnosis of multidrug-resistant tuberculosis (MDR-TB) [4]. This resistance is primarily developed through mutations in *rpoB* of *Mycobacterium tuberculosis* (MTB). In general, 96.1% of RIF resistance worldwide is associated with *rpoB* mutations, and >90% of these mutations are located in the 81 bp RIF resistance determining the region (RRDR) of *rpoB* [5,6] (see Fig. 1).

Various culture-based and molecular methods are used for drug susceptibility testing in MTB. Phenotypic drug susceptibility testing (DST) is still considered the gold standard. Culture-based methods are time-consuming and deemed to be a significant obstacle for prompt diagnosis of MDR TB, its management and control [7]. RIF DST is

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